The transcriptional co-activator NCOA6 promotes estrogen-induced GREB1 transcription by recruiting ERα and enhancing enhancer–promoter interactions

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Estrogen and its cognate receptor, ERα, regulate cell proliferation, differentiation, and carcinogenesis in the endometrium by controlling gene transcription. ERα requires co-activators to mediate transcription via mechanisms that are largely uncharacterized. Herein, using growth-regulating estrogen receptor binding 1 (GREB1) as an ERα target gene in Ishikawa cells, we demonstrate that nuclear receptor co-activator 6 (NCOA6) is essential for estradiol (E2)/ERα–activated GREB1 transcription. We found that NCOA6 associates with the *GREB1* promoter and enhancer in an E2-independent manner and that NCOA6 knockout reduces chromatin looping, enhancer–promoter interactions, and basal GREB1 expression in the absence of E2. In the presence of E2, ERα bound the GREB1 enhancer and also associated with its promoter, and p300, myeloid/lymphoid or mixed-lineage leukemia protein 4 (MLL4), and RNA polymerase II were recruited to the GREB1 enhancer and promoter. Consequently, the levels of the histone modifications H3K4me1/3, H3K9ac, and H3K27ac were significantly increased; enhancer and promoter regions were transcribed; and GREB1 mRNA was robustly transcribed. NCOA6 knockout reduced ERα recruitment and abolished all of the aforementioned E2-induced events, making GREB1 completely insensitive to E2 induction. We also found that GREB1-deficient Ishikawa cells are much more resistant to chemotherapy and that human endometrial cancers with low GREB1 expression predict poor overall survival. These results indicate that NCOA6 has an essential role in ERα-mediated transcription by increasing enhancer–promoter interactions through chromatin looping and by recruiting RNA polymerase II and the histone-code modifiers p300 and MLL4. Moreover, GREB1 loss may predict chemoresistance of endometrial cancer.

The uterus, where the embryo implants and develops, is an estrogen-regulated essential reproductive organ (1). During the proliferative phase of the menstrual cycle, the increased level of 17β-estradiol (E2)[2] induces a rapid growth of the uterine endometrium consisting of both epithelial and stromal cells (1). Endometrial tissues with estrogen overexposure are predisposed to endometrial hyperplasia and carcinogenesis (2). Estrogen promotes endometrial growth mainly through binding to the estrogen receptor α (ERα) (3). Either estrogen or ERα deficiency causes uterine hypoplasia, which results in a failure of uterine decidualization and embryo implantation (4). Interestingly, although both endometrial epithelial and stromal cells express ERα, estrogen-induced epithelial proliferation is indirectly regulated by ERα in the stromal cells that release paracrine factors upon estrogen stimulation (5). In the uterus, ERα expressed in the epithelial cells mainly mediates the estrogen-regulated epithelial differentiation, function, and survival (6). In endometrial carcinoma cells, ERα is often required for maintaining the proliferation and differentiation status of these cancer cells. Inhibition of ERα function may slow down cancer cell proliferation but may induce dedifferentiation and resistance to anti-estrogen therapy (7, 8). However, it is still not clear how ERα exactly regulates its individual target genes in endometrial carcinoma cells.

Estrogen-bound ERα forms a dimer complex that translocates into the nucleus, where it binds the DNA estrogen-responsive element (ERE) in an enhancer or a promoter of its target gene to regulate mRNA transcription. As other nuclear receptors, ERα relies on the recruitments of co-activators such as the three members of the steroid receptor co-activator (SRC) family, p300, CREB-binding protein (CBP), and CARM1 to mediate transcriptional activity (1, 3, 9). In addition to re-programmed histone modifications by the recruited CBP and p300 histone acetyltransferases and CARM1 methyltransferase, co-activators may also play a role in the reorganization of chromatin loops to enhance enhancer and promoter interaction. It has

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This article contains Fig. S1 and Tables S1–S3.

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[2] The abbreviations used are: E2, 17β-estradiol; CBP, CREB-binding protein; PPAR, peroxisome proliferator-activated receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; qPCR, quantitative PCR; TSS, transcriptional start site; V-Ctrl, vector-transfected Ishikawa control; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt; ERα, estrogen receptor α; RXR, retinoid X receptor; AR, androgen receptor; ERE, estrogen-responsive element; NR, nuclear receptor; pRNA, promoter RNA; eRNA, enhancer RNA; pol, polymerase; CREB, cAMP-response element-binding protein; SRC, steroid receptor co-activator; P-Ctrl, parental Ishikawa control; DMEM, Dulbecco's modified Eagle's medium.

recently been demonstrated that SRC-3 can promote estrogen/ERα-mediated transcription by re-organizing estrogen/ERα-induced chromatin looping in breast cancer cells (10). However, the mechanisms for many other co-regulators in the regulation of endogenous target gene transcription by estrogen/ERα remain largely unclear.

The nuclear receptor co-activator 6 (NCOA6), also known as AIB3, ASC-2, PRIP, NRC, TRBP, and RAP250 (11–16), is a transcriptional co-regulator that can interact with multiple nuclear receptors (NRs), including ERα, peroxisome proliferator-activated receptor γ (PPAR γ), retinoid X receptor (RXR), retinoic acid receptor, thyroid hormone receptors, glucocorticoid receptor, liver X receptor, vitamin D receptor, and androgen receptor (AR) (12, 14, 17, 18). Based on reporter gene assays carried out in cultured cells, NCOA6 can promote the transcriptional activities of these NRs and certain other transcription factors such as CREB, AP-1, NF-κB, SRF, CEBPα, and E2F1 (12, 19–21). NCOA6 is a component of ASCOM complex that also contains MLL3 and MLL4 lysine methyltransferases for modifying H3K4 when recruited to the chromatin (22, 23). Furthermore, NCOA6 has been shown to interact with 53BP1 to mediate p53 function (23), with RB to mediate AR function (18), with PPAR-binding protein (also known as TRAP220 or MED1) to mediate PPAR function (13), and with SRC-1/CBP co-activator complex or COAA to mediate NR transcriptional functions (14, 24, 25). However, the role of NCOA6 in chromatin-looping configuration has not been studied.

We have shown that NCOA6 is expressed in many tissues, including neurons in the brain, mammary gland epithelial cells, pancreatic islet cells, and endometrial epithelial and stromal cells (26). Germline knockout of Ncoa6 in mice causes embryonic lethality (27). Furthermore, heterozygous knockout of Ncoa6 in mice accelerated mammary gland tumor growth induced by the polyoma middle T oncoprotein, probably due to the compromised tumor suppressor function of PPAR and RXR (28). Moreover, conditional knockout of Ncoa6 in the mouse endometrium increases ERα protein in the stromal cells and SRC-3 expression in the epithelial cells, resulting in estrogen super-sensitivity, overproliferation of epithelial cells, and failure of embryo implantation. The loss of Ncoa6 causes ERα accumulation because Ncoa6 enhances ERα ubiquitination to accelerate its degradation (29). These findings indicate that NCOA6 plays pleiotropic physiological roles in development and estrogen-regulated organ functions. However, the molecular mechanisms responsible for NCOA6 to mediate ERα transcriptional function have not been studied with any endogenous estrogen/ERα target genes.

The growth-regulating estrogen receptor–binding 1 *GREB1* is an early estrogen-responsive gene in breast cancer cells (30). In the uterus, *GREB1* is highly expressed in the endometrium, and its expression levels fluctuate in accordance with estrogen levels through the woman's reproductive age (31). Similarly, positive correlation between GREB1 expression levels and ERα activation was also observed in endometrial cancer cells (32). As an ERα target gene, previous studies have identified EREs at −35-, −21-, −9.5-, −1.6-, and +6-kb positions from the transcriptional start site (TSS) of the human *GREB1* gene in breast cancer cells (10, 33, 34). The −35-kb site is considered as the major enhancer (10). All of these ERE regions are associated with ERα, SRC-3, RNA polymerase II (pol II), and increased histone acetylation upon estrogen treatment. The chromatin loops formed among the −21-, −9.5-, and −1.6-kb and TSS regions are detected in the presence of estradiol in MCF-7 breast cancer cells (33). A chromatin loop between the −35-kb ERE and the +6-kb region (the TSS of an isoform) of the *GREB1* gene was also identified (10). However, the chromatin looping between the enhancer at −35-kb ERE and the major promoter at −1.6 kb has not been defined. The role and molecular mechanisms of NCOA6 in estrogen/ERα-regulated *GREB1* transcription are also unknown.

In this study, we used *GREB1* as a model of ERα target genes in endometrial cancer cells to understand how NCOA6 regulates estrogen/ERα-activated gene transcription by enhancing chromatin looping, facilitating ERα, p300, and pol II recruitments, modifying histone acetylation and methylation, and promoting enhancer–promoter contact. Our findings also suggest that the NCOA6-dependent *GREB1* expression may help to maintain the chemotherapy sensitivity of endometrial cancer cells.

Results

NCOA6 is required for baseline and estrogen-induced GREB1 expression

GREB1 is a well-established target gene of estrogen-activated ERα (35). To study the role of NCOA6 in the expression of genes such as *GREB1* regulated by E2-activated ERα, we generated two *NCOA6* knockout (KO) clones (N6-KO1 and N6-KO2) from Ishikawa cells derived from a human endometrial carcinoma (36) by using the CRISPR/Cas9 gene-editing system to create InDels in exon 6 (37). Ishikawa cells contain three *NCOA6* alleles, and all three alleles were disrupted by frameshifting mutations in both KO clones as confirmed by sequencing analysis (Fig. 1*A*). We also examined the DNA sequences of five predicted potentially-exotic off-targeting sites, including chr19:−1952860, chr13:+50129778, chr14:+5247272, chr4:+119239571, and chr7:+92238268 in both KO cell lines, and we found no mutations at these sites (data not shown).

As expected, NCOA6 protein was present in parental Ishikawa control (P-Ctrl) cells and empty vector-transfected Ishikawa control (V-Ctrl) cells but absent in N6-KO1 and N6-KO2 cells cultured in medium with full serum (Fig. 1*B*). In the estrogen-free medium with charcoal-stripped serum, vehicle-treated P-Ctrl and V-Ctrl cells expressed *GREB1* mRNA at a basal level, and this basal level was reduced by 50% in N6-KO1 and N6-KO2 cells, indicating that NCOA6 is required for maintaining basal level expression of GREB1 in the absence of estrogen in Ishikawa cells (Fig. 1*C*). After E2 treatment for 24 h, *GREB1* mRNA expression was robustly induced in P-Ctrl and V-Ctrl cells. However, *GREB1* mRNA expression failed to respond to E2 treatment in both N6-KO1 and N6-KO2 cells (Fig. 1*C*). In the absence of E2 treatment, we detected similar basal levels of GREB1 protein in N6-KO1 and N6-KO2 cells *versus* P-Ctrl and V-Ctrl cells, which were not proportional to their mRNA expression ratios and might be attributed to vari-

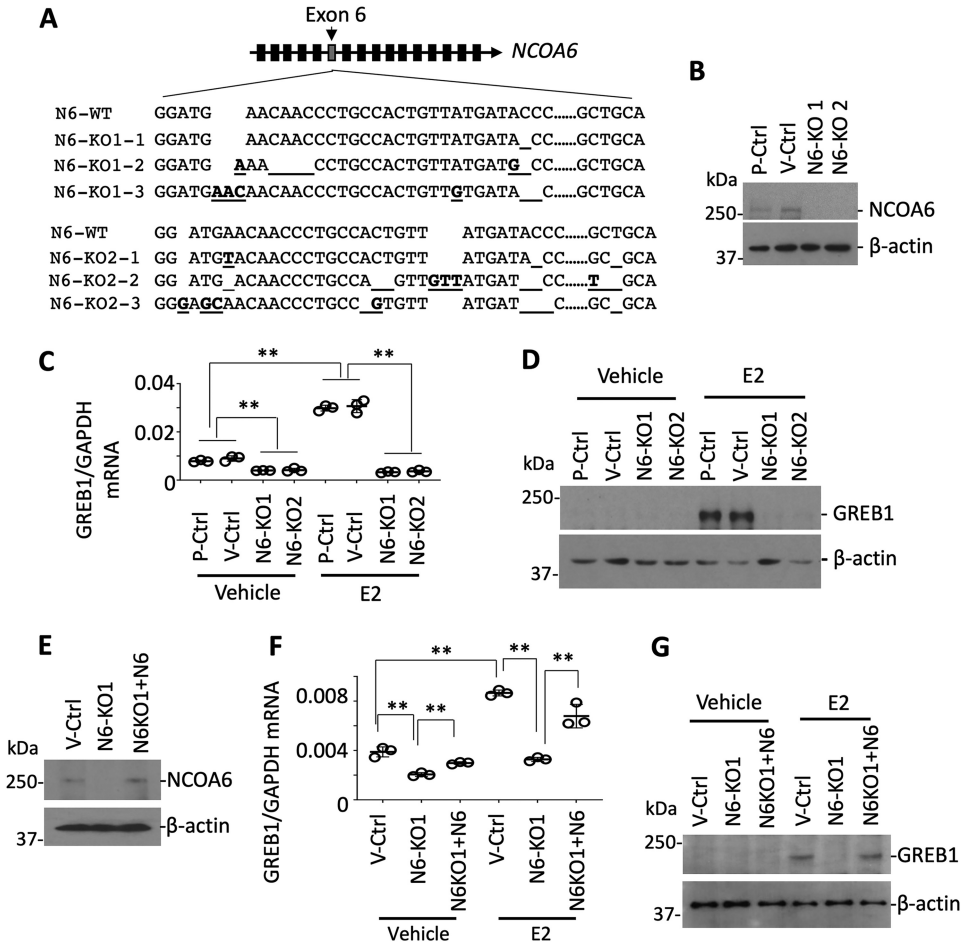

Figure 1. NCOA6 is required for baseline and E2-induced GREB1 expression. *A,* InDels identified in the 6th exon of the three *NCOA6* alleles caused by CRISPR/Cas9-mediated double-strand break and nonhomologous end-joining DNA repair in N6-KO1/2 Ishikawa cell lines. All of these InDels disrupted NCOA6 protein by shifting the amino acid reading frame. *B,* Western blot analysis confirmed the absence of NCOA6 protein in N6-KO1/2 cell lines. β-Actin was assayed as a loading control. *C,* RT-qPCR measurement of the *GREB1* mRNA expression levels in parent control (*P-Ctrl*), vector control (*V-Ctrl*), and N6-KO1/2 cell lines treated with vehicle (ethanol) or E2. GAPDH was used as an internal control. **, $p < 0.01$. *D,* Western blot analysis of the GREB1 protein in vehicle or E2-treated parent control, vector control, and N6-KO1/2 cells. Cells were cultured in phenol red-free medium for 72 h and then treated with vehicle (ethanol) or 1 nM E2 for 24 h. *E,* Western blot analysis of NCOA6 in vector control, N6-KO1, and N6KO1+N6 cells. N6KO1+N6 cells were derived from N6-KO1 cells by stable expression of NCOA6 from transfection of the pCDNA3β-NCOA6 vector with a G418-resistant marker. *F* and *G,* qPCR and Western blotting measurements of GREB1 mRNA (*F*) and protein (*G*) in vector control, N6-KO1, and N6KO1+N6 cells treated with vehicle or E2 for 24 h. The relative expression levels of *GREB1* mRNA were normalized to *GAPDH* mRNA levels. **, $p < 0.01$. β-Actin was used as a loading control.

able protein degradation rates in NCOA6 WT and KO cells. Upon E2 treatment, GREB1 protein drastically increased in P-Ctrl and V-Ctrl cells but showed no obvious increase in N6-KO1 and N6-KO2 cells (Fig. 1*D*). To further validate the essential role of NCOA6 in GREB1 expression, we restored NCOA6 expression with an NCOA6-expressing plasmid in N6-KO1 cells, which we designated as N6KO1+N6 cells (Fig. 1*E*). We found that restored NCOA6 expression rescued both GREB1 mRNA and protein expression in these cells to levels similar to that in V-Ctrl cells with WT NCOA6 either in the absence or presence of E2 treatment (Fig. 1, *F* and *G*). In addition, we also knocked out *NCOA6* in RL95-2 cells, which is another ERα-positive endometrial carcinoma cell line. Again, knockout of *NCOA6* abolished E2-induced GREB1 mRNA and protein expression in RL95-2 cells (Fig. S1). These results indicate that NCOA6 is absolutely required for E2-induced GREB1 expression in human endometrial carcinoma cells.

NCOA6 is associated with both the promoter and enhancer of the GREB1 gene in an estrogen-independent manner and NCOA6 preoccupancy is required for efficient recruitment of ERα

Previous studies reported four estrogen-response elements (EREs) for binding ERα at −35-, −21-, −9.5-, and −1.6-kb regions from the TSS of the *GREB1* gene in MCF-7 cells (10, 33, 34). In Ishikawa cells, there were only three ERα-binding peaks at −35.4, −1.6, and +6 kb of the *GREB1* gene identified by ChIP-Seq assays (Fig. 2*A*) (38). Our ChIP assays could only confirm a strong ERα binding at the −35.4-kb enhancer and a weaker ERα binding at the −1.6-kb promoter region of the *GREB1* gene (39) in an E2-dependent manner. Interestingly, the ERα binding was reduced by 70% at the enhancer and to the background levels at the promoter in N6-KO1 cells (Fig. 2*B*). Because we have validated the regulatory relationship between NCOA6 and GREB1 expression in N6-KO1 cells, we chose to do most experiments with this cell line for saving resources.

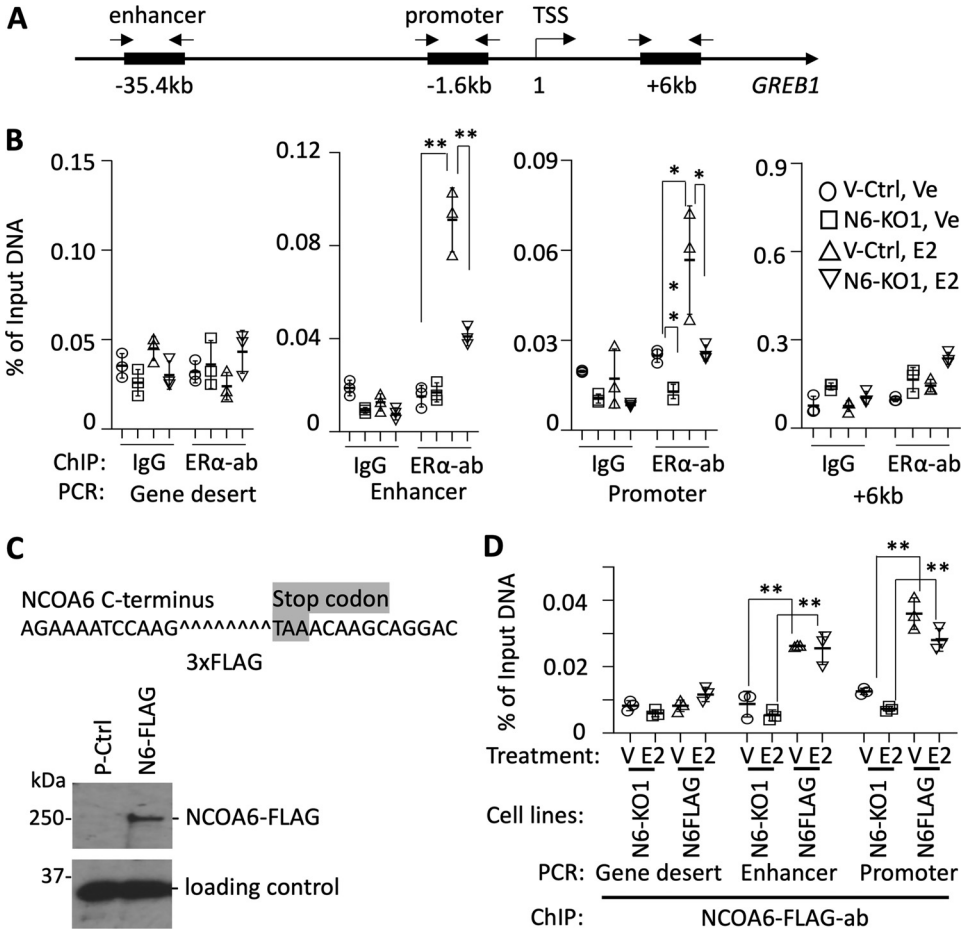

Figure 2. NCOA6 is associated with both *GREB1* promoter and enhancer in an estrogen-independent manner, and NCOA6 preoccupancy is required for efficient recruitment of ERα. *A,* locations of the *GREB1* enhancer, promoter, TSS, the three putative ERα-binding sites at −35.4, −1.6, and +6 kb, and the three primer pairs (*arrows*) used for ChIP assays. *B,* ChIP assays for ERα recruitment to the GREB1 enhancer, promoter, and +6-kb site shown in *A* in V-Ctrl and N6-KO1 cells treated with vehicle (*Ve*) or E2 (10 nM) for 45 min as indicated. A gene desert region and IgG were used as negative controls. *, $p < 0.05$; **, $p < 0.01$. *C,* strategy for knocking in the 3×FLAG tag sequence into the C-terminal end of NCOA6 by the CRISPR/Cas9/single-strand DNA donor gene-editing system, and Western blot analysis of NCOA6-FLAG protein in N6-FLAG cells. Parent control (*P-Ctrl*) cells served as a control. A nonspecific band was used as a loading control. *D,* ChIP assays for NCOA6 recruitment to the *GREB1* enhancer and promoter in NCOA6-FLAG cells treated with vehicle (*V*) or E2 (10 nM) for 45 min. N6-KO1 cells and a gene desert region served as negative controls. **, $p < 0.01$.

These results indicate that NCOA6 facilitates ERα recruitment to its binding sites of the *GREB1* gene.

Because FLAG-tagged protein offers many benefits for ChIP assay such as high specificity, optimized protocol, and bypass of ChIP grade antibody for specific proteins, we knocked-in a 3×FLAG-coding sequence to the C-terminal end of the endogenous NCOA6 protein in Ishikawa cells, designated as N6-FLAG cells (Fig. 2*C*). ChIP assays using FLAG antibody revealed that NCOA6-FLAG is associated with both the enhancer and the promoter of the *GREB1* gene in either absence or presence of E2 treatment (Fig. 2*D*). These results indicate that NCOA6 is recruited to the *GREB1* enhancer and promoter in an ERα-independent manner, because the recruitment of ERα to the *GREB1* enhancer and promoter depends on E2 treatment.

NCOA6 is essential for programming an active configuration of the GREB1 enhancer and promoter

Next, we assessed the functional impact of NCOA6 on the activities of the *GREB1* enhancer and promoter. In the absence of E2, KO of *NCOA6* decreased the basal levels of both enhancer RNA (eRNA) and the RNA transcripts of the promoter (pRNA). In the presence of E2, both eRNA and pRNA were robustly increased in V-Ctrl Ishikawa cells with NCOA6, but the levels of both eRNA and pRNA expression showed no changes in N6-KO1 cells (Fig. 3*A*). In agreement with the changes of eRNA and pRNA, we also found that E2 treatment significantly increased RNA pol II recruitment to both the enhancer and promoter regions in V-Ctrl Ishikawa cells, but its recruitment was abolished in N6-KO1 cells (Fig. 3*B*). These results indicate that *NCOA6* KO compromised the activities of the *GREB1* enhancer and promoter.

To understand why NCOA6 is required for the activities of the *GREB1* enhancer and promoter, we examined the levels of H3K4me1, H3K4me3, H3K9ac, and H3K27ac, which are positively correlated with active enhancers and promoters (40–42). In the absence of E2, the levels of H3K4me1, H3K4me3, and H3K9ac at the enhancer showed no significant differences in V-Ctrl and N6-KO1 cells, whereas the level of H3K27ac at the enhancer in N6-KO1 cells was reduced to 25% that in V-Ctrl cells. E2 treatment significantly increased the levels of H3K4me1, H3K4me3, H3K9ac, and H3K27ac at the enhancer

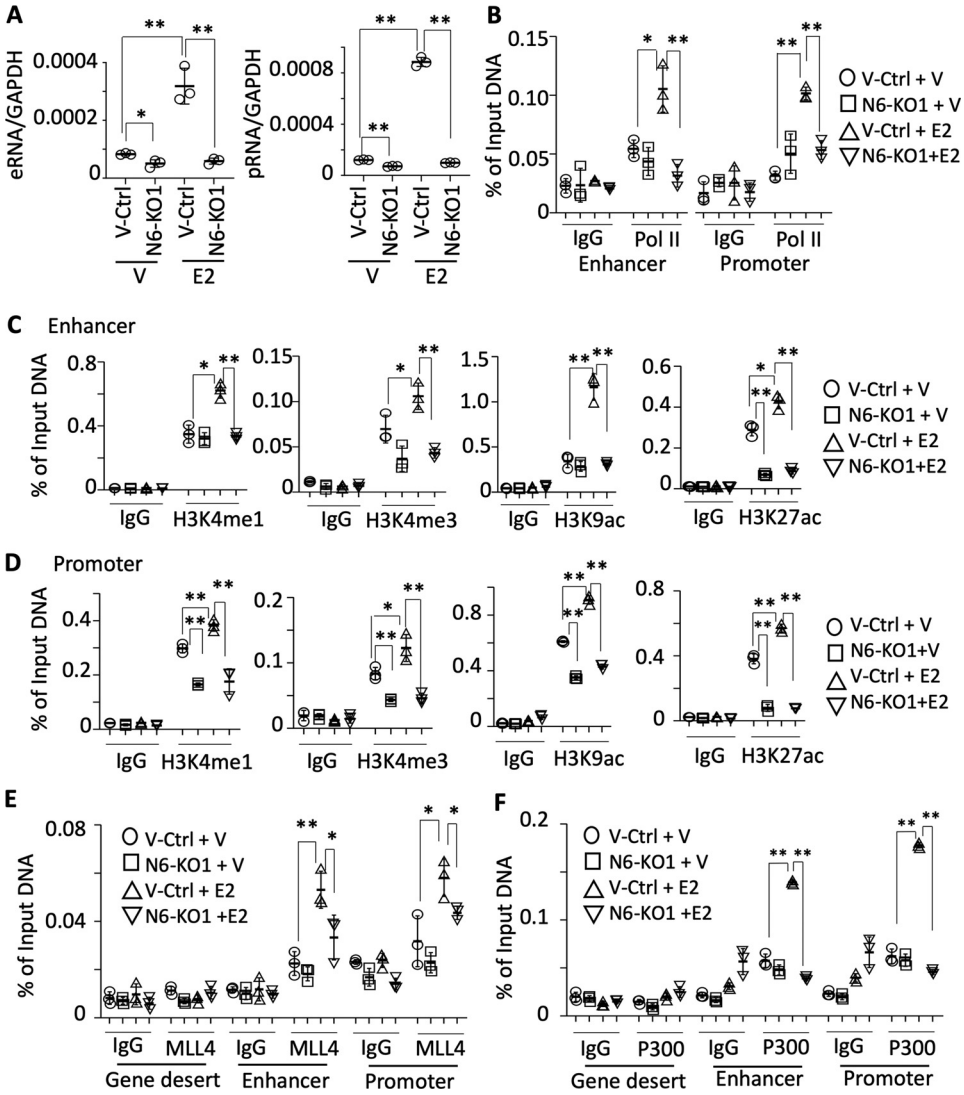

Figure 3. NCOA6 is essential for programming an active configuration of *GREB1* enhancer and promoter. *A,* measurement of eRNA and pRNA by RT-qPCR in V-Ctrl and N6-KO1 cells treated with vehicle or 10 nM E2 for 24 h. Relative eRNA and pRNA expression levels were normalized to GAPDH mRNA. *, $p < 0.05$; **, $p < 0.01$. *B,* ChIP-qPCR assays for measuring pol II recruitment to the GREB1 enhancer and promoter in V-Ctrl and N6-KO1 cells treated with vehicle or E2 (10 nM) for 45-min. IgG was used as a negative control *versus* pol II antibody. *, $p < 0.05$; **, $p < 0.01$. *C* and *D,* ChIP-qPCR assays for measuring the levels of H3K4me1/3, H3K9ac, and H3K27ac at the *GREB1* enhancer (*C*) and promoter (*D*) in V-Ctrl and N6-KO1 cells treated with vehicle (*V*) or E2 (10 nM) for 45 min as indicated. IgG was used as a negative control. *, $p < 0.05$; **, $p < 0.01$. *E* and *F,* ChIP-qPCR assays for measuring MLL4 (*E*) and p300 (*F*) recruitments to the GREB1 enhancer and promoter in V-Ctrl and N6-KO1 cells treated with vehicle (*V*) or E2 (10 nM) for 45 min. A gene desert region and IgG served as negative controls. *, $p < 0.05$; **, $p < 0.01$.

in V-Ctrl cells, but it failed to induce any increases in the levels of these histone codes at the enhancer in N6-KO1 cells (Fig. 3*C*). At the *GREB1* promoter, the levels of all four histone codes were significantly lower in N6-KO1 cells *versus* V-Ctrl cells in the absence of E2. In the presence of E2, all of these histone codes were significantly increased in V-Ctrl cells, but these histone codes were not increased in N6-KO1 cells (Fig. 3*D*). These results demonstrate that NCOA6 is required for maintaining the basal levels of H3K27ac at the enhancer and H3K4me1/3, H3K9ac, and H3K27ac at the promoter in the absence of E2, whereas NCOA6 is essential for the E2-induced increases in all four examined histone codes at the promoter.

Because NCOA6 and ERα interact with MLL4 to methylate H3K4 and p300 to acetylate H3K27, respectively (15, 43–45), we further assayed the effects of NCOA6 KO on MLL4 and p300 recruitments at the *GREB1* enhancer and promoter. Without E2 treatment, almost no MLL4 and p300 were recruited to either the *GREB1* enhancer or the promoter. E2 treatment robustly-induced MLL4 and p300 association with the *GREB1* enhancer and promoter in V-Ctrl cells. However, NCOA6 KO largely diminished E2-induced MLL4 recruitment and completely abolished p300 recruitment to the enhancer and promoter (Fig. 3, *E* and *F*). These results demonstrate that NCOA6 is required for E2-induced MLL4 and p300 recruitments to the *GREB1* enhancer and promoter.

NCOA6 enhances the interaction between the GREB1 enhancer and promoter through increasing chromatin looping in an E2-independent manner

Because NCOA6 is associated with both the enhancer and promoter of *GREB1* and this association is correlated with transcriptionally-active histone codes, we further evaluated the

contribution of NCOA6 to the enhancer and promoter interaction. We performed 3C assays with Ishikawa cells by cross-linking chromatin, digesting with the apoI restriction enzyme, ligating the digested cohesive DNA ends, and detecting the ligated junctions of DNA fragments by PCR and DNA sequencing. By screening a 117.2-kb chromatin region from −39,832 to 77,371 bp of the *GREB1* gene by 40 PCRs using combinatorial primer pairs of the 21 primers, we only detected one chromatin loop between the enhancer and the promoter regions by PCR using primer 2 at the enhancer and primer 11 at the promoter. DNA sequence analysis of this PCR-amplified fragment identified a ligated apoI restriction enzyme site at −35,698 and −538 bp of the *GREB1* gene (Fig. 4*A*). To validate and quantitatively measure the amount of this chromatin looping, we performed a 3C assay with another restriction enzyme, BamHI, followed by qPCR measurement. Again, the chromatin loop was detected between the enhancer and the promoter regions, and the ligated junction was at −33,016 and −8222 bp (Fig. 4*B*). This chromatin loop was detected in either the absence or presence of E2 treatment with a small increase in the presence of E2 treatment in V-Ctrl cells. Interestingly, the amount of this chromatin looping was significantly reduced in N6-KO1 cells *versus* that in Ishikawa V-Ctrl cells either with or without E2 treatment (Fig. 4*C*). We next performed *in vitro* looping assays by mixing a biotin-labeled double-strand DNA (dsDNA) fragment of the *GREB1* enhancer, an unlabeled dsDNA fragment of the *GREB1* promoter, and nuclear extracts of V-Ctrl or N6-KO1 cells. When mixed with the nuclear extracts of V-Ctrl cells with NCOA6 expression, the unlabeled promoter dsDNA was efficiently coprecipitated by the biotin-labeled enhancer dsDNA, and when mixed with the nuclear extracts of N6-KO1 cells without NCOA6 expression, the biotin-labeled enhancer pulled down much less unlabeled promoter dsDNA (Fig. 4*D*). We also obtained consistent results from reciprocal *in vitro* looping assays by mixing a biotin-labeled promoter dsDNA of *GREB1*, an unlabeled dsDNA fragment of *GREB1* enhancer and nuclear extracts of V-Ctrl or N6-KO1 cells (Fig. 4*E*). These results demonstrate that the *GREB1* enhancer loops to the promoter in an E2-independent manner and NCOA6 significantly increases this chromatin looping to promote the interaction of the *GREB1* enhancer and promoter, although NCOA6 is non-essential for initiating this chromatin looping.

Deletion of either the enhancer or the promoter core sequences of the GREB1 gene decreases chromatin looping

The exact looping sites between the *GREB1* enhancer and promoter are currently unknown. To examine whether the enhancer and promoter sequences are required for the looping, we deleted the enhancer region from −35,588 to −34,959 bp or the promoter region from −1908 to −1221 bp where both NCOA6 and ERα bind to in Ishikawa cells by co-expressing Cas9 with two gRNAs flanking the enhancer or the promoter region. PCR analysis confirmed the homozygous deletion of the enhancer or the promoter sequences in Ishikawa cells (Fig. 5*A*). Our 3C–qPCR assays revealed that deletion of either the enhancer or the promoter reduced chromatin looping in an E2-independent manner (Fig. 5*B*). These results suggest that the backbone of the chromatin looping is formed outside of the enhancer and promoter core sequences in an E2-independent manner and that the interaction between the enhancer and the promoter facilitates this chromatin looping.

We also performed ChIP assays to assess the relationships among the enhancer- and the promoter-bound NCOA6 and ERα. Surprisingly, deletion of the promoter reduced the enhancer-associated NCOA6 by 65% (Fig. 5*C*), whereas deletion of the enhancer only reduced the promoter-associated NCOA6 by 20% (Fig. 5*D*). In contrast, deletion of the promoter only slightly reduced the E2-induced ERα binding to the enhancer (Fig. 5*E*), whereas deletion of the enhancer almost completely diminished the E2-induced ERα binding to the promoter (Fig. 5*F*). These results suggest that NCOA6 and E2/ERα are primarily associated with the promoter and the enhancer, respectively, and that the majority of the enhancer-associated NCOA6 and the promoter-associated E2/ERα detected by ChIP assays may be due to a spatial interaction between the enhancer and the promoter.

Both the ERα-bound enhancer and the NCOA6-associated promoter are required for transcriptional activation of the GREB1 gene

Deletion of the ERα-bound enhancer decreased the basal levels of H3K4me3 and H3K9ac as well as the E2-induced increases in H3K4me1, H3K4me3, H3K9ac, and H3K27ac at the promoter region in Ishikawa cells (Fig. 6*A*). Deletion of the NCOA6-associated promoter almost completely abolished the E2-induced increases in H3K4me1, H3K4me3, H3K9ac, and H3K27ac at the enhancer (Fig. 6*B*). Without the enhancer region, pRNAs were not synthesized in the absence or presence of E2. The pol II association with the promoter was reduced in the absence of E2, and E2 treatment failed to induce pol II recruitment to the promoter. Without the promoter region, eRNAs were not produced, and E2-induced pol II recruitment to the enhancer was also abolished (Fig. 6, *C* and *D*). Importantly, either deletion of the enhancer or the promoter completely silenced the E2-stimulated expression of the GREB1 mRNA and protein (Fig. 6, *E* and *F*). These results demonstrate that both the ERα-bound enhancer and the NCOA6-associated promoter are required for programming transcriptionally-active histone codes, recruiting pol II, and synthesizing eRNAs and pRNAs at both the enhancer and the promoter regions, which results in transcriptional activation of the *GREB1* gene.

Cells with GREB1 down-regulation caused by NCOA6 KO, GREB1 enhancer deletion, or GREB1 promoter deletion are resistant to chemotherapy drugs

In agreement with previous studies reporting a role of GREB1 in promoting cell proliferation (46, 47), Ishikawa cells with low *GREB1* expression caused by deletion of its enhancer or promoter showed slower growth than control Ishikawa cells. However, N6-KO1 cells that express low GREB1 exhibited a similar growth rate as that of control Ishikawa cells (Fig. 7*A*), which could be related to a counterbalanced regulation of cell growth by *GREB1*, one of the NCOA6-regulated genes, and other NCOA6-regulated genes that have a cell growth–promoting function. To examine the role of GREB1 in the responses of endometrial carcinoma cells to chemotherapy

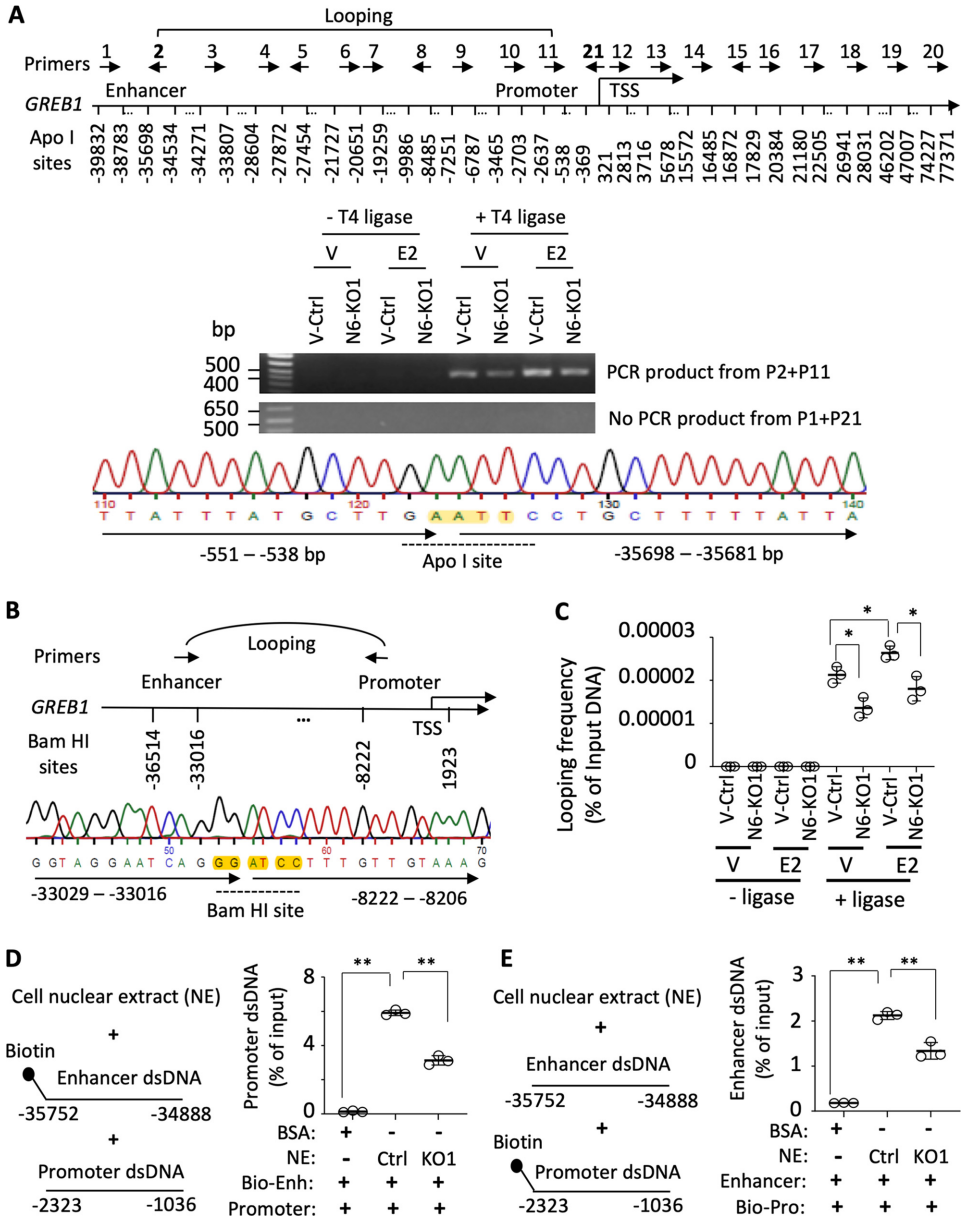

Figure 4. NCOA6 enhances chromatin looping between the enhancer and promoter of the *GREB1* gene in an E2-independent manner. *A,* screening of the chromatin loops along the *GREB1* gene by 3C-PCR. V-Ctrl and N6-KO1 cells were cultured in estrogen-free medium for 72 h and then treated with vehicle (*V*) or 10 nM E2 for 45 min. Cross-linked chromatin was digested with apoI and ligated by T4 ligase. Samples without addition of T4 ligase served as negative controls. The locations of apoI-cutting sites and the primers (*arrows*) used for PCR are indicated. PCRs were performed by pairing primer 2 with each of the other primers and by pairing primer 21 with each of the other primers. Among these PCRs, only primer 2 paired with primer 11 detected a chromatin loop (*upper gel image*). All other PCRs did not detect any chromatin loop (*lower gel image* and data not shown). The sequence of PCR product detected by primers 2 and 11 confirmed a chromatin loop digested by apoI and then ligated by T4 ligase. *B,* confirmation of the chromatin looping between the *GREB1* enhancer and promoter by 3C-PCR. Ishikawa cells were treated with 10 nM E2 for 45 min. Cross-linked and extracted chromatin was digested by BamHI and ligated with T4 ligase. Samples without addition of T4 ligase were used as a negative control. The locations of PCR primers and BamHI-cutting sites are indicated. A chromatin loop between the enhancer and promoter was detected by PCR in a ligation-dependent manner. Sequence analysis of the PCR product confirmed a ligated junction between BamHI sites at −33,016 and −8222 bp of the *GREB1* gene. *C,* qPCR measurement of chromatin loops formed between the GREB1 enhancer and promoter in V-Ctrl and N6-KO1 cells treated with vehicle (*V*) or 10 nM E2 for 45 min. Chromatin was digested with BamHI and ligated with T4 ligase as described in *B*. *, $p < 0.05$; **, $p < 0.01$. *D* and *E, in vitro* assays to detect chromatin-looping formation between the *GREB1* enhancer and promoter. Biotin-labeled enhancer and unlabeled promoter dsDNAs (*D*) or biotin-labeled promoter and unlabeled enhancer dsDNAs (*E*) were mixed with BSA, V-Ctrl (*Ctrl*) cell nuclear protein extracts, or N6-KO1 (*KO1*) cell nuclear protein extracts at 4 °C for 4 h. DNA–protein complex was co-precipitated by pulling down bio-enhancer or bio-promoter dsDNA using streptavidin beads. Co-precipitated promoter DNA (*D*) or enhancer DNA (*E*) were measured by qPCR and normalized to each unlabeled DNA input. *, $p < 0.05$; **, $p < 0.01$.

drugs, we treated V-Ctrl Ishikawa, N6-KO1, enhancer-deleted Ishikawa, and promoter-deleted Ishikawa cells with different concentrations of paclitaxel and docetaxel, the two commonly used chemotherapy drugs for treating cancer. The survival rates of N6-KO1, enhancer-deleted Ishikawa, and promoter-deleted Ishikawa cells were significantly higher than that of V-Ctrl Ishikawa cells under either drug treatment (Fig. 7*B*). The degrees of paclitaxel-induced cell apoptosis were also much less severe in N6-KO1, enhancer-deleted Ishikawa, and promoter-deleted Ishikawa cells *versus* control Ishikawa cells as detected

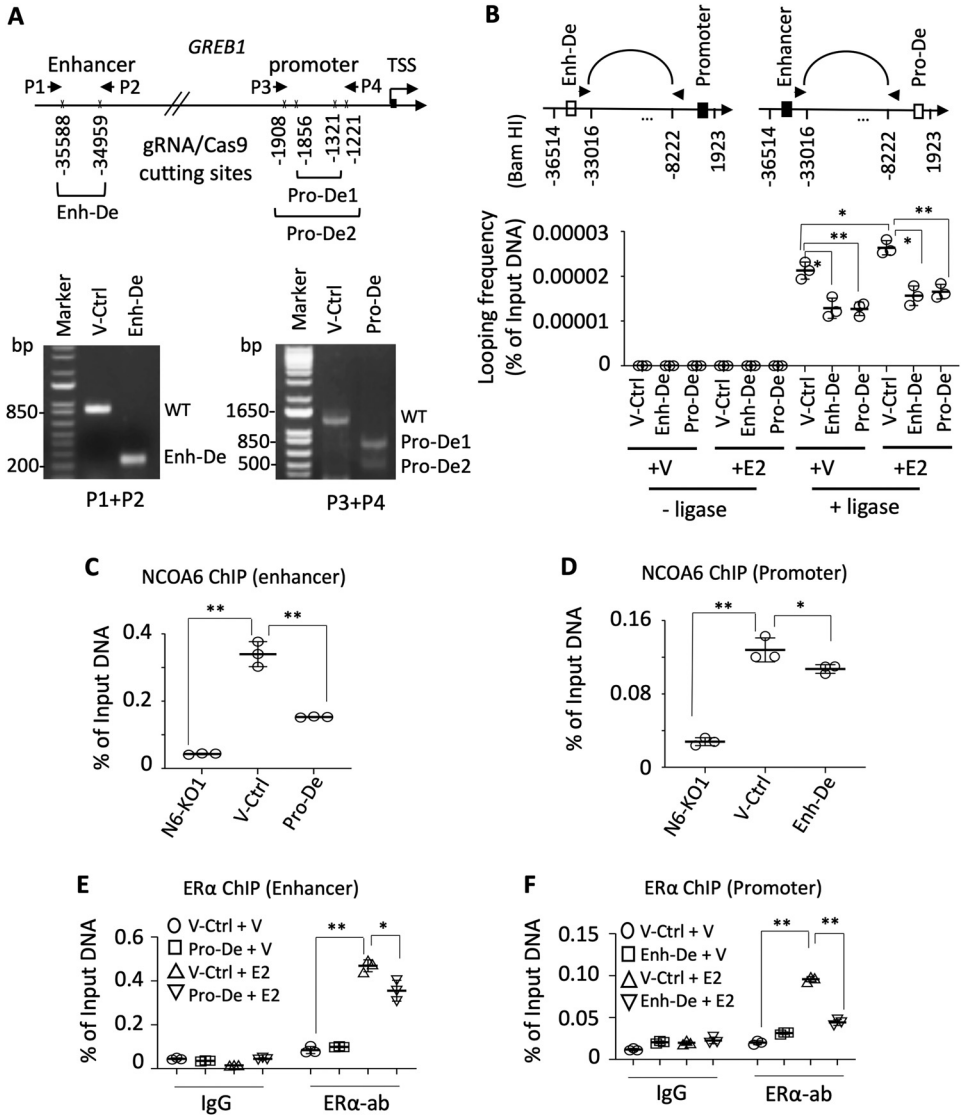

Figure 5. Deletion of the *GREB1* enhancer or the promoter core sequences influences NCOA6 and ERα recruitments and decreases chromatin looping. *A,* generation of enhancer core sequence-deleted (*Enh-De*) and promoter core sequence-deleted (*Pro-De*) Ishikawa cell lines by using the CRISPR/Cas9 gene editing system. *Arrows* indicate the locations of PCR primers. In Enh-De cells, both copies of the *GREB1* enhancer were deleted by using a pair of gRNAs targeting −35,588 and −34,959 sites in one experiment. In Pro-De cells, the first copy and the second copy of the *GREB1* promoter were deleted by using a pair of gRNAs targeting −1856 and −1321 sites and a pair of gRNAs targeting −1980 and −1221 sites in two serial experiments, respectively. The Enh-De and Pro-De cells were identified by PCR using primers P1/P2 and P3/P4, respectively. *B,* 3C– qPCR measurement of the chromatin loop in V-Ctrl, Enh-De, and Pro-De cells treated with vehicle (*V*) or 10 nM E2 for 45 min. *, $p < 0.05$; **, $p < 0.01$. *C* and *D,* ChIP-qPCR analysis of NCOA6 recruitments to the *GREB1* enhancer or promoter in N6-KO1, V-Ctrl, Pro-De, and/or Enh-De cells. ChIP assays were carried out with NCOA6 antibody and N6-KO1 cells served as a negative control. *, $p < 0.05$; **, $p < 0.01$. *E* and *F,* ChIP-qPCR assays of ERα recruitments to the GREB1 enhancer or promoter in V-Ctrl, Pro-De, and/or Enh-De cells treated with vehicle (*V*) or 10 nM E2 for 45 min. *ERα-ab*, ERα antibody. *, $p < 0.05$; **, $p < 0.01$.

by staining the annexin V and assaying the cleaved caspase 3 (Fig. 7, *C* and *D*). In agreement with this finding, bio-computational analysis of the TCGA data sets (48) revealed that endometrial carcinoma patients with low *GREB1* mRNA expression were associated with shorter survival time when compared with endometrial carcinoma patients with high GREB1 expression (Fig. 7*E*). These results indicate that although GREB1 is required for fast growth of endometrial cancer cells, its loss actually defines a worse grade of cancer cells resistant to chemotherapy and poor clinical outcome.

Discussion

NRs for steroid hormones, thyroid hormones, vitamin D, and retinoic acid are ligand-inducible transcription factors involved in the regulation of numerous biological and pathological processes. After a long history of pursuing the mechanisms for hormonal action and NR signaling, it is now known that DNA-associated NRs recruit a variety of co-activators and/or co-repressors to regulate gene expression. These co-regulators mediate NR transcriptional activities mainly by modulation of the enhancer–promoter contact and assembly of transcriptional initiation and/or elongation machineries through reprogramming the epigenetic configurations of DNA and histones, changing chromatin topology, and/or re-organizing chromatin loops. Because each gene has different sequence-specific association of different transcription factors and their interactive proteins and each cell type may express different co-regulators, a NR may regulate the same target gene differently in different

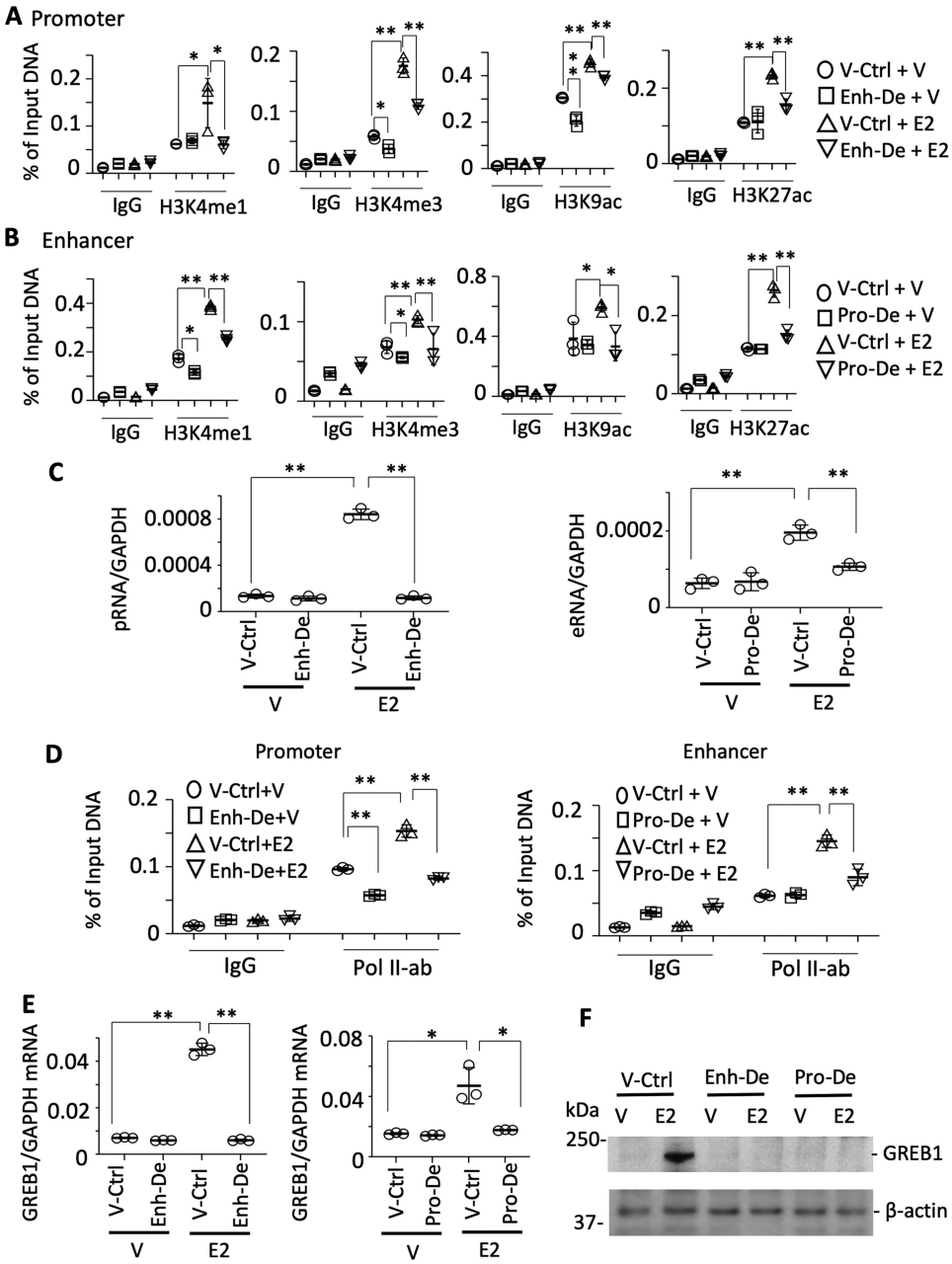

Figure 6. Both the ERα-bound enhancer and the NCOA6-associated promoter are required for transcriptional activation of *GREB1*. *A,* ChIP-qPCR assays performed with antibodies against H3K3me1/3, H3K9ac, and H3K27ac and primers and the *GREB1* promoter-specific PCR primers and TaqMan probe in V-Ctrl and Enh-De cells treated with vehicle (*V*) or E2 for 45 min. The same IgG control was used in all four parts of panel *A* with data from experiments performed at the same time. *, $p < 0.05$; **, $p < 0.01$. *B,* ChIP-qPCR assays performed with antibodies against H3K3me1/3, H3K9ac, and H3K27ac and the GREB1 enhancer-specific PCR primers and TaqMan probe in V-Ctrl and Pro-De cells treated with vehicle (*V*) or E2 for 45 min. The same IgG control was used in all four parts of panel *B* with data from experiments performed at the same time. *, $p < 0.05$; **, $p < 0.01$. *C,* qPCR measurement of the *GREB1* pRNA and eRNA in V-Ctrl, Enh-De, and Pro-De cells treated with vehicle (*V*) or E2. The data were normalized with GAPDH mRNA. **, $p < 0.01$. *D*, ChIP-qPCR measurement of pol II recruitments to the *GREB1* promoter and enhancer in V-Ctrl, Enh-De, and Pro-De cells treated with vehicle (*V*) or E2. **, $p < 0.01$. *E* and *F,* RT-qPCR (*E*) and Western blotting (*F*) analyses of GREB1 expression in V-Ctrl, Enh-De, and Pro-De cells treated with vehicle (*V*) or E2. The relative GREB1 mRNA expression levels were normalized to GAPDH mRNA expression levels. β-Actin was used as a loading control.

types of cells or regulate different target genes differently in the same cell type. Given these complexities for NR-regulated gene expression, we still have a long way to go to understand the exact molecular mechanisms responsible for NR/co-regulator-mediated gene transcription.

Previous studies showed that NRs interact with NCOA6, and NCOA6 was recruited to the chromatin by NRs (12, 14, 17, 18). In this study, we found that NCOA6 is associated with the *GREB1* promoter and enhancer in an E2/ERα-independent manner. Deletion of the enhancer does not affect NCOA6 recruitment at the promoter, but deletion of the promoter abolished NCOA6 recruitment at the enhancer. However, ERα is associated with *GREB1* enhancer and promoter in an E2-dependent manner, and NCOA6 also enhances ERα recruitment to both enhancer and promoter in an E2-dependent manner. Deletion of the promoter only slightly decreases ERα at the

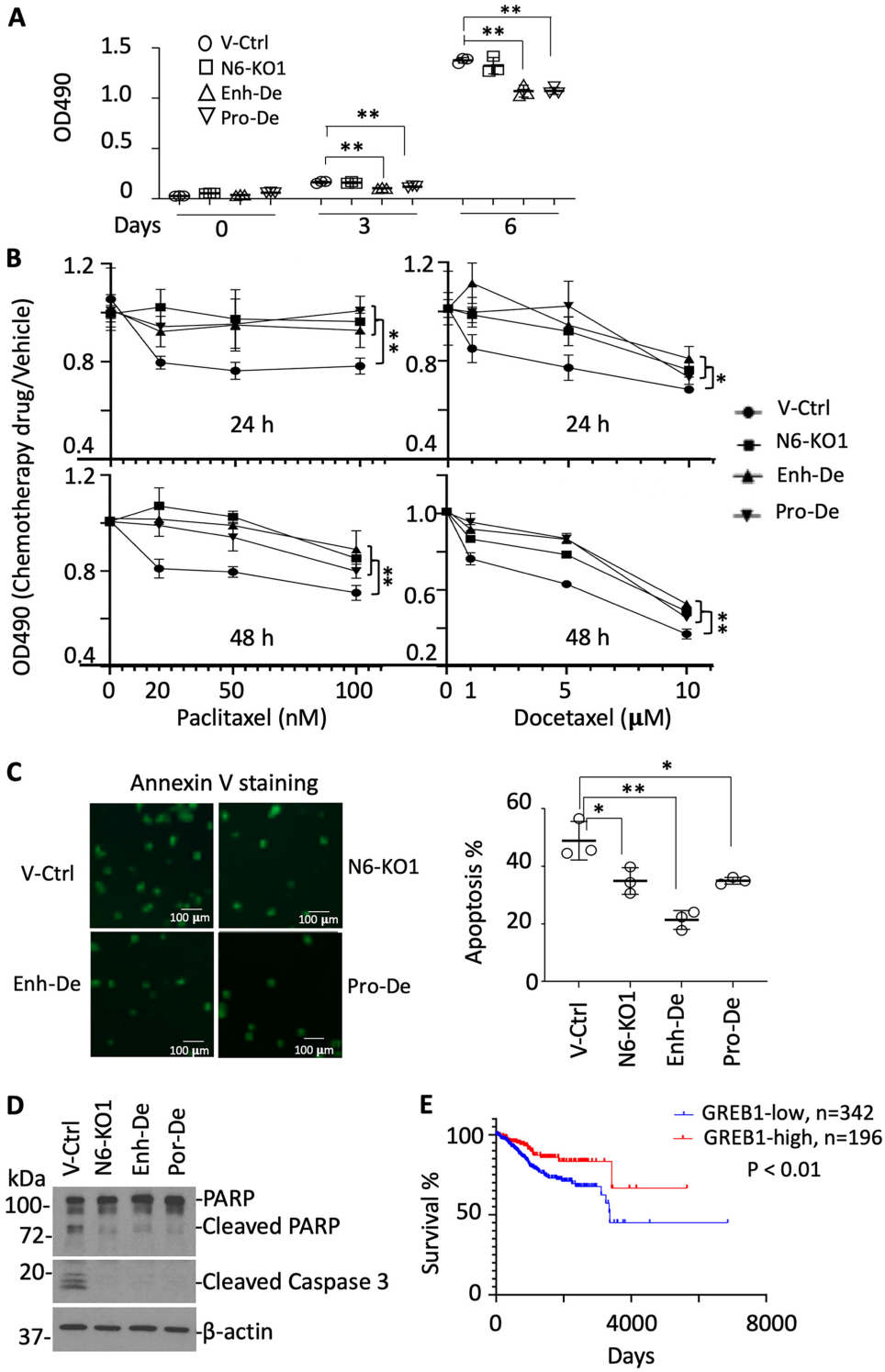

Figure 7. GREB1 loss causes chemotherapy resistance. *A,* cell growth assays. V-Ctrl, N6-KO1, Enh-De, and Pro-De cells were cultured in estrogen-free medium and treated with E2 in 96-well plates for the days indicated before cell viability was measured by MTS assay. **, $p < 0.01$. *B,* effects of paclitaxel or docetaxel on the viability of V-Ctrl, N6-KO1, Enh-De, and Pro-De cells. 2000 of the indicated cells were cultured in each well of the 96-well plate and treated with different doses of paclitaxel or docetaxel for 24 or 48 h. Cell viability was measured by MTS assay. *, $p < 0.05$; **, $p < 0.01$. *C,* annexin V staining to detect apoptotic cells. V-Ctrl, N6-KO1, Enh-De, and Pro-De cells were treated with 50 nM paclitaxel for 24 h. Cell apoptosis was measured by annexin V staining. Apoptotic cell percentages were determined by counting annexin V–positive cell number *versus* total cell number. *, $p < 0.05$; **, $p < 0.01$. *D,* Western blot analysis of cleaved PARP and cleaved caspase 3 for assessing apoptosis. The indicated cells were treated with 50 nM paclitaxel for 24 h before being assayed by Western blotting. β-Actin was used as a loading control. *E,* higher *GREB1* mRNA expression in endometrial cancer is associated with a better overall survival. TCGA UCEC patient's data were downloaded from OncoLnc and grouped according to the mean expression level of *GREB1*. Overall survival was statistically analyzed by log rank test.

enhancer, whereas deletion of the enhancer nearly abolished ERα recruitment to the promoter. These findings suggest that NCOA6 is primarily associated with the promoter, and E2/ERα primarily binds to the enhancer. The NCOA6 detected at the enhancer in the absence and presence of E2 and the ERα detected at the promoter in the presence of E2 may have

resulted from the enhancer–promoter contact in the absence and presence of E2. Importantly, it is the NCOA6 pre-occupied at the promoter and enhancer in the absence of E2 that aids in ERα recruitment to the enhancer and promoter upon E2 treatment.

It is generally considered that multiple co-activators such as the SRC family members support NR transcriptional function in an additive manner, so that one co-activator loss only causes partial decrease in NR-mediated gene expression (49). In this study, we found that knockout of NCOA6 in Ishikawa cells completely abolished E2/ERα-dependent *GREB1* expression. This finding demonstrates that NCOA6 is an essential co-activator for ERα-mediated *GREB1* transcription in this cell context, indicating that a single co-activator can be a determinant of NR transcriptional activity. This causal role of NCOA6 is consistent with our observations showing that knockout of NCOA6 diminished E2-induced MLL4 and p300 recruitments to the *GREB1* enhancer and promoter, the MLL4 and p300-mediated histone codes for transcriptional activation, including H3K4me1/3, H3K9ac, and H3K27ac, and the pol II recruitment to the enhancer and promoter. Because NCOA6 knockout only partially reduces E2-induced ERα recruitment to the enhancer but completely inhibits p300 recruitment to the enhancer and promoter, we conclude that the interaction of NCOA6 with E2/ERα is required for p300 recruitment, and it is this interaction brings p300 to both enhancer and promoter for increasing the H3K9ac and H3K27ac, resulting in pol II recruitment to the enhancer and promoter and the synthesis of eRNA and pRNA for transcriptional activation.

There is a chromatin looping backbone linked between a site near the enhancer and a site near the promoter, which was detected after the chromatin was cut by apoI or BamHI. This looping frame is independent of NCOA6, E2/ERα, and the core sequences of the enhancer and promoter since it is still present under the NCOA6 knockout, estrogen-free, enhancer-deleted, and promoter-deleted conditions, suggesting that this looping backbone may be just an infrastructure facilitating enhancer–promoter interaction and itself is incapable of activating *GREB1* transcription. In contrast, this looping can be enhanced by NCOA6 in an E2/ERα-independent manner and by ERα in an E2-dependent manner, suggesting that E2/ERα/NCOA6-promoted *GREB1* transcription is related to their roles in enhancing chromatin looping. We speculate that this chromatin looping is enhanced by the enhancer–promoter interaction that is pre-existing in the absence of NCOA6 and E2 and further enhanced by NCOA6 and E2/ERα recruitment, because the enhancer and the promoter DNAs are co-precipitated when mixed with nuclear proteins from NCOA6 knockout cells without E2 treatment and the co-precipitation efficiency was further increased when mixed with nuclear proteins from control cells with NCOA6 expression. The protein–protein interaction between the enhancer-associated E2/ERα and the promoter-associated NCOA6 may also play a role in enhancing enhancer–promoter interaction. A previous study has shown that NCOA6 interacts with MED1, and therefore, NCOA6 might associate with the promoter through the mediator complex near the promoter (50). In addition, a recent study demonstrated that JMJD6 bridges the interaction between the E2-bound ERα at the enhancer and the MED12 in the mediator complex near the promoter to facilitate enhancer–promoter interaction for transcriptional activation of E2/ERα target genes (51). Another study reported that the E2-induced ERα/SRC-3 complex can also enhance the enhancer–promoter interaction via reorganizing the configuration of chromatin looping (10). Although the enhancer–promoter contact enhanced by these protein interactions can work together to regulate the levels of *GREB1* transcription, this enhanced enhancer–promoter interaction may not be the determinant for turning on and off the *GREB1* transcription because depletion of JMJD6 or SRC-3 only partially decreases *GREB1* expression (10, 51). As aforementioned, NCOA6-mediated p300 recruitment may play a major role for E2/ERα-activated *GREB1* transcription.

Taken together, the model for E2-induced transcriptional activation of *GREB1* can be postulated in Fig. 8. In the absence of E2, the enhancer and promoter have a loose contact maintained by a chromatin loop and the components of the mediator complex. NCOA6 may be recruited to the enhancer and promoter through interacting with MED1. This configuration maintains the baseline expression of *GREB1* (Fig. 8*A*). In the presence of E2, E2-bound ERα binds to the enhancer, which recruits SRC-3 and JMJD6 to the enhancer and also interacts with the pre-existing NCOA6. In turn, SRC-3 and NCOA6 recruit p300 and/or MLL4 to the co-activator complex for re-programming histone codes, whereas NCOA6 and JMJD6 also enhance enhancer–promoter contact via interacting with MED1 and MED12, respectively. These serial events result in high frequency of chromatin looping, recruitment of pol II, synthesis of eRNA and pRNA, and robustly increased GREB1 transcription (Fig. 8*B*). In the absence of both E2 and NCOA6, ERα and its co-activator complex is not recruited; the chromatin looping is less active, and the enhancer–promoter contact is loose. Thus, *GREB1* expression at baseline can be further decreased (Fig. 8*C*). In the presence of E2 but the absence of NCOA6, ERα can be still recruited to the enhancer, although at a lesser amount, and SRC-3 and JMJD6 may also be accordingly recruited. However, p300 and MLL4 are missing from the co-activator complex, and the histone codes remain to be transcriptionally inactive. Thus, E2/ERα becomes incapable to induce *GREB1* transcription in the NCOA6 knockout cells (Fig. 8*D*).

The NCOA6 knockout, the enhancer-deleted, or the promoter-deleted Ishikawa cells only express basal level GREB1, and GREB1 expression in these cells does not respond to estrogen. In agreement with the cell growth data from previous studies (32, 46, 47), the cells with deleted enhancer or promoter exhibit a lower proliferation rate when compared with control cells. However, the cells with NCOA6 knockout showed similar cell growth rate as control cells, which could be attributed to the effects of NCOA6 knockout on other NCOA6-regulated genes. More importantly, these low GREB1-expressing Ishikawa endometrial carcinoma cells are significantly more resistant to chemotherapy drug-induced cell apoptosis *versus* control cells with normal GREB1 expression. This notion is consistent with some previous findings. For example, GREB1 expression levels are positively correlated with the sensitivity of endocrine ther-

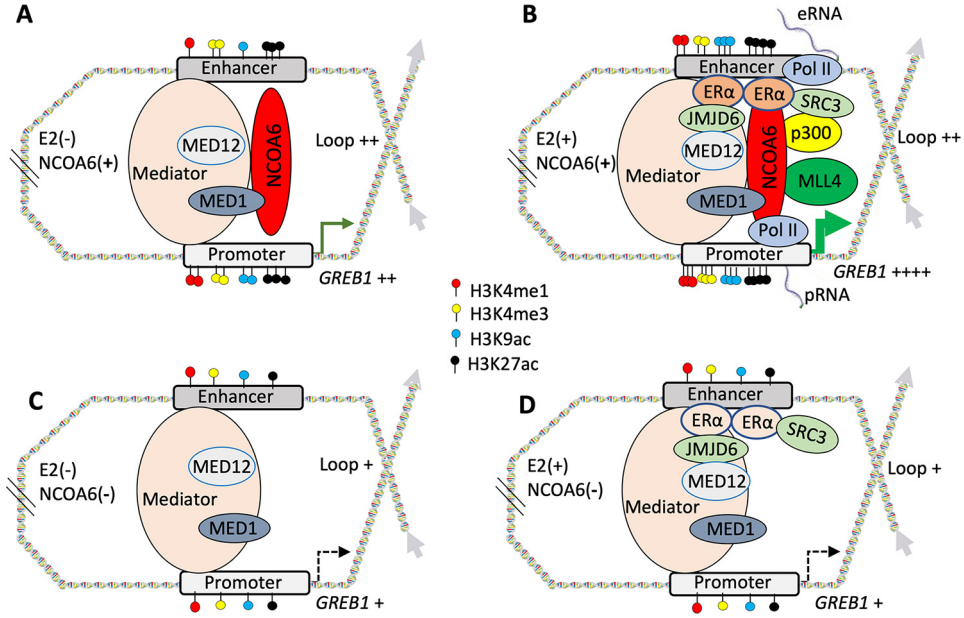

Figure 8. Model for E2-bound ERα-induced gene transcription in NCOA6 WT and knockout Ishikawa cells. *A,* in the absence of E2, the chromatin-looping backbone exists, and NCOA6 is associated with the enhancer and promoter, probably through interacting with MED1 in the mediator complex. *GREB1* is expressed at the baseline level. The levels of individual histone codes are indicated. *B,* in the presence of E2, ERα binds to the enhancer and forms protein complex with NCOA6, SRC-3, and JMJD6. NCOA6 and SRC-3 recruit p300, and NCOA6 also recruits MLL4 to remodeling histone codes. In addition, NCOA6 interacts with MED1, and JMJD6 interacts with MED12 to facilitate enhancer–promoter cross-talk. pol II is recruited to promoter and enhancer, and eRNA and pRNA are synthesized. Chromatin looping is also enhanced. *GREB1* is expressed at a high level. *C,* in the absence of both E2 and NCOA6, the chromatin-looping, enhancer–promoter interaction, the levels of H3K4me1/3, H3K9ac, and H3K27ac, and the baseline expression level of *GREB1* are decreased. *D,* in the presence of E2 but absence of NCOA6, ERα, SRC-3, and JMJD6 might be recruited, but NCOA6, p300, and MLL4 are missing from the co-activator complex. The chromatin looping, histone codes, and *GREB1* expression level remain the same low levels as shown in *C*. In this case, E2/ERα is incapable to activate *GREB1* transcription.

apy in MCF7-derived breast cancer cells (39, 52). Ovarian cancer with hypomethylation of the *GREB1* gene (higher *GREB1* expression) is correlated with good disease-free survival, which was thought to be related to the beneficial role of GREB1 in chemosensitivity because patients with advanced stage ovarian cancer are usually treated with carboplatin and Taxol (53). *GREB1* expression was also found to be down-regulated in ovarian cancer stem cells (54), suggesting that loss of GREB1 expression may promote ovarian cancer cell stemness and increase their resistance to chemotherapy (54, 55). In agreement with this notion, the patients with endometrial cancers expressing low-level GREB1 also exhibit a worse disease-free survival rate *versus* patients with endometrial cancers expressing high-level GREB1. These results suggest that the GREB1 expression level may serve as prognostic marker of endometrial carcinomas.

Materials and methods

Cell culture and E2 treatment

Ishikawa and RL95-2 human endometrial cancer cells were cultured in DMEM/F12 and DMEM, respectively. Either medium contains penicillin-streptomycin and 10% of fetal bovine serum. For estrogen-free culture conditions, cells were cultured in phenol red-free medium with 5% charcoal-stripped serum for 72 h, and then the cells were treated with 10 nM E2 or vehicle (ethanol) for 45 min or with 1 nM E2 or vehicle for 24 h.

Western blotting

Cells were lysed in RIPA buffer containing 50 mM Tris-hydrochloric acid (pH 7.5), 150 mM sodium chloride, 1% sodium deoxycholate, 4 mM EDTA, 1% Nonidet P-40, and proteinase inhibitors. Protein concentration was measured by using Pierce™ BCA protein assay kit (23225, Thermo Fisher Scientific, Waltham, Middlesex, MA). Proteins (20 μg) in each cell lysate were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Membranes were probed with the following primary antibodies: NCOA6 (HPA004198, Sigma), β-actin (A5441, Sigma), GREB1 (ab72999, Abcam, Cambridge, MA), PARP (9542s, Cell Signaling Technology, Danvers, MA), or caspase-3 (96642, Cell Signaling Technology). Membranes were then incubated with a horseradish peroxidase–conjugated secondary antibody (STAR207P/STAR208P, Bio-Rad). Immunoreactivities were visualized by using the reagents producing chemiluminescence (32106, Thermo Fisher Scientific).

Generation of NCOA6 knockout cell lines

To disrupt the exon 6 of the NCOA6 gene in Ishikawa cells, a gRNA was designed by using the method described by Ran et al. (37). The DNA fragment (5′-GCCACTGTTATGATACCCCCGGG) coding the gRNA was cloned into the PX459 vector for expressing both gRNA and Cas9. Ishikawa cells were transfected with PX459 vector or PX459-gRNA vector by using Lipofectamine 3000 (L3000015, Thermo Fisher Scientific) according to the manufacturer's instructions. Transfected cells were transiently growth-selected in the medium containing 2 μg/ml puromycin for 48 h to eliminate the untransfected cells. Survived clones were individually isolated and screened by Western blotting using NCOA6 antibody. Two primers (5′-GGCAACAGAGCGAGACCCTGTCAAA and 5′-TACCTGACTGAGAAGCAGGGCGAGG) flanking the targeted region

in exon 6 were used in PCR to amplify the DNA fragments for sequencing analysis. Potential exotic off-targeting sites were also predicted by using the same method and locus-specific primer pairs (supplementary Table S1) were designed for PCR to amplify DNA regions containing the top five potential off-targeting sites for sequence analysis.

To disrupt the exon 5 of the *NCOA6* gene in RL95-2 cells, another gRNA (5′-GCGGGATTTCCTATGGCAAG) was designed and cloned into PX459 vector for expressing both gRNA and Cas9. A pair of primers (5′-TGCTATTAACCTGG-CTTTGGC and 5′-TATGCCATGAACCCCCACCTA) flanking the targeted region in exon 5 was used in PCR to amplify the DNA fragments for sequencing analysis. Other procedures were the same as described above for Ishikawa cells.

Generation of the NCOA6-FLAG knock-in Ishikawa cell line

The DNA fragment (5′-CGCAGTCCTGCTTGTTTACT-TGG) for a gRNA targeting the N-terminal coding sequence of the *NCOA6* gene was cloned into the PX459 vector. A single-strand DNA fragment with a 3×FLAG-coding sequence flanked by a 67-bp 5′ homologous arm before the stop codon and a 67-bp 3′ homologous arm after the stop codon of the *NCOA6* gene were used as a donor template for homology-directed DNA repair (supplementary Table S2). Ishikawa cells were transfected with the PX459-gRNA vector and the donor DNA by using Lipofectamine 3000 and growth-selected in medium containing 2 μg/ml puromycin for 48 h. Individual clones were screened for FLAG insertion by PCR using two specific primers (5′-ACCTCTGTCACATTCCAATTCT and 5′-CCATTGCACTTTATGAAACAGGT), Western blotting using FLAG and NCOA6 antibody, and DNA sequencing analysis.

qPCR

Total RNA was isolated using TRIzol reagent (15596018, Thermo Fisher Scientific) and converted to cDNA using the reverse transcriptase core kit (4368813, Thermo Fisher Scientific). qPCR was performed to measure cDNA concentration by using the matched universal TaqMan probes (4683633001, Sigma), gene-specific primers, and qPCR MasterMix Plus kit (05-QP2X-03-075+, Eurogentec, Seraing, Belgium). Primers for *GREB1* were 5′-ACAATGGCCACAATGCTCTT and 5′-TGATTGGAGAATTCCGTGAAG. Primers for *GAPDH* were 5′-AGCCACATCGCTCAGACAC and 5′-GCCCAATACGA-CCAAATCC. Probes were no. 76 for *GREB1* and no. 60 for *GAPDH*.

Measurement of the GREB1 enhancer RNA (eRNA) and promoter RNA transcript (pRNA)

The *GREB1* eRNA and pRNA were measured as described previously (56). Total RNA was isolated using TRIzol reagent (15596018, Thermo Fisher Scientific), treated with DNase I (DN25, Sigma), and then converted to cDNA using the reverse transcriptase core kit (4368813, Thermo Fisher Scientific). eRNA/pRNA levels were measured by qPCR using matched universal TaqMan probes (4683633001, Sigma), specific primers, and the qPCR MasterMix Plus kit (05-QP2X-03-075+, Eurogentec, Seraing, Belgium). The sequences of primers for measuring eRNA were 5′-gagctgaccttgtggtaggc and 5′-gctg-acagaggagacaaaacg. The sequences of primers for measuring pRNA were 5′-GACCTAGAAGCAACCAAAATACTTCT and 5′-AAGGCAGCAAACTTGTTTAGGTA. The probes were no. 23 for eRNA and no. 84 for pRNA. *GAPDH* mRNA was measured to serve as an internal control for normalizing the relative levels of eRNA and pRNA.

ChIP–qPCR

The DNA-bound proteins were cross-linked using 1% formaldehyde for 10 min and quenched with 0.125 M glycine for 5 min. Cells were lysed with ChIP lysis buffer containing 50 mM HEPES-KOH (pH 7.5), 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, and proteinase inhibitors. Chromatin was sonicated to fragment sizes mainly between 200 and 1000 bp. Equal amounts of cross-linked chromatin were immunoprecipitated using antibodies against ERα (ab108398, Abcam, Cambridge, MA), FLAG (F1804, Sigma), RNA pol II (PLA0127, Sigma), H3K4me1 (39297, Active Motif, Carlsbad, CA), H3K4me3 (39159, Active Motif), H3K9ac (39585, Active Motif), H3K27ac (39133, Active Motif), P300 (sc-48343, Santa Cruz Biotechnology, Dallas, TX), or MLL4 (AP6183a, Abgent, San Diego, CA). Equal amounts of normal rabbit IgG (ab37415, Abcam) were used as a negative control. Immunoprecipitated DNA samples were processed and subjected to qPCR analysis using the following primers and probes: 5′-CTTGGCTTACCATGCACCTT and 5′-TGTCATTGGG-GGTTCAGTCT and probe 9 (4683633001, Sigma) for a gene desert locus as a negative control; 5′-GAGCTGACCTTGTG-GTAGGC and 5′-GCTGACAGAGGAGACAAAACG and probe 23 for the −35.4-kb *GREB1* enhancer; 5′-GACCTA-GAAGCAACCAAAATACTTCT and 5′-AAGGCAGCAA-ACTTGTTTAGGTA and probe 84 for the −1.6-kb *GREB1* promoter; and 5′-GTGGCATTTGCCATCTGAC and 5′-ATT-CAGCAGTAGCCCTTCCA and probe 43 for a putative ERα-binding site at +6-kb region of the *GREB1* gene.

3C-PCR

The basic 3C method was described previously (57). Briefly, 2×10^7 Ishikawa cells cultured in 15-cm dishes with phenol red-free medium and charcoal strip serum were treated with vehicle or 10 nM E2 for 45 min. The treated cells were cross-linked with 1% formaldehyde for 10 min and quenched with 0.125 M glycine for 5 min. Cells were then washed twice with cold phosphate-buffered saline (PBS) and collected by scraping the plates. Cells were lysed with a lysis buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 0.2% Nonidet P-40, and 1× complete protease inhibitors for 20 min on ice. Cell nuclei were washed twice with 1.2× NEB2.1 buffer, resuspended in 0.5 ml of 1.2× NEB2.1 buffer containing 0.3% SDS, and incubated at 37 °C for 1 h while shaking at 900 rpm. Triton X-100 was added to a final 2% of total sample volume, and the samples were incubated at 37 °C for an additional hour. Samples were then digested overnight with 400 units of apoI or BamHI (New England Biolabs, Ipswich, MA) at 37 °C while shaking at 900 rpm. Then, SDS was added to samples to 1.6%. The samples were incubated at 65 °C for 20 min and then diluted with 6.125 ml of 1.15× DNA ligation buffer containing 50 mM Tris-HCl (pH

7.5), 10 mM $MgCl_2$, 1 mM ATP, 10 mM DTT, and 1% Triton X-100. After incubating at 37 °C for 1 h, the samples were mixed without (negative control) or with 100 units of T4 DNA ligase (M0202S, New England Biolabs), followed by overnight incubation at 16 °C. The samples were de-proteinized and de-cross–linked by incubating overnight with 300 μg of proteinase K (3115879001, Sigma) at 65 °C. DNA was extracted with phenol-chloroform, precipitated with ethanol, and dissolved in 200 μl of H_2O. For apoI-digested samples, PCRs for detecting the ligated chromatin loops were carried out by using 21 primers (supplementary Table S3) capable of screening the 117.2-kb chromatin region from −39,832 to 77,371 bp of the *GREB1* gene. Of these PCRs, primer #2 at the enhancer and primer #21 near the promoter were paired with each of the other 20 primers, respectively, for detecting any chromatin loops between the enhancer or the promoter and other sites within this 117.2-kb chromatin region. For the BamHI-digested samples, qPCRs for measuring the ligated chromatin loops between the *GREB1* enhancer and promoter were carried out by using a primer pair of 5′-TTCACTCCCCAGTTCAAGCT and 5′-AGTTACTCTCCGGGCAAAGT and probe 86 (4683633001, Sigma). An internal control qPCR was carried out by using a primer pair of 5′-CAGGCTTAGGGCAACAGCTCTCAAA and 5′-TCCACACATGGTGTCAGAACTTTCC and probe 85.

In vitro chromatin-looping assay

In vitro chromatin-looping assay was carried out as described previously (10). The *GREB1* enhancer, promoter, biotin-labeled enhancer (Bio-enhancer), and bio-promoter double-strand DNA fragments were synthesized by PCR and purified by using GenElute™ gel extraction kit (NA1111, Sigma). Purified enhancer and bio-promoter DNA or promoter and bio-enhancer DNA (40 ng each) were mixed with 70 μg of nuclear extract proteins from V-Ctrl or N6-KO1 Ishikawa cells, followed by incubation at 4 °C for 4 h. DNA/protein complex was precipitated by using streptavidin beads (S1638, Sigma). The co-precipitated enhancer or promoter DNA was measured by qPCR using the same primers and TaqMan probes described above under "ChIP–qPCR."

Generation of Ishikawa cell lines with deleted enhancer or promoter of the GREB1 gene

To delete the enhancer core sequence, two DNA fragments (5′-GCTAATTCTAGGCTTCAAG and 5′-AACTCCATTCTTACTCCAGT) for a pair of gRNAs to target a 5′ and a 3′ sites of the enhancer were individually cloned into PX459 vectors. To delete the promoter core sequence, two DNA fragments (5′-ACTTATTTCTGGTAGGGGCC and 5′-GGACAAGCCATATCCCTAAC) for a pair of gRNAs that target two chromatin sites flanking the promoter were individually cloned into PX459 vectors. Ishikawa cells were transfected with each pair of the vectors to express gRNAs and Cas9 or with PX459 empty vector to express only Cas9 as a control by using Lipofectamine 3000. Transfected cells were transiently growth-selected in the medium containing 2 μg/ml puromycin for 48 h and then cultured in growth medium without puromycin until the formation of individual clones. Clones were isolated and expanded. Clones with enhancer deletion were screened by PCR using primers 5′-GCTGTCTCTCCTACAATGGATTGCC and 5′-TCTCTCCAGATTCAGCCATTG. Clones with promoter deletion were screened by PCR using primers 5′-TCTCCTGACCTGAAGTGATCC and 5′-TCCCATTAGTTTGGAGTTGCC. From the screening, Enh-De cells with homozygous deletion of the enhancer were obtained. However, only cells with heterozygous deletion of the promoter were identified. To obtain Pro-De cells with homozygous deletion of the promoter, another two DNA fragments (5′-TTGAAAATCAACCCGCGTAT and 5′-TAAAAGGTGCTTTACGGTCC) for another pair of gRNAs that target 5′ upstream and a 3′ downstream DNA sequences of the previous pair of gRNAs were cloned into PX459 vectors. The cells with heterozygous deletion of the promoter obtained in the first run of experiment were further transfected with these vectors. Individual clones were screened for Pro-De cells with homologous deletion of the promoter by PCR using the same pair of primers used in the first run of experiment.

Nuclear protein extraction

V-Ctrl and N6-KO1 cells grown in 15-cm dishes were scraped into PBS containing 0.5 mM EDTA. Cell membrane was broken down by a buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% Nonidet P-40 and proteinase inhibitors. Cell nuclei were collected by centrifuging at 13,000 × *g* for 5 min, and nuclear membrane was broken down by vertexing for 25 min in a buffer containing 20 mM HEPES (pH 7.9), 0.4 M sodium chloride, 1 mM EDTA, 1 mM DTT, and proteinase inhibitors. Nuclear proteins were collected after centrifuging at 13,000 × *g* for 5 min.

Cell growth assay

2000 cells were seeded in each well of the 96-well plate and cultured for the indicated growth time period. Relative cell number was assayed by incubating the cells with MTS (G3580, Promega, Fitchburg, WI) at 37 °C for 2 h and then measuring the absorbance at 490 nm on a plate reader.

Chemotherapeutic drug treatment and cell viability assay

2000 cells were seeded in each well of the 96-well plates, cultured for 24 h, and treated with vehicle or different concentrations of paclitaxel (T7402, Sigma) or docetaxel (01885, Sigma) for 24 or 48 h. The treated cells were incubated with MTS (G3580, Promega) for 2 h and then subjected to measurement of the absorbance at 490 nm on a plate reader.

Annexin V staining

V-Ctrl, N6-KO1, and enhancer-deleted and promoter-deleted cells were treated with 50 nM paclitaxel for 24 h. Apoptotic cells were measured by annexin V staining with an Alexa Fluor® 488 annexin V/dead cell apoptosis kit (V13241, Thermo Fisher Scientific) according to the manufacturer's instructions. Annexin V–stained cells were counted on images taken under a microscope.

Statistical analysis

Data were collected from several independent experiments, with three replicates performed in each experiment. Represent-

ative data from one of the experiments were presented. Scatter plots were generated by using the Prism Software. All data are expressed as the mean ± S.E. Statistical differences were determined by two-tailed Student's *t* test or one-way analysis of variance, with $p < 0.05$ being considered significant.

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