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CCAR1, a key regulator of Mediator complex recruitment to nuclear receptor transcription complexes

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Summary

DNA-bound transcription factors recruit many coactivator proteins to remodel chromatin and activate transcription. The Mediator complex is believed to recruit RNA polymerase II to most protein-encoding genes. It is generally assumed that interaction of Mediator subunits with DNA-binding transcription factors is responsible for Mediator recruitment to promoters. However, we report here that Mediator recruitment by nuclear receptors (NR) requires a new coactivator protein, CCAR1 (cell cycle and apoptosis regulator 1). CCAR1 associates with components of the Mediator and p160 coactivator complexes and is recruited to endogenous NR target genes in response to the appropriate hormone. Reduction of endogenous CCAR1 levels inhibited hormone-induced expression of endogenous NR target genes, hormone-induced recruitment of Mediator components and RNA polymerase II to target gene promoters, and estrogen-dependent growth of breast cancer cells. Thus, CCAR1 regulates expression of key proliferation inducing genes. CCAR1 also functions as a p53 coactivator, suggesting a broader role in transcriptional regulation.

Introduction

Nuclear receptors (NRs) are ligand-dependent transcriptional activators, including receptors for steroid and thyroid hormones, retinoic acid, and vitamin D, as well as orphan receptors (Tsai and O'Malley, 1994). NRs bind specific regulatory DNA sequences of target genes and recruit numerous coactivator proteins, which remodel chromatin structure and recruit and activate RNA polymerase II (Pol II). The p160 coactivators (SRC-1, GRIP1/TIF2/SRC-2, and AIB1/ACTR/RAC3/SRC-3) bind directly to hormone-activated NRs and serve as protein scaffolds for the assembly of multi-component coactivator complexes on target gene promoters (Stallcup et al., 2003). p160 coactivators recruit histone acetyltransferases (e.g. p300/CBP), histone methyltransferases (e.g. CARM1 and PRMT1), and other downstream coactivators such as the coiled-coil coactivator (CoCoA) (Kim et al., 2003; Stallcup et al., 2003). However, the mechanisms by which CoCoA and most other coactivators contribute to transcriptional activation are unknown.

The Mediator is a group of related coactivator complexes (including TRAP, DRIP, SMCC, PC2, NAT, CRSP, and ARC) composed of about 30 subunits that interact with ADs of activated NRs and other types of transcriptional activators including p53, VP16, and SP1 (Fondell et al.,

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1996; Ito et al., 1999; Kang et al., 2002; Naar et al., 1999). Mediator complex is believed to be responsible for recruiting Pol II and components of the general transcription apparatus to promoters of many genes (Blazek et al., 2005; Malik and Roeder, 2005). These findings suggest that NRs and other transcription factors recruit Mediator to target gene promoters by direct interaction, but this conclusion is challenged by results presented here.

CoCoA, the product of the *calcoco1* gene, interacts indirectly with NRs through the p160 coactivators (Kim et al., 2003). The large central coiled-coil domain interacts with the N-terminal bHLH-PAS domains of p160 coactivators, and the strong C-terminal AD is essential for its coactivator function with NRs (Kim et al., 2003; Kim et al., 2006). To further characterize the mechanism by which the CoCoA C-terminal AD contributes to transcriptional activation, we used a biochemical approach to identify cell cycle and apoptosis regulator 1 (CCAR1; also known as CARP-1) as a CoCoA AD binding protein. CCAR1 was originally identified by a functional genetic approach and implicated in retinoid-induced cell cycle arrest and apoptosis (Rishi et al., 2003). Here we show that CCAR1 can associate with components of the Mediator complex as well as CoCoA and NRs, suggesting that CCAR1 may provide a link to coordinate activities of the p160 and Mediator complexes. Indeed, CCAR1 is important for recruitment of the Mediator to target genes of estrogen receptor α (ER) and glucocorticoid receptor (GR), for target gene activation by ER and GR, and for estrogen-dependent growth of breast cancer cells. CCAR1 also acts as a coactivator for p53 and is therefore coactivator to multiple classes of transcription factors.

Results

Isolation of CCAR1

Recombinant GST-CoCoA AD (amino acids 470-691) on glutathione-Sepharose beads was used to isolate interacting proteins from MCF-7 breast cancer cell nuclear extracts. A 130-kDa protein reproducibly bound to CoCoA AD was identified by mass spectrometry as CCAR1 (data not shown). The mouse CCAR1 gene encodes an 1146-amino acid protein (Figure 1A) containing a 35-amino acid SAP domain, found in many chromatin-associated proteins (Aravind and Koonin, 2000). Consistent with our previous data, CoCoA AD bound p300 but not GRIP1 from MCF-7 cell nuclear extract, and CCAR1 also bound to CoCoA AD (Figure 1B) and to full length CoCoA in vitro (Figure 1C) and in vivo (Supplemental Figure S1A). However, CCAR1 did not bind to the coiled-coil or N-terminal domain of CoCoA (Figure 1D). CCAR1 also bound to ER and GR in a hormone-independent manner (Supplemental Figure S1B), and purified recombinant CCAR1 bound to recombinant CoCoA and ER (Supplemental Figure S1C-E), indicating direct interactions.

CCAR1 Functions as a NR Coactivator

Because CCAR1 interacts with CoCoA and NR, we tested whether it possesses coactivator activity for NRs. CCAR1 enhanced the hormone-dependent activity of endogenous (in MCF-7 cells, Figure 1E) or exogenous (in CV-1 cells, Figure 1F) ER with 3 different transiently transfected ER reporter genes in a dose-dependent manner. CCAR1 also enhanced the uninduced basal activities of MMTV(ERE)-LUC (approximately 100 fold) and 2ERE-TATA-LUC (approximately 5-10 fold), but not 2ERE-TK-LUC, in CV-1 cells (Figure 1F and Supplemental Figure S2A); in contrast to CV-1 cells, MMTV(ERE)-LUC basal activity was only modestly enhanced by CCAR1 in MCF-7 cells (Figure 1E). The uninduced basal and hormone-dependent activities of GR and thyroid hormone receptor β1 (TR) reporter genes driven by the MMTV promoter were also stimulated by CCAR1 in CV-1 cells expressing the appropriate NR (Figure 1G). Thus, CCAR1 alone can function as a coactivator for multiple NRs and can also enhance basal transcription in a promoter- and cell type-specific manner. CCAR1 cooperated with CoCoA and GRIP1 as coactivators for ER (Supplemental Figure S2B)

and also cooperated synergistically with four other coactivators: GRIP1, CoCoA, p300, and CARM1 (Supplemental Figure S2C). Synergy was highly dependent on the presence of all five coactivators and CoCoA C-terminal AD. Thus the functional interaction between CoCoA AD and CCAR1 is required for their synergistic coactivator function for ER.

Requirement of CCAR1 for ER Function and Estrogen-Dependent Growth of MCF-7 Cells

To assess the involvement of CCAR1 in ER-mediated transcription, the expression of CCAR1 was reduced by RNA interference. When CCAR1 protein levels were specifically reduced (compared with actin control) in MCF-7 cells by transfected siRNA, estrogen-induced expression of transiently transfected 2ERE-TK-LUC reporter plasmid was inhibited by more than 60% (Figure 2A). A control siRNA with a nonspecific sequence (siNS) had no effect. Similarly, estrogen-induced expression of the endogenous pS2 gene in MCF-7 cells was inhibited by the CCAR1-specific siRNA but not by the non-specific siRNA (Figure 2B). As a further test, the MCF-7tTS cell line that stably expresses the tetracycline-responsive transcriptional suppressor (tTS) was stably transfected with pTER plasmids encoding non-specific shRNA (shNS) or shRNA against CCAR1 (shCCAR1 6). Treatment of clonal derivative MCF-7tTS/shCCAR1 6-4 cells with the tetracycline analogue doxycyclin (Dox) reduced the level of endogenous CCAR1 protein, but not actin (Figure 2C, right panel). The levels of CCAR1 mRNA and estradiol (E2)-induced levels of pS2 and cathepsin D mRNAs were reduced in shCCAR1 sublines but not in control shNS4 cell lines by Dox treatment (data not shown). In eleven such independent experiments, we found a direct linear relationship between the levels of CCAR1 and pS2 mRNAs (correlation coefficient R^2=0.937, Figure 2C, left panel), indicating a direct functional relationship between CCAR1 levels and pS2 gene expression. Thus, among dozens of different coactivators implicated in transcriptional activation by NRs (Rosenfeld et al., 2006; Stallcup et al., 2003), endogenous CCAR1 was required for efficient hormonal induction of transcription by ER. In the same cell lines, reduction of endogenous CCAR1 levels by Dox treatment reduced by more than 50% the ability of a Gal4 DBD-CoCoA AD fusion protein to activate transcription of a transiently transfected reporter gene controlled by Gal4 response elements (Supplemental Figure S2D). Thus, CCAR1 also plays an important role in transcriptional activation by the CoCoA C-terminal AD.

E2 regulates mammary gland development and promotes the growth of ER-positive breast tumor cells. We therefore examined the effect of reduced CCAR1 levels on E2-stimulated MCF-7 cell proliferation in MCF-7tTS/shCCAR1 6-4 and MCF-7tTS/shNS4 cells. As expected, E2 treatment enhanced proliferation of both cell lines (Figure 2D). Inducing expression of the non-specific shRNA by Dox treatment did not affect E2-dependent or E2-independent growth of the control MCF-7tTS/shNS4 cells; but E2 stimulation of MCF-7tTS/shCCAR1 6-4 cell growth was significantly attenuated by Dox-induced knockdown of CCAR1 expression. Similarly, a shRNA (shCCAR1 7) targeting a different region of CCAR1 mRNA also efficiently inhibited E2-stimulated MCF-7 cell growth and ER function in MCF-7 cells (Supplemental Figure S3). Thus, CCAR1 contributes to E2-dependent growth (but not E2-independent growth) of breast cancer cells, presumably due to its role in ER-mediated gene regulation.

CCAR1 Is Required for Optimal Recruitment of Mediator to an ER Target Promoter

To investigate whether CCAR1 is recruited to the promoter of ER-responsive genes in a hormone-dependent manner, chromatin immunoprecipitation (ChIP) assays were performed in MCF-7 cells. ER, CoCoA, and AIB1 were recruited to the ER binding site of the pS2 gene promoter in a hormone-dependent manner, whereas the normal IgG control was not affected by E2 (Figure 3A). We also observed a 5-fold increase in pS2 promoter occupancy by CCAR1 with E2 treatment. In contrast, only background signals, equivalent to those obtained with normal IgG, were observed with or without E2 treatment for binding of the same proteins to

an irrelevant site 5 kb upstream from the transcription start site of the pS2 gene (data not shown). ChIP experiments with sequential immunoprecipitations (ChIP and ReIP) demonstrated that CCAR1 and CoCoA exist in the same complex on the pS2 promoter (Figure 3B).

Previous studies found a few proteolytic fragments of CCAR1 in purified Mediator complexes containing MED10/NUT2 or MED23 (Sato et al., 2004) or MED12 (www.nursa.org/10.1621/datasets.01001), suggesting that CCAR1 might be peripherally associated with some forms of the Mediator complex. Our CoIP experiments also revealed the association of endogenous CCAR1 with tagged MED1/TRAP220 and MED10 (Supplemental Figure S4A). Recombinant CCAR1 1-660 bound to the recombinant MED1 AB fragment (amino acids 1-670), indicating that CCAR1 can directly bind MED1 in vitro (Supplemental Figure S4B). Moreover, ChIP and ReIP experiments demonstrated that MED1 and CCAR1 are in a common complex bound to the pS2 promoter (Figure 3C). Thus CCAR1 associates at least transiently with some forms of the Mediator complex.

To assess the effect of reduced CCAR1 levels on transcription complex assembly on the pS2 promoter, MCF-7 cells were transfected with siRNAs. Reduction of CCAR1 levels by siRNA had no measurable effect on the cellular levels of ER, AIB1, CoCoA, p300, MED1, MED6, Pol II, and tubulin (Figure 3D). However, reduction in CCAR1 levels substantially compromised the E2-dependent recruitment of CCAR1, two components of the Mediator complex (MED1 and MED6), and Pol II to the pS2 promoter; but it had no effect on the E2-dependent recruitment of ER, AIB1, CoCoA, and p300 (Figure 3E). Similar results were obtained using the MCF-7 cell lines containing a doxycyclin-regulated shRNA expression unit (Supplemental Figure S5). Thus ER and p160 coactivator complex components (AIB1, CoCoA, and p300) are recruited to the pS2 promoter independently of CCAR1, while recruitment of Mediator components depends upon CCAR1. Furthermore, the previously documented ability of MED1 to bind directly to ER is not sufficient for stable recruitment of Mediator to the pS2 promoter. The fact that CCAR1 can bind to ER, CoCoA, and Mediator components suggests that CCAR1 is brought to the promoter by its interaction with either ER or CoCoA and in that context helps to recruit Mediator complex, which is generally believed to play a critical role in recruitment of Pol II (Figure 4D).

In time-course ChIP assays with the MCF-7 cell lines containing a doxycyclin-regulated shRNA expression unit, ER, AIB1, and MED1 were recruited to the pS2 promoter in a cyclic fashion (Figure 3F), as reported previously (Metivier et al., 2003). Association of CCAR1 with the pS2 promoter was also cyclic, but the peak occupancy of the pS2 promoter by MED1 (60 min) was later than that for ER, AIB1, and CCAR1 (40 min). CCAR1 silencing (by Dox treatment) did not affect the cyclic recruitment of ER and AIB1 to the promoter, but the ligand-dependent recruitment of CCAR1 and MED1 to the promoter was severely affected. Thus CCAR1 and MED1 are sequentially recruited to the pS2 promoter, and CCAR1 is required for Mediator recruitment to the promoter.

Physiological Role for CCAR1 on GR Target Promoters

Since CCAR1 interacts with and functions as a coactivator for GR (Figure 1G and Supplemental Figure S1B), we further determined whether CCAR1 is required for GR function in a physiological context. We analyzed a set of three GR responsive genes, GILZ, IGFBP1, and hIAP1, in A549 cells expressing endogenous GR. Mediator and p160 coactivator are differentially required for different GR target genes in osteosarcoma U2OS cells expressing GR (Chen et al., 2006). For example, Mediator, but not GRIP1, is required for hormone-dependent activation of IGFBP1, LAD1, and IRG8 genes. GRIP1, but not Mediator, is required for GR-mediated induction of GILZ. CCAR1 expression was efficiently and specifically silenced in A549 cells at the mRNA (data not shown) and protein levels (Figure 4A) by infection with shRNA-encoding lentivirus. Upon CCAR1 silencing, basal and DEX-induced

expression of IGFBP1 and hIAP1 mRNAs was substantially and reproducibly decreased; in contrast, Dex-dependent expression of GILZ was only slightly and inconsistently affected by CCAR1 silencing (Figure 4B and data not shown). Thus, CCAR1 is required for optimal transcriptional activation of some but not all GR target genes.

To examine the mechanistic contributions of CCAR1, ChIP assays were performed for the GR binding sites in the IGFBP1 promoter in A549 cells (Figure 4C). GR, CCAR1, MED1, and Pol II occupied the IGFBP1 promoter in a Dex-dependent manner, whereas the background signal with normal IgG was not changed by Dex treatment. CCAR1 silencing did not affect GR recruitment, but hormone-dependent promoter occupancy by CCAR1, MED1, and Pol II was reproducibly reduced. Thus, optimal recruitment of MED1 and Pol II depends on the presence of CCAR1.

CCAR1 and CoCoA Act as Coactivators for p53

To explore a broader role for CCAR1 as a coactivator, we investigated possible physical and functional interactions with the cell cycle and apoptosis-regulating transcription factor p53. GST-CCAR1 and GST-CoCoA bound specifically to p53 synthesized in vitro (Figure 5A). In transient transfection assays used to test for coactivator function, over-expression of either CCAR1 or CoCoA enhanced expression of a reporter plasmid controlled by p53 binding sites in a p53-dependent manner in p53-null SAOS2 osteosarcoma cells (Figure 5B). Together, CCAR1 and CoCoA enhanced p53 activity synergistically (Figure 5C), and they also functioned synergistically as coactivators with p300 (Figure 5D). In MCF-7 cell lines containing a Dox-regulated shRNA expression unit, CCAR1 silencing by Dox treatment reduced the transcriptional activity of Gal4 DBD fused to p53 (Supplemental Figure S2E). Thus, CCAR1 plays an important role in transcriptional activation by p53.

To assess the physiological role of CCAR1 and CoCoA as p53 coactivators, we examined the p53 target gene p21, which is activated by p53 in response to DNA damage. The DNA-damaging agent etoposide caused recruitment of p53, CCAR1, and CoCoA to the region containing the p53 binding site on the p21 promoter in MCF-7 cells (Figure 6A). However, etoposide caused no recruitment of these proteins to the pS2 promoter, which is activated by estradiol but not by etoposide. Furthermore, when endogenous levels of CCAR1 or CoCoA were reduced by siRNA transfection in U2OS osteosarcoma cells, the induction of p21 mRNA levels was compromised (Figure 6B). Thus, endogenous CCAR1 and CoCoA are recruited to the p21 promoter in response to DNA damage and are required for efficient expression of p21 mRNA in response to DNA damage. These results indicate that CCAR1 and CoCoA serve as coactivators for p53 as well as NRs, suggesting a broader role for CoCoA and CCAR1 in transcriptional regulation by various classes of transcription factors.

Discussion

The Role of CCAR1 in Transcriptional Activation by NRs and p53

Coactivators have specific domains that anchor them to the promoter through protein-protein interactions and other domains that act upon the chromatin or transcription machinery. p160 coactivators bind directly to NRs through their LXXLL motifs and use three ADs to recruit other coactivators, including histone acetyltransferases, histone methyltransferases, and CoCoA (Stallcup et al., 2003). The coiled-coil domain of CoCoA anchors it to the p160 protein (Kim et al., 2003), and the CoCoA C-terminal AD recruits p300 (Kim et al., 2006) and CCAR1 (Figure 1).

CCAR1 functions as a coactivator for NRs in transient reporter gene assays (Figure 1 and Supplemental Figure S2), and its synergy with other coactivators depends on the presence of

CoCoA AD (Supplemental Figure S2C), thus demonstrating the importance of CoCoA-CCAR1 binding. CCAR1 is recruited to the promoters of endogenous NR target genes (Figures 3 and 4); and depletion of endogenous CCAR1 protein caused reduced hormone-induced expression of NR target genes (Figures 2 and 4), attenuated E2-dpendent growth of breast cancer cells (Figure 2D and Supplemental Figure S3), and significantly reduced hormone-dependent recruitment of Mediator components and pol II to NR target promoters (Figures 3 and 4 and Supplemental Figure S5). Thus CCAR1 plays a physiological role in NR function.

CCAR1 has previously been implicated in cell cycle arrest and apoptosis in response to retinoids and DNA damaging agents in ER-negative breast cancer cells (Rishi et al., 2003). A recent study showed that knockdown of CCAR1/CARP-1 resulted in inhibition of etoposide-induced apoptosis by an unknown mechanism (supplementary information in Kim et al., 2008). Here we show that CCAR1 bound to p53, enhanced p53 activation of reporter genes, was recruited to a p53 target gene promoter, and was required for endogenous target gene activation by p53 (Figures 5 & 6 and Supplemental Figure S2E). These results indicate an important role for CCAR1 in the function of p53 and may explain the role of CCAR1 in supporting apoptosis. Thus, CCAR1 appears to have a dual role, supporting estrogen-induced cell proliferation and also supporting apoptosis induced by DNA damage and other cellular insults.

Physical and Functional Association of CCAR1 with Mediator

CCAR1 was identified by MudPIT analysis in two of six Mediator preparations (each isolated through a different epitope tagged component) (Sato et al., 2004), but the lower percent sequence coverage of CCAR1 than for other Mediator components suggests that it may be associated with Mediator at substoichiometric levels. A similar study also found CCAR1 with MED12/TRAP230 complexes (www.nursa.org/10.1621/datasets.01001). Here we show in vivo association of CCAR1 with Mediator complexes (Supplemental Figure S4) and the association of CCAR1 with MED1 on the pS2 promoter (Figure 3C). Mediator is believed to play a critical role in transcription of many genes by recruiting basal transcription factors and Pol II to specific promoters; it is required for basal and activated transcription of most protein-encoding genes in yeast and mammalian cells (Acevedo and Kraus, 2003; Baek et al., 2002; Blazek et al., 2005; Malik and Roeder, 2005).

TRAP220/MED1-containing Mediator complex is recruited to ER target gene promoters and is required for ER-dependent transactivation and estrogen-dependent growth of breast cancer cells (Zhang et al., 2005). CCAR1 may contribute to transcriptional activation by NRs through multiple mechanisms; however, we show here that CCAR1 facilitates recruitment of MED1 and other Mediator components to ER- and GR-regulated promoters but was not required for recruitment of ER, AIB1, p300, CoCoA, and GR (Figure 3E, Figure 4C, and Supplemental Figure S5). Thus, although ER and GR can interact directly with Mediator complex through the MED1 subunit (Chen and Roeder, 2007; Chen et al., 2006; Kang et al., 2002), our results indicate that CCAR1 is required for efficient recruitment of Mediator to at least some NR target genes. Further study is required to determine whether a similar mechanism pertains to the role of CCAR1 as a coactivator for p53.

Consistent with our observations (Figure 3F), recent ChIP analyses found that p160 coactivators and MED1 are sequentially recruited to NR target promoters after ligand treatment (Burakov et al., 2002; Metivier et al., 2003; Sharma and Fondell, 2002). Thus, it is possible that chromatin remodeling by p160 coactivator complexes and recruitment of CCAR1 by the NR or the p160 complex are required for Mediator recruitment. This model may explain why Mediator complex supports transcription in vitro from naked DNA templates more efficiently than from chromatized templates and also may explain functional synergies on chromatin

templates between Mediator and p300 or p160 coactivator complexes containing p300 (Acevedo and Kraus, 2003; Baek et al., 2002; Huang et al., 2003; Roeder, 2005).

Recent studies showed that NR coactivator PGC-1α interacts with and stimulates the function of both p300 and Mediator in NR-mediated transcription and also suggested that PGC-1α may play a role in mediating the transition between NR/p300-dependent chromatin remodeling and NR/Mediator-dependent transcription (Roeder, 2005; Wallberg et al., 2003). A more recent study showed that the MED1 subunit of the Mediator complex is essential for PPARγ-stimulated adipogenesis (Ge et al., 2008). However, a strong, direct interaction of PPARγ with Mediator through the LXXLL motifs of MED1 was not required either for PPARγ target gene expression or for PPARγ–stimulated adipogenesis in cultured fibroblasts. The minimal region required for MED1 function in adipogenesis is mapped to the N-terminal region (lacking the NR boxes) that mediated the incorporation of MED1 into the Mediator complex. Thus several previous studies are consistent with the idea that additional coactivators which interact physically and functionally with Mediator complexes are required to facilitate recruitment and/or function of the Mediator complex. Our results linking CCAR1 with Mediator recruitment provide a molecular mechanism which may explain at least some of these previous observations; i.e. CCAR1 facilitates recruitment of Mediator complex to the promoter of target genes in natural chromatin templates by providing a physical link between p160 coactivators and the Mediator complex (Figure 4D).

Experimental Procedures

Isolation of CCAR1

For details, see Supplemental data.

Plasmids and Lentivirus Production

For details, see Supplemental data.

Immunoblot and Protein Interaction Assays

Immunoblot, GST pull-down, and coimmunoprecipitation assays were performed as described previously (Kim et al., 2003). See Supplemental data for details.

Cell Culture, Transient Transfection, and Cell Proliferation Assays

For details, see Supplemental data.

Chromatin Immunoprecipitation (ChIP) Assays

ChIP and ChIP-ReIP assays were performed as described previously (Kim et al., 2003). qPCR was performed with Brilliant SYBR Green QPCR Master Mix (Stratagene). Each experiment was repeated independently at least three times. For details of antibodies and PCR primers used in ChIP assays, see Supplemental data.

RNA Interference and qRT-PCR

For details, see Supplemental data. Each experiment was repeated independently at least three times.

Establishment of Stable Cells Expressing Tet-Inducible shRNAs

For details, see Supplemental data.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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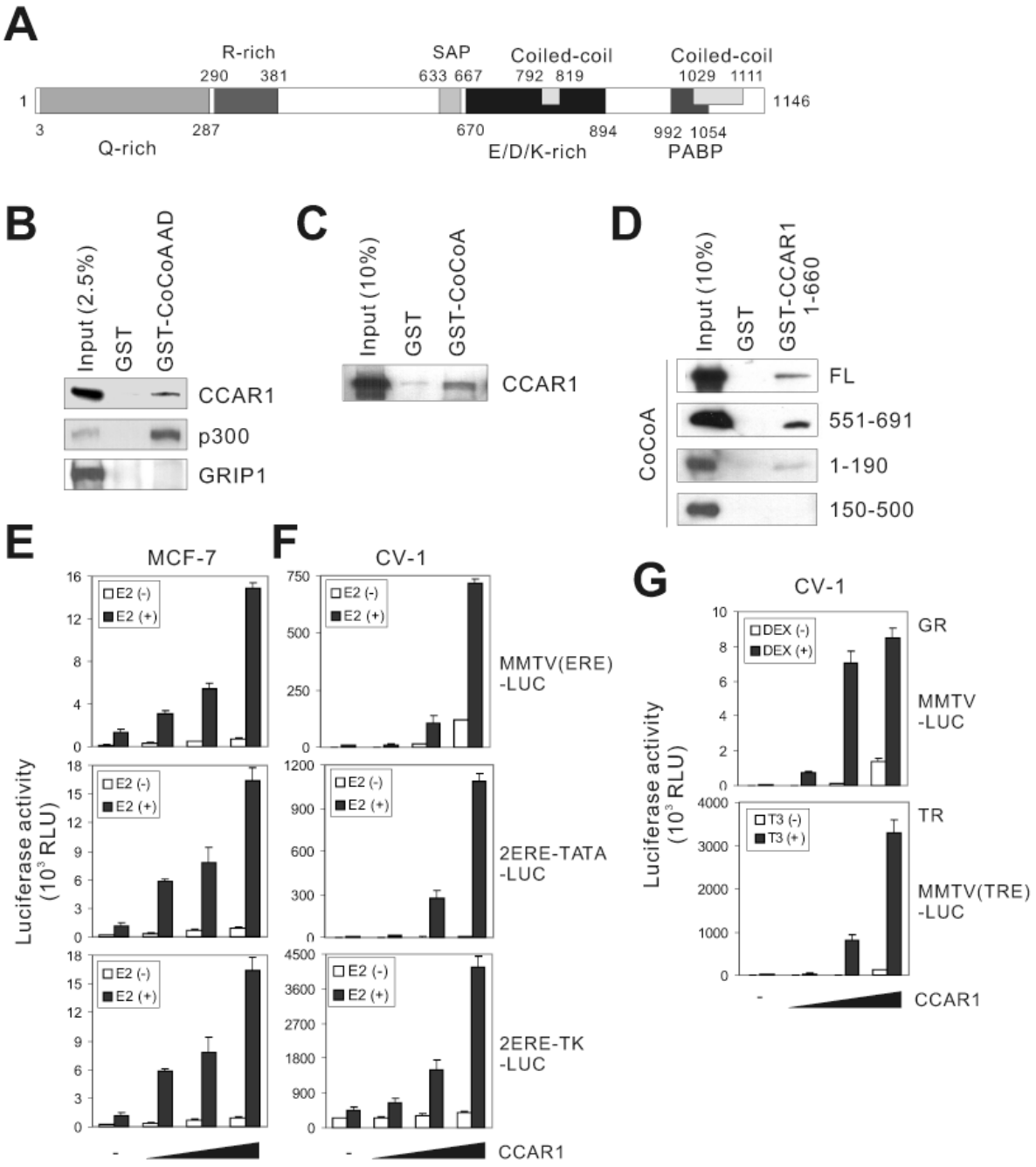

Figure 1. CCAR1 Interacts with CoCoA AD and Functions as a NR Coactivator

(A) Schematic representation of CCAR1 structure. SAP, SAP domain; PABP, poly A binding protein homology region.

(B) MCF-7 cell nuclear extracts were incubated with immobilized GST-CoCoA AD. The bound proteins were analyzed by immunoblot; the CCAR1 antibody was 435A.

(C) HA-tagged CCAR1 synthesized in vitro was incubated with equal amounts of GST or GST-CoCoA protein bound to beads. Bound proteins were analyzed by immunoblot with anti-HA antibody.

(D) GST pull-down assays were performed as described in (C) using in vitro translated HA-CoCoA or its fragments and GST or GST-CCAR1 1-660 bound to beads.

(E) MCF-7 cells in 12-well plates were transfected with luciferase reporter plasmids (200 ng) containing estrogen-responsive elements (ERE) in combination with variable amounts (300, 600, and 900 ng) of pSG5.HAb-CCAR1 and grown in medium containing or lacking 100 nM estradiol (E2) before conducting luciferase assays on cell extracts. Results shown are mean and SD of triplicate points.

(F) Transient transfections using ERE-reporters and pHE0 encoding ER (2 ng) in CV-1 cells were performed as described in (E).

(G) CV-1 cells were transfected with plasmids as indicated below and grown in medium containing 100 nM dexamethasone (DEX) for GR or 100 nM triiodothyronine (T3) for TR. Reporter plasmids (200 ng): MMTV-LUC for GR, MMTV(TRE)-LUC for TR. Expression vectors: pKSX (GR) (10 ng), pCMX-TR (10 ng), pSG5.HAb-CCAR1 (300, 600, and 900 ng). Results shown are mean and SD of triplicate points.

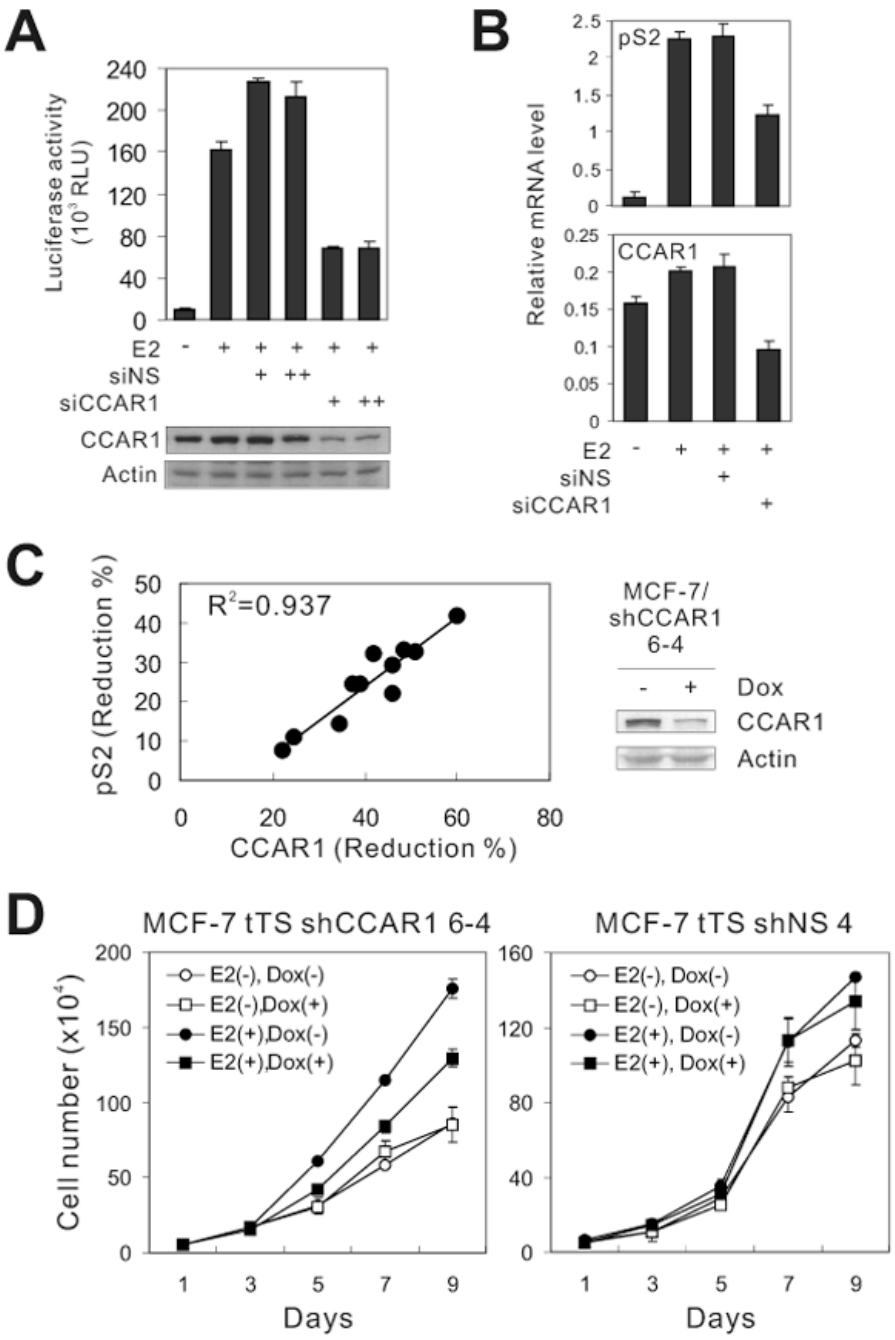

Figure 2. Requirement of CCAR1 for Estrogen-Inducible Gene Expression and Hormone-Dependent Growth of Breast Cancer Cells

(A) Knock-down by siRNA. MCF-7 cells were transfected with 2ERE-TK-LUC and 20 (+) or 40 (++) pmole of either the CCAR1 siRNA duplex or non-specific (NS) siRNA duplex, as indicated. 48 hr after transfection, cells were treated with 100 nM E2 or untreated and harvested after an additional 24 hr for luciferase assays and immunoblot analysis with CCAR1 antibody 435A. Results shown are mean and SD of triplicate points.

(B) MCF-7 cells were transfected with the indicated siRNA (20 pmole) and treated or untreated with E2 as in (A). Total RNA was prepared, and real-time qRT-PCR was performed. CCAR1

and pS2 mRNA levels are normalized to β-actin mRNA levels, and results shown are mean and SD from triplicate reactions.

(C) Statistical correlation of endogenous CCAR1 and pS2 mRNA levels. MCF-7tTS/shCCAR1 6-4 cells were cultured in the absence or presence of doxycyclin (Dox) for 48 hr, treated with E2 for 24 hr, and collected for the preparation of total RNAs. Real-time qRT-PCR analyses were performed on RNA from 11 independent experiments. The protein levels of CCAR1 and actin were determined by immunoblot; the CCAR1 antibody was 435A.

(D) CCAR1 is required for E2-dependent growth of MCF-7 cells. MCF-7tTS cell lines in 12 well plates were cultured in the absence or presence of 10 nM E2 and 1 μg/ml Dox, as indicated. Viable cells were counted by trypan blue staining. Results shown are mean and SD of triplicate wells in a single experiment and are representative of three independent experiments.

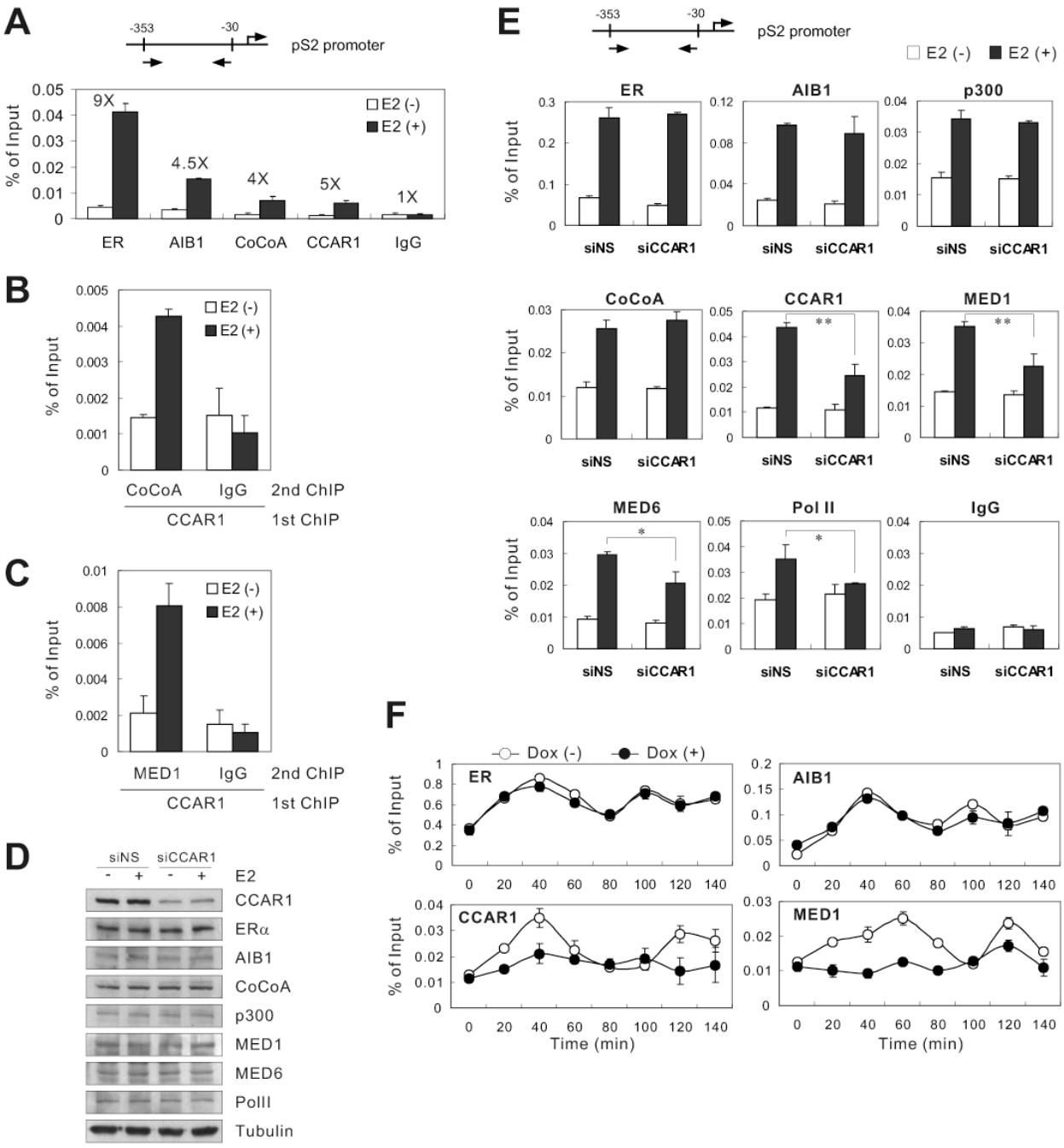

Figure 3. CCAR1 Is Required for Optimal Recruitment of Mediator to the pS2 Promoter

(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. The CCAR1 antibody used was an equal mixture of N306 and C1128. qPCR analyses were performed using primers for the pS2 promoter. The results are shown as percentage of input and are the mean and SD from triplicate reactions. Numbers above the bars indicate fold increase in pS2 promoter occupancy with E2 treatment.

(B and C) ChIP and ReIP assays, using the indicated antibodies, were performed as described in (A) and (Kim et al., 2003). The CCAR1 antibody used was 435A.

(D and E) CCAR1 is required for recruitment of Mediator and Pol II to the pS2 promoter. Specific siRNAs targeting CCAR1 or control non-specific (NS) siRNAs were transfected into MCF-7 cells 72 hr before E2 stimulation. ChIP assays were performed as in (A). Protein levels were monitored by immunoblot using the indicated antibodies. The CCAR1 antibody used was 435A. * indicates $p<0.05$ and ** indicates $p<0.01$ with Student's *t*-test on triplicate PCR reactions from a single experiment. *p* values from paired, two-tailed t-tests for ChIP results from *n* independent experiments comparing RNAi against CCAR1 or against non-specific (NS) targets were: for CCAR1 recruitment to pS2 promoter in the presence of E2, $p = 0.00016$ (n = 9); for MED1 recruitment, $p = 0.0011$ (n = 9); for MED6 recruitment, $p = 0.034$ (n = 6); for pol II recruitment, $p = 0.037$ (n = 5).

(F) Cyclic and sequential recruitment of ER and its coactivators. ChIP assays were performed as in Supplemental Figure S5, except that MCF-7 cells were treated with E2 for the indicated times. The CCAR1 antibody used was 435A.

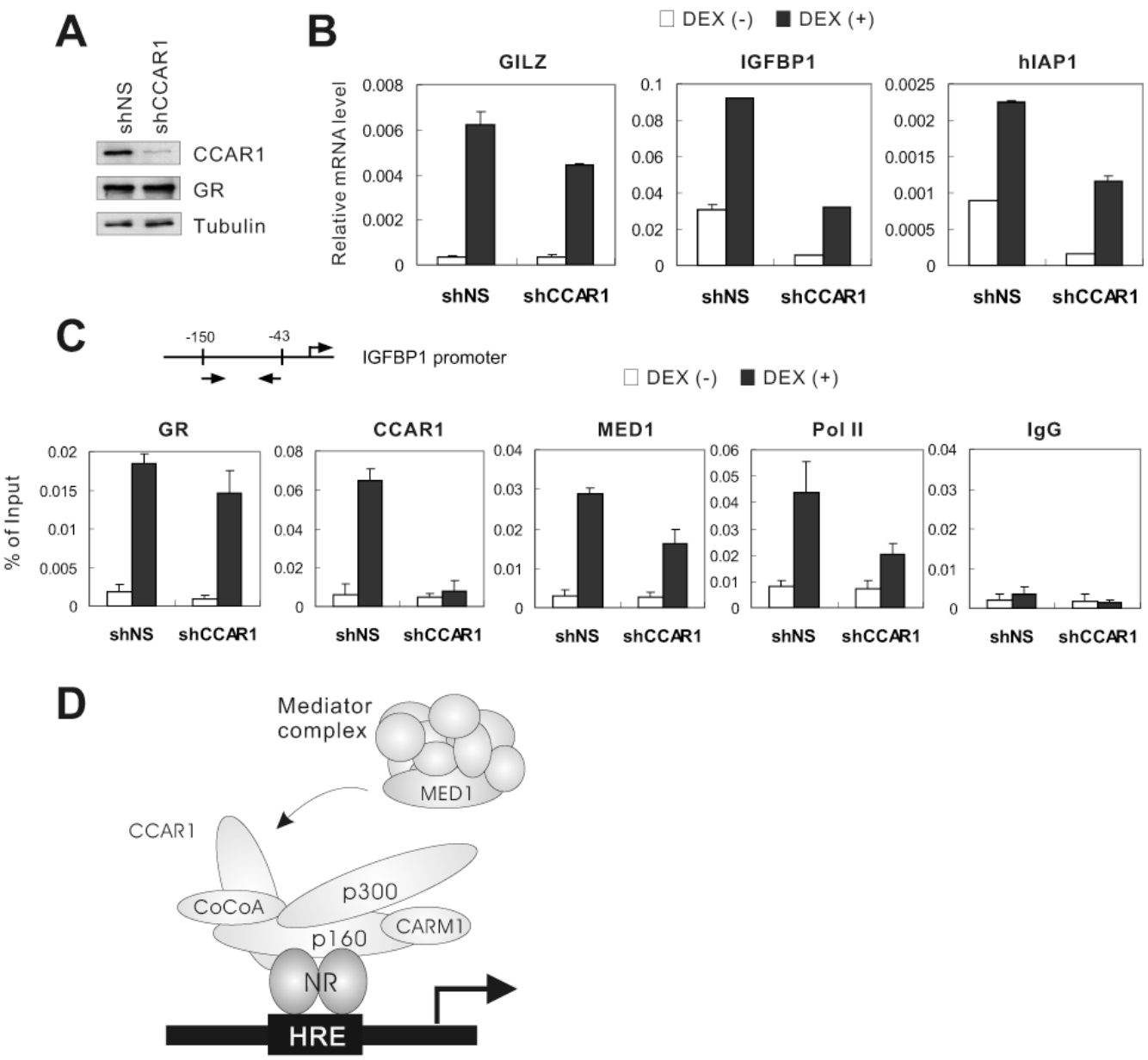

Figure 4. CCAR1 Is Required for GR Target Gene Expression and Optimal Recruitment of Mediator to a GR Target Gene Promoter

(A) Immunoblot analyses of A549 cells infected with a lentivirus expressing a non-specific (NS) or CCAR1 shRNA. The CCAR1 antibody used was 435A.

(B) A549 cells expressing a NS or CCAR1 shRNA were treated with 100 nM DEX or ethanol vehicle for 2 hr. The indicated mRNA levels were determined by qRT-PCR as in Figure 2B.

(C) Occupancy of the IGFBP1 promoter by Mediator (MED1) and Pol II. A549 cells infected with lentivirus encoding a non-specific (NS) or CCAR1 shRNA were treated with 100 nM DEX for 2 hr. ChIP assays were performed as in Figure 3A. The CCAR1 antibody used was 270A.

(D) The proposed role of CCAR1 in coordinating p160 coactivator and Mediator complexes. CCAR1 associates with DNA-bound NRs either directly or through interaction with CoCoA.

CCAR1 recruits Mediator, which helps to recruit and activate RNA polymerase II and its basal transcription factors.

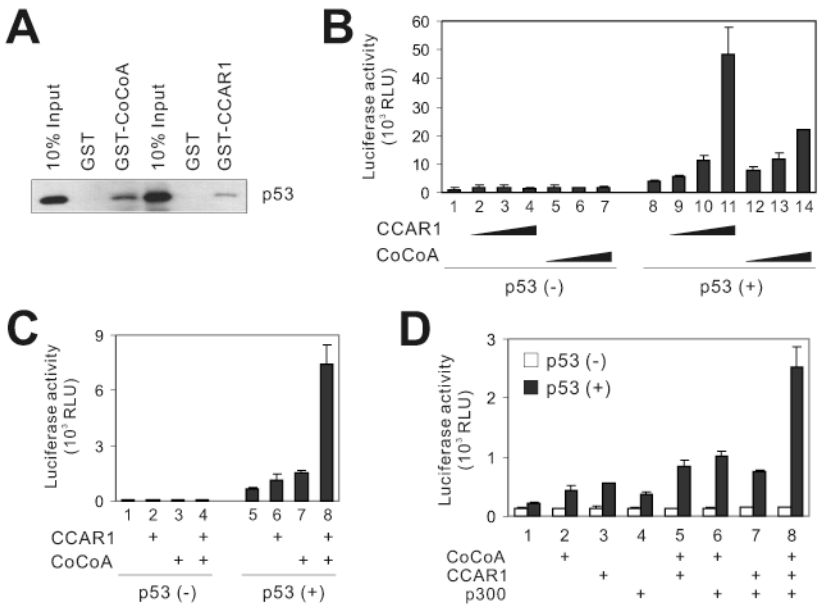

Figure 5. CCAR1 and CoCoA Act as Coactivators for p53

(A) HA-tagged p53 synthesized in vitro was incubated with equal amounts of GST, GST-CCAR1, or GST-CoCoA protein bound to beads. Bound proteins were analyzed by immunoblot with anti-HA antibody.

(B) Coactivator activity of CCAR1 and CoCoA for p53. SAOS2 cells (p53-null) in 12-well dishes were transfected with pSG5.HA-p53 (2 ng) and pG13-LUC (200 ng) in combination with variable amounts (200, 400, and 800 ng) of pSG5.HAb-CCAR1 or pSG5.HA-CoCoA. Cell extracts were assayed for luciferase activity. Results shown are mean and SD of triplicate points.

(C) CCAR1 cooperates with CoCoA to enhance p53 activity. SAOS2 cells were transfected with pG13-LUC (200 ng), pSG5.HA-p53 (2 ng), pSG5.HAb-CCAR1 (200 ng), and pSG5.HA-CoCoA (200 ng), and luciferase activity of cell extracts was determined.

(D) Synergistic enhancement of p53 activity under low-p53 conditions, which facilitate multiple-coactivator synergy. SAOS2 cells were transfected with pG13-LUC (200 ng), pSG5.HA-p53 (0.2 ng), pSG5.HAb-CCAR1 (200 ng), pSG5.HA-CoCoA (200 ng), and pCMV-p300 (200 ng).

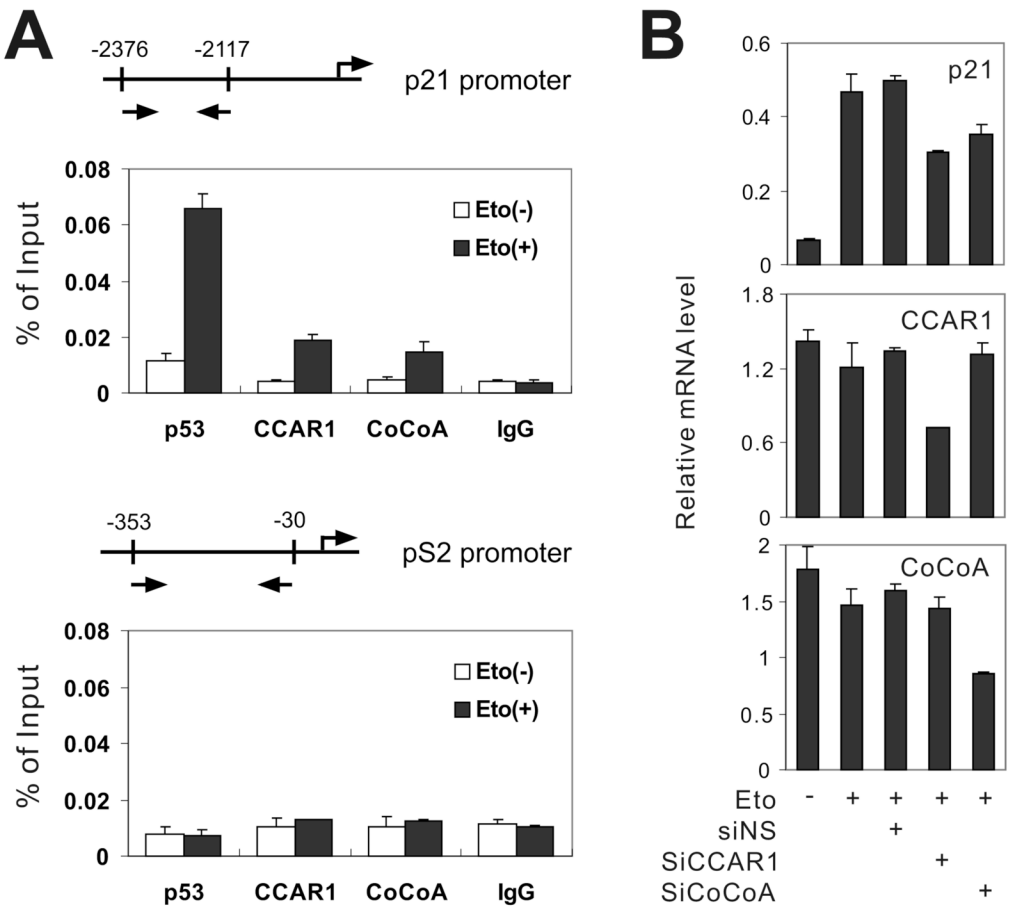

Figure 6. Physiological Role for CCAR1 and CoCoA on the p53-Responsive p21 Promoter

(A) CCAR1 and CoCoA are recruited to the endogenous p21 promoter. Crosslinked, sheared chromatin from MCF-7 cells treated with or without 10 μM etoposide (Eto) for 2 hr was immunoprecipitated with the indicated antibodies. Real-time PCR analyses were performed using primers for the p21 promoter (nucleotide -2290 to -2074 relative to transcription start site) or pS2 promoter. The results are shown as percentage of input and are the mean and SD from triplicate PCR reactions. Results shown are from a single experiment and are representative of 3 independent experiments.

(B) CCAR1 and CoCoA are required for p53-mediated transcription. U2OS cells were transfected with the indicated siRNA (40 pmole) and treated or untreated with 10 μM etoposide (Eto) for 18 hr. Total RNA was prepared, and real-time qRT-PCR was performed to determine p21, CCAR1, CoCoA, and β-actin mRNA levels. Results shown are normalized to β-actin mRNA levels, are mean and SD from triplicate PCR reactions, and are representative of 3 independent experiments. NS, non-specific.