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TITLE: ROLE OF ART-27, A NOVEL ANDROGEN RECEPTOR COACTIVATOR, IN
NORMAL PROSTATE AND PROSTATE CANCER

PRINCIPAL INVESTIGATOR: Wenhui Li, Ph.D.

CONTRACTING ORGANIZATION: New York University, School of Medicine
New York, NY 10016

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14. ABSTRACT Androgen receptor (AR), a hormone-dependent transcription factor, plays a role in the growth of normal and malignant prostate cells. Androgen Receptor Trapped clone-27 (ART-27), a recently identified AR N-terminal coactivator, may interact with the receptor modulating its activity and affecting cell growth. Here we examined the effect of 13 naturally occurring AR N-terminal mutations on the transcriptional response of the receptor to ART-27. It was found that, one of these mutation, AR P340L, a somatic alteration associated with prostate cancer, although interact more avidly with ART27, paradoxically decreases AR transcription. This may represent a novel mechanism of pathogenesis whereby increased AR-coactivator association negatively regulates AR activity and biological response. Previous studies have shown ART-27 is expressed in normal adult human prostate in the luminal epithelial cells but not in the undifferentiated precursor cells, and is negligibly expressed in prostate cancer. Understanding the regulation of ART-27 gene transcription will help us to elucidate the role of ART-27 in prostate and the cancer development. We hence have mapped ART-27 promoter region and identified a minimal cis-element with a strong basal activity and its likely binding factor CREB/ATF. Functional significance of CREB/ATF in ART-27 regulation will be under further investigation.					
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Introduction

The Androgen receptor (AR) is a hormone-dependent transcription factor involved in the regulation of both normal and malignant prostate cell growth. However, the precise mechanisms by which AR regulates normal prostate development and initiates prostate cancer have yet to be elucidated. It is believed that co-factors (coactivators and corepressors) that interact with the AR and modulate its activity play an important role in these processes. ART-27, as a newly identified AR coactivator, could be such a modulator. In my previous report, we have shown that some mutated AR, found in some prostate cancer and androgen insensitive syndrome patients, has altered ART27:AR interaction. This provides important genetic evidence for the role of ART27 in human disease, such as prostate cancer.

Histoimmunochemistry studies have just shown that in normal adult human prostate, ART-27 is expressed in luminal epithelial cells, but not in the stroma. During prostate development, ART-27 is expressed in differentiated luminal epithelial cells, but not in their undifferentiated precursors. Further, ART-27 expression levels are negligible in human prostate cancer and increased expression of ART-27 in the androgen-responsive LNCaP prostate cancer cell line inhibits androgen-mediated cellular proliferation [1]. All these suggest a possible role for ART-27 in AR-mediated growth suppression and differentiation of the prostate epithelium. It is conceivable that understanding how ART-27 is regulated on transcription level will help us to design a new target for prostate cancer treatment.

Body

Task 1 Determine if AR N-terminal mutations that have been identified in prostate and androgen insensitivity syndrome (AIS) affect ART-27 binding or function.

A manuscript has been published based on this part of work. A copy was attached in the Appendices.

Task 2 (Original) To create ART-27 null mice by targeted mutagenesis and assess the consequences of lack of ART-27 on prostate development.

In the proposal I have reported that I had constructed ART-27 targeting vector (knock-out) based on the sequence isolated from mouse genomic DAN library, and also obtained two embryonic stem (ES) cell clones which incorporated ART-27 knock-out gene. The project was carried out further by injecting blastocysts with ES clones and transplanted into pseudopregnant female mice; a litter of chimera mice had been born. The birth of chimera mice indicated successfulness of DNA recombination. However further examination of the targeted ART-27 genome revealed it was likely a pseudogene as the predicted transcribed sequence had an early stop codon. Searching the newly updated genbank database confirmed the finding. The true ART-27 gene was mapped to chromosome X, like it in humans, not chromosome 15 which the whole construct was based on, which also meant that the whole efforts had to be abandoned because of the wrongful targeting construct. Since engineering of transgenic mouse from the very beginning will take more than two years, but I only had one year term remaining, I had to adjust my SOM to meet the deadline.

Task 2 (revised) Mapping ART-27 promoter regions.

The gene encoding human ART-27 was identified on X-chromosome by searching the human genome database. Different lengths of upstream pieces of hART-27 transcript were PCR out, sequenced, and cloned upstream of a luciferase reporter gene, and used in transcription studies using HeLa extracts. The data demonstrated that promoter fragment length correlated with luciferase production with shorter lengths associated with increased luciferase accumulation down to the -389 base pair (bp) fragment. Similar data were obtained from transient

transfections in a rat epithelial cell line, NRP152. Deletion of the ART-27 promoter sequence section -288bp to -249bp reduced luciferase activity from 90% to 8% when compared to the full-length promoter construct indicating that this is an important area for transcription. Through nucleotide substitution analysis it was determined that the core CRE binding motif sequence, 5/-TACGTCAT-3', was critical to the regulation of transcription of the ART-27 gene. Several members of the CRE-binding protein (CREB)/activating transcription factor (ATF) family of transcription factors as well as members of the CCAAT/enhancer-binding protein (C/EBP) family recognize and bind to the core CRE binding motif. Data generated by electrophoretic mobility shift assay (EMSA) using the CREB/ATF or C/EBP consensus binding sequences as competitors demonstrate that a CREB/ATF family member bound to this sequence in the ART-27 promoter. This conclusion was substantiated by supershift assay results showing that anti-CREB/ATF antibodies not anti-C/EBP caused a band shift. The antibody was specific for the CREB/ATF family of transcription factors not for individual members. Additional preliminary data indicate that ART-27 protein expression was upregulated during differentiation in NRP152 epithelial cell lines when cultured in differentiation medium.

Please see appended publication for details of work, results and findings.

Key research Accomplishments

1. Constructed a series of point mutations in the androgen receptor (AR) N-terminus that correspond to alterations observed in androgen insensitivity syndrome and prostate cancer by site directed mutagenesis.
2. Analyzed the effects of these mutations on AR-mediated transcriptional activation and expression in cultured cells.
3. Analyzed the interaction of AR point mutations with ART-27.
4. Analyzed the effect of AR point mutations on ART-27-dependent transcriptional activation.
5. Analyzed the human ART-27 promoter region and identified a minimal promoter.
6. Identified CREB/ATF as a major regulator of ART-27 minimal promoter.

Reportable Outcomes

Poster Abstract "An androgen receptor mutation isolated from a prostate cancer patient displays aberrant ART-27 coactivator function" by Wenhui Li, Susan K. Logan and Michael J. Garabedian at 2004 Keystone Symposium "Nuclear Receptor:Steroid Sisters", Denver, CO.

Li W, Cavasotto C, Cardozo T, Ha S, Dang T, Taneja S, Logan S and Garabedian M (2005) Androgen receptor mutations identified in prostate cancer and androgen insensitivity syndrome display aberrant ART-27 coactivator function. Mol Endocrinol. 19(9): 2273-2282

Nwachukwu JC*, Li W* (*co-first author), Pineda-Torra I, Huang HY, Ruoff R, Shapiro E, Taneja SS, Logan SK, Garabedian MJ (2007). Transcriptional Regulation of the Androgen Receptor Cofactor ART-27. Mol Endocrinol. 21(12):2864-76

Conclusions

It has been proposed that aberrant interactions between the AR and its coregulators contribute to prostate cancer; however, evidenced linking abnormal receptor:cofactor interaction to disease is scant. Here we found that an AR somatic alteration in a prostate cancer (AR P340L) displays reduced response to ART-27 coactivation relative to the wild type AR, whereas its response to the p160 class of coactivators was not affected. Despite the fact that AR P340L shows decrease transcriptional activation in response to ART-27, more ART-27 associates with AR P340L as compared to the wild type receptor. Thus, P340L promotes a more avid AR:ART-27 interaction, suggesting that aberrant AR-coactivator association interferes with normal ART-27 coactivator function resulting in suppression of AR activity. This represents a novel mechanism of pathogenesis whereby an AR mutation reduces AR susceptibility to its coactivator ART-27, which normally suppresses cellular proliferation, and underscores the importance of loss of ART-27 in oncogenesis.

Analysis of the human ART-27 showed that a -263bp fragment was sufficient to confer strong basal promoter activity in HeLa and NRP152 cells. We have identified CREB/ATF as binding protein for this region. These work provided the foundation of further analysis of functional importance of CREB/ATF in regulation of ART-27 transcription.

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Appendices

Androgen Receptor Mutations Identified in Prostate Cancer and Androgen Insensitivity Syndrome Display Aberrant ART-27 Coactivator Function

Wenhui Li, Claudio N. Cavasotto, Timothy Cardozo, Susan Ha, Thoa Dang, Samir S. Taneja, Susan K. Logan, and Michael J. Garabedian

Departments of Microbiology (W.L., T.D., M.J.G.), Urology (W.L., S.H., T.D., S.S.T., S.K.L., M.J.G.), and Pharmacology (S.K.L., T.C.), New York University Cancer Institute, New York University School of Medicine, New York, New York 10016; and Molsoft (C.N.C.), La Jolla, California 92037

The transcriptional activity of the androgen receptor (AR) is modulated by interactions with coregulatory molecules. It has been proposed that aberrant interactions between AR and its coregulators may contribute to diseases related to AR activity, such as prostate cancer and androgen insensitivity syndrome (AIS); however, evidence linking abnormal receptor-cofactor interactions to disease is scant. ART-27 is a recently identified AR N-terminal coactivator that is associated with AR-mediated growth inhibition. Here we analyze a number of naturally occurring AR mutations identified in prostate cancer and AIS for their ability to affect AR response to ART-27. Although the vast majority of AR mutations appeared capable of increased activation in response to ART-27, an AR mutation identified in prostate cancer (AR P340L) and AIS (AR

E2K) show reduced transcriptional responses to ART-27, whereas their response to the p160 class of coactivators was not diminished. Relative to the wild-type receptor, less ART-27 protein associated with the AR E2K substitution, consistent with reduced transcriptional response. Surprisingly, more ART-27 associated with AR P340L, despite the fact that the mutation decreased transcriptional activation in response to ART-27. Our findings suggest that aberrant AR-coactivator association interferes with normal ART-27 coactivator function, resulting in suppression of AR activity, and may contribute to the pathogenesis of diseases related to alterations in AR activity, such as prostate cancer and AIS. (*Molecular Endocrinology* 19: 2273–2282, 2005)

THE ANDROGEN RECEPTOR (AR) is a transcriptional regulatory protein that transduces the signaling information conveyed by androgens (1). Upon androgen binding, the hormone-AR complex enters the nucleus, associates with specific DNA sequences, and modulates transcription initiation from nearby promoters (2). Activation of AR is essential for the maintenance of the prostate gland in adult males, and, in the absence of androgens, the prostate shrinks to a rudimentary form. For this reason, nonsteroidal anti-androgens are frequently used in conjunction with LHRH agonists to lower circulating androgen to treat advanced prostate cancer (3).

The transcriptional activation functions (AFs) of AR (4, 5) represent surfaces capable of interaction with

general transcription factors and additional transcriptional regulatory factors termed coactivators. Coactivators have been identified that interact with the AR N-terminal AF-1 and the C-terminal AF-2 region to enhance AR-dependent gene transcription (6, 7). AR also interacts with the general transcription factor TF_{II}F (8) as well as the cyclin-dependent kinase-activating kinase of TF_{II}H (9).

AR trapped clone-27 (ART-27) was identified in our laboratory as an AR N-terminal coactivator (10). ART-27 binds to a region of AR encompassing AF-1a and AF-1b and activates AR-dependent transcription in a dose-dependent manner in cell-based assays. Endogenous ART-27 interacts with AR in nuclear extracts of LNCaP cells, and velocity gradient sedimentation of nuclear extracts suggests that native ART-27 is part of a multiprotein complex.

Indeed, the components of the ART-27 complex have recently been identified by mass spectrometric analysis of ART-27-associated proteins from HeLa whole-cell lysates (11). ART-27 associates with proteins that include RBP5, a subunit shared by RNA polymerases I, II, and III, an RBP5 binding protein called unconventional prefoldin RBP5 interactor and the ATPase/helicase TIP48 and TIP49, as well as other unidentified proteins. Thus, ART-27 appears to be part of a large multiprotein complex in human cells that

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Abbreviations: AF, Activation function; AIS, androgen insufficiency syndrome; AR, androgen receptor; ARR, androgen responsive region; ART-27, AR trapped clone-27; CAIS, complete AIS; DTT, dithiothreitol; GRIP, glucocorticoid receptor interacting protein; HA, hemagglutinin; MMTV, mouse mammary tumor virus; PAIS, partial AIS; SDS, sodium dodecyl sulfate; SRC, steroid receptor coactivator; TBS, Tris-buffered saline.

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includes proteins that function in transcriptional regulation.

We have also shown that in normal adult human prostate, ART-27 protein is expressed in luminal epithelial cells, in contrast to the stroma, where ART-27 is not expressed (12). During prostate development in humans, ART-27 is expressed in differentiated luminal epithelial cells but is not detected in undifferentiated epithelial cell precursors, suggesting a role for ART-27 in AR-mediated growth suppression and differentiation. Consistent with a growth-suppressive function, ART-27 expression levels are negligible in human prostate cancer, and regulated expression of ART-27 in the androgen-sensitive LNCaP prostate cancer cell line inhibits androgen-mediated cellular proliferation (12). These findings suggest that ART-27 affects AR target genes important to prostate growth suppression and/or differentiation.

To examine the physiological contribution of ART-27 to AR-dependent processes, we examined a set of naturally occurring AR N-terminal mutations identified in prostate cancer and androgen insensitivity syndrome (AIS) for effects on the AR transcriptional response to ART-27. AR mutations have been identified in up to 50% of advanced, metastatic prostate cancers, suggesting that AR mutations may confer a growth advantage to the cells (13). In principle, AR somatic alterations associated with prostate cancer may represent gain-of-function mutations that enhance AR interaction with coactivators involved in cell proliferation. Alternatively, if coactivators such as ART-27 confer AR-dependent growth suppression and differentiation, the function of ART-27 may be compromised in certain receptor mutations isolated from prostate cancer patients. Interestingly, a growing list of mutations localizing to the N terminus is emerging, suggesting an important role for the AR N terminus and associated factors in prostate cancer.

Naturally occurring mutations are also found in individuals with AIS. There is a broad range of androgen insensitivity from complete AIS (CAIS) to partial AIS (PAIS). In CAIS, tissues are insensitive to androgen, whereas in PAIS tissues vary in their sensitivity to hormone. CAIS individuals are genetically male, yet phenotypically appear female as a result of a loss in AR activity, whereas a spectrum of phenotypic changes occur in PAIS patients depending on the severity of the defect in AR function (14, 15). The AR N-terminal mutations isolated from AIS individuals are presumed to produce alterations in AR function perhaps as a consequence of abnormal AR-coactivator interactions. Here we analyze naturally occurring AR mutations identified in prostate cancer and AIS for their ability to functionally interact with ART-27.

RESULTS

To examine the physiological contribution of ART-27 to AR-dependent processes, we tested a set of natu-

rally occurring AR N-terminal mutations identified in prostate cancer and AIS for their effect on the AR transcriptional response to ART-27. We focused on AR N-terminal mutations because ART-27 has been shown to bind exclusively to this region of the receptor (10). Eleven AR mutations, spanning amino acids 2–491, were made in the N terminus. Of these mutations, five were mutations identified in human AIS (E2K, Q194R, N233K, L255P, and G491S), and six were mutations from individuals with prostate cancer (K180R, E198G, M266T, P269S, S334P, and P340L) (Fig. 1A).

It is conceivable that these AR mutations induce changes in the receptor structure, such that unstructured regions would adopt structural features or, alternatively, that structured domains would be disrupted. Therefore, we analyzed the ability of the AR mutations to alter AR structure before embarking on the functional analysis. Initially, we analyzed the secondary structure of the wild-type AR N terminus from a multiple alignment among ARs from different species (Fig. 1A). This approach can achieve greater than 75% accuracy (16, 17) and revealed 16 regions with predicted structural features. Although the majority of the AR N terminus appears unstructured, this is in agreement with biophysical studies (18). Of the AR mutations analyzed, the majority had no apparent effect on the secondary structure of the AR N terminus (data not shown). The two mutations that did change the predicted structure of the AR N terminus were AR E2K, a germ line mutation identified in a patient with PAIS (19), and AR P340L, a somatic mutation identified in a localized prostate cancer (20). Analysis of the low-energy conformations (within 10 kcal/mol) accumulated during extensive peptide simulations of the AR residues 1–25 shows that the first 10 residues form an α -helical fold in the E2K mutant, whereas this region is largely unstructured in the wild-type AR (Fig. 1, B and C). The sequence-based prediction in Fig. 1A and the helical fold observed in residues 16–25 of the wild-type AR in Fig. 1B are in agreement with a recent report suggesting that AR amino acids 16–36 fold into a long amphipathic α -helix (21). Interestingly, the tertiary structure simulation for the AR 331–355 region shows a dramatic preference for wild-type peptide to adopt an α -helical fold at its C terminus between residues 341–355, whereas the P340L mutation folds into a α -helix near the N terminus flanked by residues 331–345 (Fig. 1, D and E). Therefore, the E2K and P340L mutations generate new structural elements, suggesting that they may represent functionally relevant alterations.

The AR mutants were initially analyzed for their ability to affect AR transcriptional activity as compared with wild-type AR in a cell-based assay using an AR-responsive mouse mammary tumor virus (MMTV)-luciferase reporter (Fig. 2A). The AR alterations E2K, Q194R, and P340L exhibited lower AR transcriptional activation, whereas the remaining mutations, K180R, E198G, N233K, L255P, M266T, P269S, S334P, and

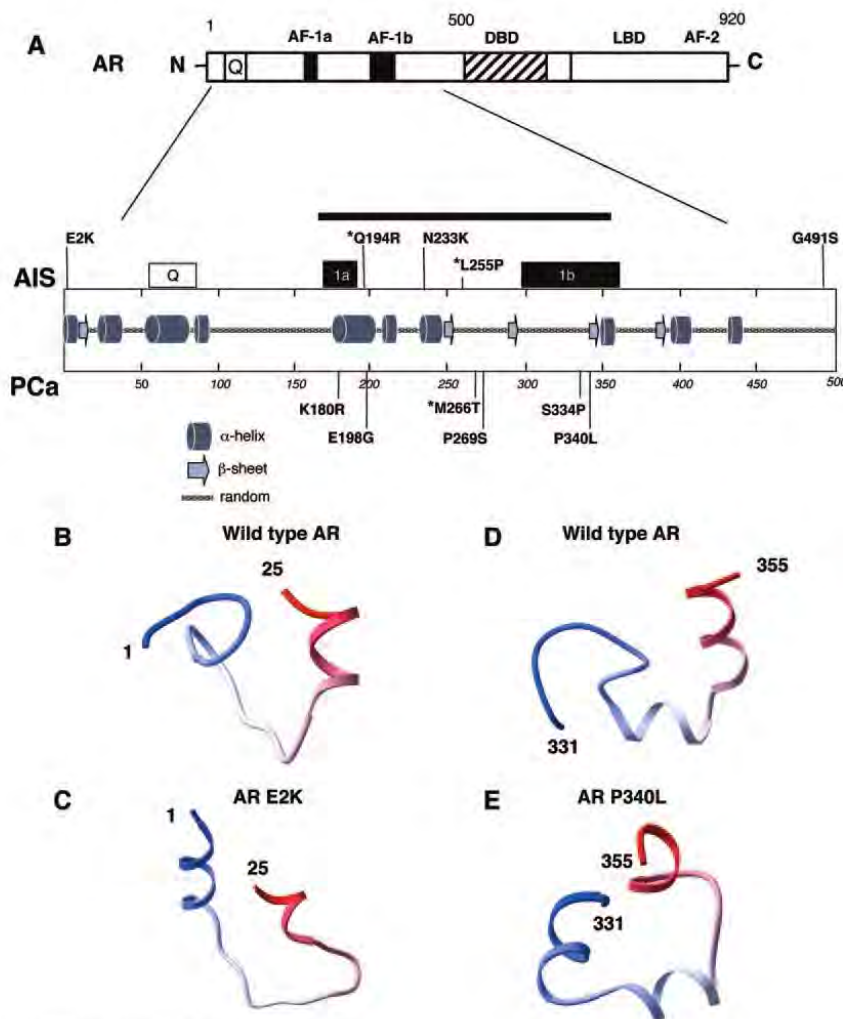


Fig. 1. AR Functional Domains and Predicted Structure

A, Schematic diagram of the functional domains of the human AR. Shown are a poly-glutamine stretch (Q), AF-1a and AF-1b (black), the DNA binding domain (DBD) (hatched), and the ligand binding domain (LBD) and AF-2. The predicted secondary structure of the AR N terminus is also shown (bottom panel). The thick black line above the structure alignment depicts the ART-27 binding region. The AR N-terminal mutations identified in androgen insensitivity (AIS) (top) and prostate cancer (PCa) (bottom) are shown. A list of AR point mutations can be found at <http://www2.mcgill.ca/androgendb/>. The secondary structure content for the AR N terminus from a multiple alignment comparing AR from eight different species to the human AR; these include AR from rat (P15207) (38), mouse (P19091) (39), dog (Q9TT90) (40), rabbit (P49699) (41), lemur (O97776) (42), chimpanzee (O97775) (42), macaque (O97952) (42), and baboon (O97960) (42). Gray cylinders represent α -helices; gray arrows are β -sheets, and stippled gray lines are unstructured. AR mutants with a single asterisk denote an alteration that was found in conjunction with another mutation outside the AR N terminus. Predicted tertiary structures of wild-type AR (1–25) (panel B) and AR (1–25) E2K (panel C), wild-type AR (331–355) (panel D), and AR (331–355) P340L (panel E) are shown. The N-terminal regions are in red and C-terminal segments are shaded blue.

G491S, did not appear to significantly affect AR activity relative to the wild-type receptor. Immunoblot analysis revealed that both wild-type AR and all of the

receptor variants are stabilized in the presence of R1881 and that some variability in AR protein expression is also observed among the mutant receptors

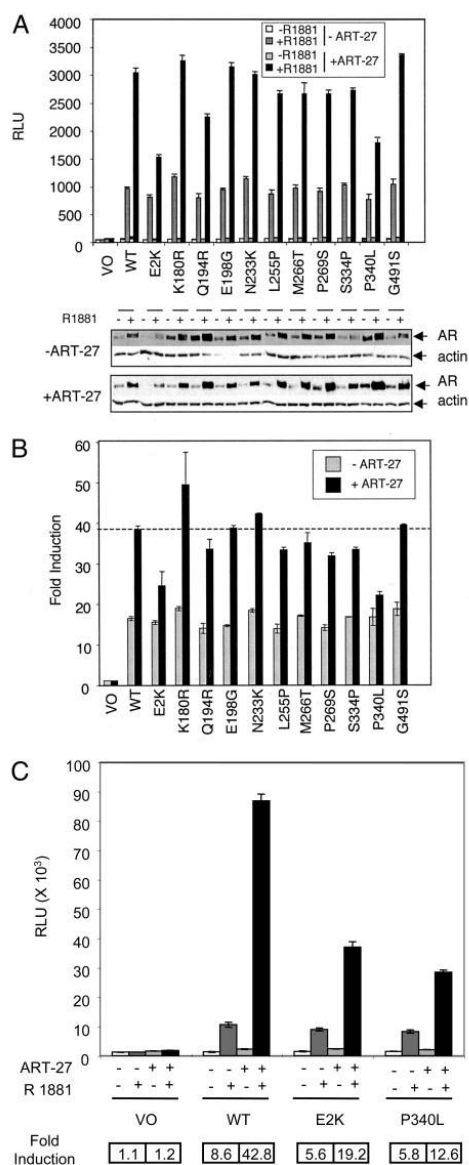


Fig. 2. Transcriptional Response of AR Mutants to ART-27

A, Top panel: HeLa cells were transiently transfected as described in *Materials and Methods* with the indicated AR expression vector or vector only (VO), MMTV-luciferase reporter gene, and CMV-LacZ, together with pcDNA3:HA-ART-27 (+ART-27) or pcDNA3 (-ART-27). Cells were treated with 100 nM R1881 or an ethanol vehicle, and AR transcriptional activation was assayed for luciferase activity, normalized to β -galactosidase activity and expressed as relative light units (RLU). **Bottom panel:** Whole-cell extracts were prepared from transfected cells, and the expression of AR variants was analyzed by Western blotting

(Fig. 2A). For example, the Q194R and P340L display elevated AR protein expression relative to wild-type receptor, but show lower receptor transcriptional activation, indicating that the decreased AR activity is not a result of reduced AR protein expression. In contrast, E2K, which also shows reduced AR-dependent activity, shows lower steady state levels of AR protein compared with the wild-type receptor, and this may contribute to the lower activity observed in the transcriptional activation assay. The AR E2K mutation, originally identified from a PAIS patient, has been previously shown to exhibit reduced receptor expression as a result of inefficient translation (19). Despite some variability of AR expression, a majority of the mutants exhibit activity comparable to wild-type AR. Thus, a subset of AR mutants affects AR transcriptional activity.

Next we examined the effect of ART-27 overexpression on wild-type and mutant AR transcriptional activation. The transcriptional activity of the wild-type AR was increased approximately 3-fold by ART-27 overexpression (Fig. 2A). Interestingly, the AR mutations E2K, Q194R, and P340L displayed reduced ART-27-dependent receptor transcriptional enhancement, whereas the majority of the other alterations appeared capable of increased activation in response to ART-27. Thus, of all the AR mutants tested, E2K, Q194R, and P340L appear to have a reduced capacity to utilize ART-27 as a coactivator.

In addition to measuring total hormone-dependent AR activity, we also compared the "fold-induction" or the AR transcriptional response to ligand in the presence and absence of ART-27 (Fig. 2B). The relative fold-induction of AR in response to ART-27 is constant over a range of AR concentrations and, therefore, is a valid means of comparison among the receptor mutants that vary in expression (supplemental Fig. 1 published as supplemental data on The Endocrine Society's Journals Online web site at <http://mend.endojournals.org>). The AR alterations E2K and P340L showed a decreased fold induction in response to overexpressed ART-27. In contrast, the Q194R mutation maintained a fold induction in response to ART-27

using an AR or an actin antibody, which serves as a control for loading. **B,** The relative fold-induction in response to 100 nM R1881 of each AR mutant in the absence (gray bars) and presence (black bars) of ART-27. Shown is a single experiment done in duplicate with the error bars representing the range of the mean. The experiment was repeated three times with similar results. **C,** Effect of the E2K and P340L mutations on the AR transcriptional response of ARR3 to ART-27. HeLa cells were transiently transfected with the rat probasin ARR reporter construct, ARR₃-TK-luciferase, along with the wild-type AR (WT), AR E2K (E2K), AR P340L (P340L), or the empty expression vector (VO) in the absence and presence of ART-27. Luciferase activity was determined in the absence and presence and 100 nM R1881, as described previously. Shown is a representative experiment done in duplicate and repeated three times with similar results.

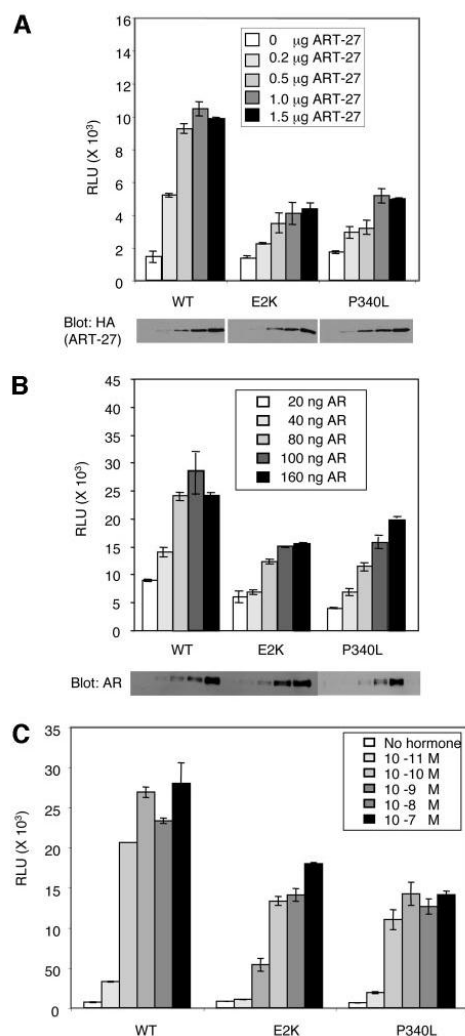


Fig. 3. Diminished Response of AR E2K and AR P340L to ART-27 Is Not Restored by Increasing ART-27, AR, or Hormone Levels

A. Transcriptional response of WT AR, AR E2K, and AR P340L to increasing levels of ART-27. HeLa cells in six-well plates (1.5×10^5 cells per well) were transfected with an MMTV-luciferase reporter construct (100 ng), the AR derivatives (WT, E2K, or P340L; 200 ng), and increasing concentrations of ART-27 (0, 0.2, 0.5, 1.0, or 1.5 μ g). Cells were treated with 100 nM R1881, and luciferase activity was assayed as described in *Materials and Methods*, normalized to β -galactosidase activity, and expressed as relative light units (RLU). Western blot (*bottom*) shows the expression of ART-27. **B.** Transcriptional response of AR to ART-27 as a function of receptor concentration. HeLa cells in 24-well plates (3×10^4 cells per well) were transfected with ART-27 (100 ng) and increasing amounts of AR (20, 40, 80, 100, and 160 ng).

that was similar to wild-type AR. This suggests that the Q194R alteration has a more general effect on AR transcriptional activation, whereas the impact of E2K and P340L alterations appear specific to ART-27-mediated AR activation and will be the focus of our subsequent experiments (Fig. 2B).

We next examined the effect of the E2K and P340L substitutions on the receptor transcriptional response to ART-27 at other promoters and regulatory elements. Transcriptional activity of the mutations was compared with wild-type AR in a cell-based assay using the ARR₃-luciferase reporter from the androgen responsive region (ARR) of the rat probasin promoter (22) and the synthetic TAT₃-luciferase reporter. A similar reduction was also observed with the AR E2K and P340L mutations at the ARR₃-luciferase reporter (Fig. 2C) and from the synthetic TAT₃-luciferase reporter (data not shown). This indicates that the effect of the E2K and P340L substitutions on the receptor transcriptional response to ART-27 is evident at distinct androgen response elements and promoter elements.

To determine whether the decreased activity of AR E2K and AR P340L would be overcome by increasing the levels of ART-27 relative to receptor, we expressed wild-type receptor and the AR mutants E2K and P340L in the presence of increasing concentrations of ART-27 (Fig. 3A). Wild-type AR shows increased levels of AR transcriptional activation in response to ART-27, consistent with previous observation. On the other hand, AR E2K and AR P340L show a greatly diminished response in comparison to wild type at all the concentrations of ART-27 tested (Fig. 3A). Thus, increasing ART-27 protein levels cannot compensate for the receptor defects.

To determine the impact of AR expression levels on transcriptional activation, we titrated the amount of wild-type or mutant receptor and evaluated the ability to activate transcription (Fig. 3B). The AR E2K mutant cannot activate transcription to the level of wild-type receptor at any of the levels tested (Fig. 3B), consistent with its identification previously as a partial loss-of-function mutation from a PAIS patient (19). AR P340L also displays decreased responsiveness to ART-27 at all AR concentrations tested relative to the wild-type AR. Our findings indicate that AR E2K and AR P340L are defective in response to ART-27 at levels of receptor comparable to the wild-type AR.

Cells were treated with 100 nM R1881 and AR transcriptional response from the MMTV-luciferase reporter gene was determined as above. Western blot (*bottom*) shows the expression of the AR variants. **C.** Increasing hormone levels do not compensate for the diminished transcriptional response of E2K and P340L to ART-27. HeLa cells were transfected with 40 ng of each AR variant and 100 ng of ART-27 and 100 ng MMTV-luciferase. Cells were then treated with the indicated concentration of R1881 and luciferase activity determined as above. WT, Wild type.

To evaluate the effect of hormone concentration on the activity of the AR E2K and AR P340L mutants relative to wild-type AR, cells were cotransfected with the AR or AR mutants and ART-27, and treated with hormone ranging from 10^{-7} to 10^{-11} M (Fig. 3C). The results indicate that both AR E2K and AR P340L show reduced receptor transcriptional activity at all hormone concentrations tested. Interestingly, AR E2K showed a more dramatic reduction in AR activity at lower hormone concentrations. For example, whereas the wild-type AR and AR P340L achieved 80% of maximal activation in response to 10^{-10} M R1881, AR E2K achieved only 30% of its maximal activity at this same hormone concentration (Fig. 3C). Therefore, increased hormone concentration does not compensate for the decreased AR transcriptional activity of AR E2K and AR P340L to ART-27. Thus, increasing ART-27, AR, or hormone concentration does not compensate for the defect in the AR E2K and P340L.

To determine whether these AR mutants show decreased responsiveness to coactivators other than ART-27, cells were transfected with wild-type AR, AR E2K, and AR P340L along with ART-27 or the p160 coactivators, glucocorticoid receptor-interacting protein 1 (GRIP-1) or steroid receptor coactivator 1 (SRC-1) (23, 24). As before, the AR P340L substitution showed a diminished capacity to respond to ART-27 relative to the wild-type AR (Fig. 4). In contrast, the AR transcriptional response to SRC-1 or GRIP-1 was not affected by the AR P340L substitution relative to the

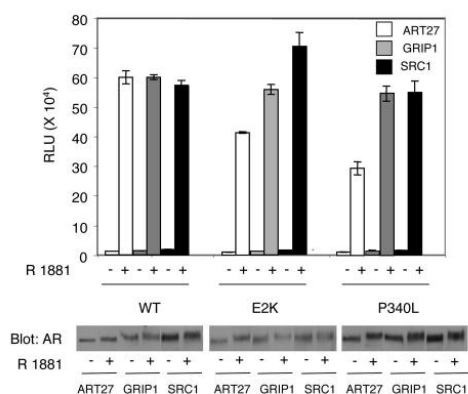


Fig. 4. Specificity of the Mutant AR Transcriptional Response to ART-27

HeLa cells were transiently transfected with the indicated AR derivatives, the MMTV-luciferase reporter gene, and ART-27, GRIP-1, or SRC-1 expression constructs. Cells were treated with 100 nM R1881 or an ethanol vehicle, and luciferase activity was determined as described in Fig. 2. Shown is a representative of three independent experiments done in triplicate with the error bars representing the SE. Western blot (bottom) shows the expression of the AR derivatives. RLU, Relative light units.

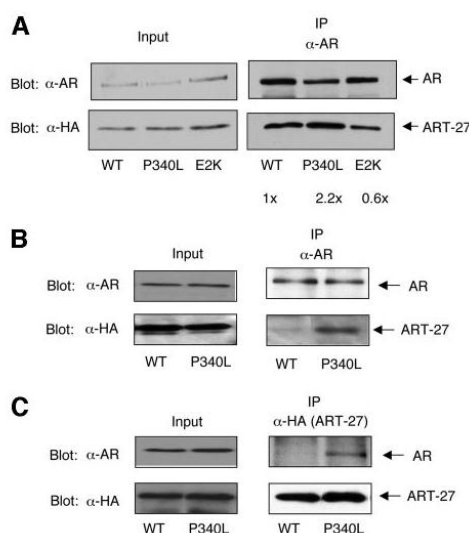


Fig. 5. Aberrant Binding of ART-27 to AR P340L and AR E2K Mutations

A, HeLa cells were transfected with HA-ART-27 and either the wild-type AR, the AR E2K, or AR P340L, treated with 100 nM R1881 for 2 h, and were immunoprecipitated with an AR antibody from nuclear extracts under low-stringency conditions as described in *Materials and Methods*. Associated proteins were resolved by SDS-PAGE. HA-ART-27 associated with AR was detected by immunoblotting with an antibody against HA. The *left panel* shows the expression of AR and ART-27 before immunoprecipitation (*input*) and the *right panel* reveals the ART-27 that was immunoprecipitated with AR (IP). The total amount of AR immunoprecipitated (*bottom panel*) was used to standardize the amount of associated ART-27 by densitometry, with the wild-type AR:ART-27 ratio arbitrarily set as 1. B and C, HeLa cells were transfected as above, and whole cell lysates were prepared in RIPA buffer (high-stringency conditions), and reciprocal immunoprecipitations were performed using antibodies against AR (panel B) or HA (ART-27) (panel C). The *left panel* shows the expression of AR and ART-27 before immunoprecipitation (*input*). Associated proteins were resolved by SDS-PAGE and revealed by immunoblotting with the corresponding AR or ART-27 (HA) antibody. Shown are representative experiments that were repeated three times with similar results. WT, Wild type.

wild-type receptor. Analysis of the AR E2K mutation again showed a reduced ability to respond to ART-27 (Fig. 4). Surprisingly, AR E2K shows an increase in transcriptional activity in response to SRC-1 (Fig. 4). Overall, our findings indicate that the E2K and P340L mutations selectively affect AR functional interactions with ART-27.

We next tested the AR mutants for physical interaction with ART-27 by coimmunoprecipitation. HeLa cells were transfected with a hemagglutinin (HA)-tagged version of ART-27 along with expression vectors for either the wild-type AR, AR E2K, or AR P340L. Cells were treated with hormone and lysed under con-

ditions that preserve the interaction between AR and ART-27. AR immunoprecipitates were analyzed by immunoblotting with an HA antibody specific for the HA-tagged ART-27. As seen in Fig. 5A, ART-27 was coimmunoprecipitated with both wild-type AR, AR E2K, and AR P340L. About half the level of ART-27 was detected in association with AR E2K as compared with the wild-type receptor. Thus, the AR E2K alteration reduces interaction with ART-27. In contrast, AR P340L showed an increase in its association with ART-27, despite its inability to enhance AR transcription. To investigate whether the increased ART-27 associated with AR P340L mutant might reflect a tighter binding, reciprocal immunoprecipitation experiments between AR and ART-27 were performed under more stringent conditions. Under these circumstances, little ART-27 is detected in association with the wild-type AR (Fig. 5B). In contrast, ART-27 is readily detected in association with the AR P340L substitution, indicating that the interaction of AR P340L with ART-27 is more stable than that of the wild-type receptor. This finding suggests that the AR P340L mutant is binding inappropriately to ART-27 and thus fails to facilitate AR-dependent transcriptional activation.

DISCUSSION

Although AR plays a role in normal and malignant prostate cell function, the impact of AR N-terminal mutations in the etiology of prostate cancer and AIS is not clear (25). One possibility is that the AR N-terminal mutations increase or decrease interaction with AR cofactors. AR mutations in prostate cancer could enhance the function of a cofactor involved in AR-dependent proliferation or inhibit the activity of a cofactor mediating AR-dependent differentiation. The cofactors themselves could also be altered and collaborate with AR mutations to promote cellular proliferation. A recent report indicates that recurrent prostate cancers express higher than normal levels of the p160 coactivators, transcriptional intermediary factor-2/GRIP-1 and SRC-1 (26). In addition, the expression levels of multiple AR coactivators varied between normal and malignant prostate tissue samples (27–29). Further, our group has found that ART-27 protein levels are decreased in prostate cancer, suggesting a role for ART-27 in growth inhibition (12). The AR N-terminal mutations in AIS, which presumably reduce AR activity *in vivo*, could also result from an alteration in cofactor binding. Recently, a patient with AIS was described whose cells lacked AR transcriptional activity, probably through the loss of an as-yet-unidentified AR N-terminal cofactor (30). This underscores the importance of the AR N terminus and associated factors in AIS.

Previous studies have shown that the AR E2K mutation decreases receptor translation, resulting in lower steady state AR levels (19). Our results also

indicate that this mutant shows a diminished interaction and transcriptional response to ART-27. Because the AR E2K mutation is located outside of the ART-27 binding region (Fig. 1A) and induces a local conformational change (Fig. 1C), we suggest that the change in conformation affects the global architecture of the receptor, which reduces ART-27 binding (Fig. 5). The unexpected finding that the AR E2K displays an enhanced transcriptional response to SRC-1 is consistent with the notion that the E2K mutation affects global AR conformation. Such changes in the AR response to coactivators may be an important determinant in the AIS phenotype.

Our results also indicate that the ability of ART-27 to function as an AR coactivator is greatly decreased by the P340L substitution. Although the expectation was that this reduced activity is a result of diminished ART-27 binding to the receptor, this is not the case (Fig. 4). Instead, our findings demonstrate that the AR P340L mutant associates more avidly with ART-27 (Fig. 5). In principle, increased ART-27 binding to AR could affect the association of a different regulatory cofactor. In support of this idea, AR P340L lies near a stretch of amino acids that has been shown to interact with TF_{II}F, a component of the basal transcription machinery consisting of two subunits, RAP74 and RAP30 (8, 31). Elegant work from the McEwan laboratory (8) has revealed that RAP74 interacts with AR at multiple sites including two motifs (PSTLSL) located between residues 159–164 and 340–345 in the AR N terminus. It is possible that the AR P340L mutation creates a new surface for ART-27 binding and eliminates a motif existing in the wild-type AR for cofactor binding, which is consistent with the structure prediction (Fig. 1E). This could explain the tendency of AR P340L mutant to exhibit increased ART-27 binding, but decreased AR activity.

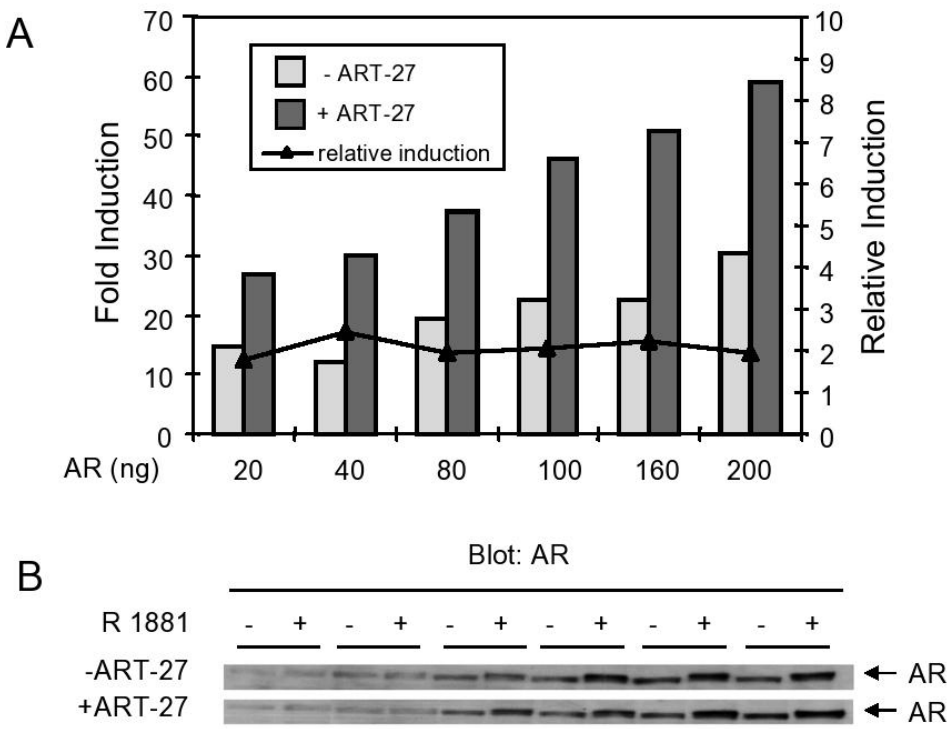
Another possibility is that ART-27 functions as a chaperone to help "load" TF_{II}F (or another factor) onto the receptor or maintain AR in a conformation competent for cofactor binding. Once this is accomplished, ART-27 would then dissociate from the receptor. In the AR P340L mutant, however, ART-27 would be unable to correctly place the cofactor onto the receptor or promote a receptor conformation compatible with cofactor binding and would neither dissociate nor coactivate. This idea is not inconceivable because ART-27 shows homology to prefoldins, which are small molecular weight proteins that assemble into molecular chaperone complexes to affect protein folding. Recently, ART-27 has been shown to be part of a transcriptional regulatory complex that contains an unconventional prefoldin that controls a transcription program in response to nutrient deprivation (11). This highlights the biological relevance of prefoldin-type proteins in the regulation of gene expression.

Our recent studies indicate that ART-27 is present in normal adult prostate but is absent in prostate cancer (12). Further, examination of ART-27 protein expression in prostate development demonstrates that

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Supplementary Figure 1



Supplemental Data Figure legends

Supplemental Figure 1

Fold-induction of AR by ART-27 is independent of AR protein concentration

A) HeLa cells in 24-well plates (3×10^4 cells/well) were transfected with the indicated amount of AR in the absence (-); or presence (+) of ART-27 as indicated. Fold-induction is determined as described in the Experimental Procedures. The relative induction by ART-27 over the range of AR concentrations examined (fold induction -ART27/+ART-27) is shown by a black line. Results shown are from a representative of three experiments done in duplicate and represent the mean. B) Western blot of AR from transfected cells in the absence (top panel) and presence (bottom panel) of ART-27. Note that fold-induction of AR by ART-27 is largely independent of the level of AR protein expression.

Transcriptional Regulation of the Androgen Receptor Cofactor Androgen Receptor Trapped Clone-27

Jerome C. Nwachukwu,* Wenhui Li,* Inés Pineda-Torra, Hong Ying Huang, Rachel Ruoff, Ellen Shapiro, Samir S. Taneja, Susan K. Logan, and Michael J. Garabedian

Departments of Microbiology (J.C.N., W.L., I.P.-T., M.J.G.), Pharmacology (J.C.N., R.R., S.K.L.), and Urology (H.Y.H., R.R., E.S., S.S.T., S.K.L., M.J.G.), New York University Cancer Institute, New York University (NYU) School of Medicine, New York, New York 10016

Cofactors modulate nuclear receptor activity and impact human health and disease, yet surprisingly little is known about their transcriptional regulation. Androgen receptor trapped clone-27 (ART-27) is a cofactor that binds to androgen receptor (AR) amino terminus and modulates AR-dependent transcription. Interestingly, ART-27 displays both a cell type- and developmental stage-specific expression pattern. However, the *cis*-acting elements and *trans*-acting factors affecting ART-27 gene expression have not been elucidated. We found that ART-27 gene expression is repressed and its promoter is histone H3-K27 tri-methylated in human embryonic kidney cells, but not prostate cells, and the histone deacetylase inhibitor, trichostatin A, relieves this inhibition. The DNA response elements that control the induction of ART-27 gene expression were also characterized. The major *cis*-acting element corresponds to a consensus

cAMP-responsive element (CRE) and binds the CRE-binding protein (CREB) as shown by EMSA and chromatin immunoprecipitation assays. Furthermore, ART-27 promoter activity is induced upon CREB overexpression. Epidermal growth factor, which activates CREB via phosphorylation, also induces ART-27 expression, whereas a reduction in CREB phosphorylation or expression blocks this induction in prostate cells. In human prostate development, both epithelial and stromal cells express CREB; however, active phosphorylated CREB is restricted to epithelial cells where ART-27 is expressed. Based on these findings, we propose a transcriptional regulatory circuit for the developmental expression of ART-27 that includes repression by chromatin modification through a trichostatin A-sensitive factor and activation upon growth factor stimulation via CREB. (*Molecular Endocrinology* 21: 2864–2876, 2007)

THE NUCLEAR RECEPTOR superfamily consists of evolutionarily conserved, ligand-activated transcription factors that regulate various biological processes. Nuclear receptors typically activate transcription by binding DNA regulatory regions containing hormone-responsive elements, recruiting specific co-

activator complexes upon ligand-binding, and directing assembly of transcription-initiation complexes at the promoters of target genes (1, 2). Coactivators are essential to nuclear receptor function (3) by enhancing nuclear receptor activity through multiple mechanisms including posttranscriptional modification of the nuclear receptor and nearby histones and through chromatin remodeling (1).

Recent evidence indicates that transcriptional regulation of coactivators is critical to nuclear receptor function. The E2F family of transcription factors, which control genes involved in cell cycle progression (4), regulates some coactivators. For example, steroid receptor coactivator-3 (SRC-3), which promotes tumor growth in breast cancer, is induced by E2F1 (5). Interestingly, both E2F1 and SRC-3 drive overexpression of SRC-3 in breast cancer (5).

Other coactivators are targeted by the cAMP-responsive element (CRE)-binding protein (CREB), a transcription factor that controls cell differentiation and cell survival (6–8). For instance, peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α) is a master regulator of energy metabolism and mitochondrial biogenesis and transducers of regulated CREB-binding proteins (TORCs) stimulate mitochondria-

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* J.C.N. and W.L. contributed equally to this work.

Abbreviations: Ac, Acetylated; AR, androgen receptor; ART-27, androgen receptor trapped clone-27; ATF-1, activating transcription factor-1; C/EBP α , CCAAT/enhancer-binding protein- α ; ChIP, chromatin immunoprecipitation; CRE, cAMP-responsive element; CREB, CRE-binding protein; DMSO, dimethylsulfoxide; EGF, epidermal growth factor; EGFR, EGF receptor; FBS, fetal bovine serum; HDAC, histone deacetylase; 3Me, trimethylated; pCREB, phosphorylated CREB; PGC-1 α , PRC, peroxisome proliferator-activated receptor- γ coactivator-1 α ; polycomb repressive complex; PS, penicillin-streptomycin; QPCR, quantitative PCR; RPL19, ribosomal protein L19; RNA-i, RNA interference; siRNA, small interfering RNA; SRC-3, steroid receptor coactivator-3; TSA, trichostatin A; UPS, upstream region; URI, unconventional prefoldin RPB5-interactor.

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drial gene expression by activating CREB-mediated transcription of PGC-1 α (9). In addition, mutant huntingtin protein represses CREB-mediated transcription of PGC-1 α and leads to mitochondrial dysfunction and neurodegeneration in mice models for Huntington's disease (10). However, little is known about transcriptional regulation of most nuclear receptor coactivators.

Androgen receptor trapped clone-27 (ART-27) was identified in our laboratory in a yeast two-hybrid screen for coregulators of the androgen receptor (AR) N terminus. ART-27 binds to AR residues 153–336, which encompasses the entire AF-1a and a part of the AF-1b domain. It enhances transcriptional activity of AR as well as glucocorticoid, estrogen, and thyroid hormone receptors, indicating that ART-27 is a nuclear receptor coactivator (11).

ART-27 (also known as UXT/STAP1) is a component of a large multiprotein complex that contains RNA polymerase II subunit 5, a subunit shared by all three RNA polymerases; unconventional prefoldin RPB5-interactor (URI), which plays a central role in the regulation of nutrient-sensitive; target-of-rapamycin (TOR)-dependent gene expression programs; a pair of prefoldin β -subunits; and the TATA-binding protein-interacting proteins, TIP48 and TIP49, which are ATP-dependent helicases present in various chromatin remodeling complexes (11, 12). Hence, ART-27 associates with key components of the transcriptional machinery and likely serves to link AR to the URI transcription factor complex.

In addition to its transcriptional regulatory properties, ART-27 has also been demonstrated to be a component of the centrosome (13), and its binding partner, URI, is required for DNA stability in *Caenorhabditis elegans* (14). Therefore, ART-27 may also participate in pathways that are associated with the control of genome integrity.

ART-27 function has been examined in the prostate, where AR is known to play a crucial role in both prostate development and cancer. These studies indicate that ART-27 inhibits androgen-dependent cell proliferation in LNCaP prostate cancer cells (15). Consistent with a growth-inhibitory function, ART-27 protein expression is down-regulated in prostate cancer (15). In normal prostate, ART-27 expression is cell-type specific (15). In both fetal and adult prostate, ART-27 protein expression is restricted to luminal epithelial cells (terminally differentiated secretory cells surrounding the lumen) (15).

Like most transcription cofactors, little is known about the regulation of ART-27 expression. Previous studies have used chromatin immunoprecipitation (ChIP) assays to show that ART-27 is an E2F target gene (16, 17). Some E2F family members, such as E2F6, function as a transcriptional repressor through the recruitment of a polycomb repressive complex (PRC) (18–20). Consistent with the role of E2F in repression, deletion of two E2F binding sites in the ART-27 upstream regulatory region results in activation of the promoter in human embryonic kidney 293

cells. Moreover, ART-27 mRNA levels were increased upon reduction of E2F6 by small interfering RNA (siRNA) in 293 cells (16). ART-27 is likely subject to both positive and negative regulation during development in that ART-27 protein is detected only when the developing prostate gland has proceeded from a solid mass of undifferentiated cells to a stage where differentiated luminal epithelial cells are evident (15).

Here we report the analysis of the *cis*-acting DNA response elements and *trans*-acting factors that control ART-27 gene expression. We find that transcriptional regulation of ART-27 involves cell-specific repression that is relieved by the histone deacetylase inhibitor trichostatin A (TSA) as well as CREB-mediated activation of the ART-27 promoter.

RESULTS

Cell-Specific Regulation of ART-27 by TSA and Growth Factors

ART-27 protein is expressed at high levels in differentiated prostate luminal epithelial cells, but its expression is not detectable in undifferentiated precursors and stromal cells (15). The mechanism by which ART-27 expression is restricted to luminal cells remains largely unknown. Previous studies in 293 cells have shown that E2F transcription factors bind the ART-27 promoter and that the ART-27 mRNA level is increased by reducing expression of E2F6 by siRNA (16, 17). We hypothesize that ART-27 will not be repressed in a cell type in which it is ordinarily expressed *in vivo*, such as prostate epithelial cells. To test this idea, we examined the regulation of ART-27 in the LNCaP prostate epithelial cancer cell line, because LNCaP cells retain most of their luminal characteristics in culture, including AR expression (21).

We treated 293 and LNCaP cells with TSA, a histone deacetylase inhibitor, or dimethylsulfoxide (DMSO) vehicle control for 4 h and examined ART-27 mRNA levels. TSA increases ART-27 mRNA levels in 293 cells but does not affect ART-27 mRNA levels in LNCaP cells (Fig. 1A). ART-27 insensitivity to TSA is not restricted to LNCaP cells, because it is also observed in DU145 cells, an AR-negative prostate cancer line (data not shown). These results suggest that ART-27 gene expression is suppressed by a TSA-sensitive factor in 293 but not in LNCaP cells.

To examine positive regulation of ART-27, we determined ART-27 expression levels in response to extracellular signals. We observed a dose-dependent increase in ART-27 mRNA levels after stimulation of LNCaP cells with serum (Fig. 1B). By contrast, 293 cells do not induce ART-27 mRNA upon serum stimulation (Fig. 1B). These results indicate that ART-27 mRNA expression is serum responsive in LNCaP but not 293 cells. Thus, ART-27 gene expression is repressed by TSA-susceptible factors and can be in-

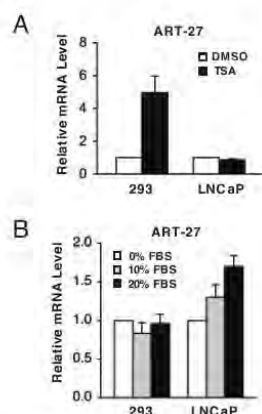


Fig. 1. Cell-Specific Regulation of ART-27 mRNA

A, Effect of TSA on ART-27 mRNA levels. 293 or LNCaP cells were deprived of serum for 24 h and treated with DMSO vehicle or 100 ng/ml TSA for 4 h. Total RNA was isolated and analyzed by RT-QPCR. Relative ART-27 mRNA levels are normalized to RPL19 and calibrated to untreated samples in each cell line, which were arbitrarily set to 1. **B,** Effect of Serum on ART-27 mRNA levels. 293 or LNCaP cells were serum starved for 24 h and then stimulated with serum for 3 h. Relative mRNA levels were determined as indicated in A.

duced by extracellular signals in cells not subject to this negative regulation.

The 5'-Regulatory Region of ART-27 Contains Positive *cis*-Acting Elements

To determine the regulatory elements that govern transcription of ART-27, about 2 kb of the ART-27 5'-regulatory region was placed upstream of a promoterless luciferase reporter gene. The ART-27-luciferase reporters containing $-2065/+124$, $-965/+124$, $-533/+124$, $-383/+124$, $-154/+124$, and $+16/+124$ were transfected into HeLa cells and assayed for luciferase activity (Fig. 2A). HeLa cells were used in these studies because they are easily transfected, unlike LNCaP cells. However, like LNCaP cells, ART-27 gene expression in HeLa cells is largely insensitive to negative regulation by E2F6 as it is in 293 cells (16). Few regulatory elements appear to lie within the $-2065/-383$ region of the ART-27 promoter, because constructs ending at different points within this region show equivalent activity (Fig. 2A). By comparison, an ART-27-luciferase reporter encompassing $-154/+124$ shows a 40% increase in activity, suggesting the presence of a weak inhibitory element between -383 and -154 bp (Fig. 2A). This observation is consistent with the reported location of an E2F transcription factor-binding site that is important for repression of ART-27 (16, 17). In contrast, decreased ART-27-luciferase reporter activity is observed with 5'-deletions from -154 to $+16$, suggesting that key regulatory

elements required for ART-27 promoter activity have been deleted (Fig. 2A).

To further map this regulatory region, a series of ART-27 5'-truncations from -154 to $+19$ bp were made and assayed for luciferase activity (Fig. 2B). Deletions from -154 to -53 bp did not compromise ART-27 promoter activity. In comparison, deletions from -53 to -14 bp reduce ART-27 promoter activity, suggesting that elements required for ART-27 expression lie between -53 and -14 bp upstream of the ART-27 start site of transcription.

A *cis*-Acting CRE Is Important for ART-27 Promoter Activity

To identify putative transcription factor-binding sites located between -53 and -14 bp, the DNA sequence of this region was analyzed using MatInspector software (22–24). The binding sites identified include a consensus CRE (-23 to -14), an Sp1 transcription factor-binding site (-41 to -31), and a CCAAT/enhancer-binding protein- α (C/EBP α)-binding site (-51 to -42). To test the functional relevance of these binding sites, we coexpressed CREB and Sp1 with various reporters containing or lacking their respective binding sites. Overexpression of CREB activated ART-27-luciferase reporter constructs containing the CRE (Fig. 3A). Overexpression of Sp1 also activates the ART-27 luciferase reporter (Fig. 3B). These findings suggest that basal factors, such as Sp1, and inducible factors, such as CREB, are important for induction of ART-27 expression.

The importance of the C/EBP α -binding site and the CRE for ART-27 promoter activity was assessed using ART-27-luciferase reporter constructs deleted of their respective DNA-binding elements (Fig. 4). ART-27 promoter activity was not reduced by deletion of the C/EBP α -binding site ($\Delta 1$) (Fig. 4B). In contrast, deletion of the CRE ($\Delta 2$) compromised ART-27 promoter activity by more than 50%, suggesting that the CRE but not the C/EBP α -binding site is important for ART-27 promoter activity.

We also investigated the requirement of the CRE sequence for ART-27 promoter activity by introducing a series of trinucleotide substitutions (S1–S8) spanning the CRE site and measuring the activities of the resulting ART-27-luciferase reporter constructs (Fig. 4B). ART-27 promoter activity was largely unaffected by nucleotide substitutions flanking the CRE (S1–S3, S7, and S8; Fig. 4B). However, mutations within the CRE (S4–S6) compromise promoter activity, indicating that this sequence is important for ART-27 expression. This result suggests that a sequence-specific transcription factor binding the CRE element, such as CREB, is important for the induction of ART-27 gene expression.

A CREB Family Member Binds to the CRE *in Vitro*

We next sought to determine whether this CRE serves as a binding site for CREB or another factor by EMSA.

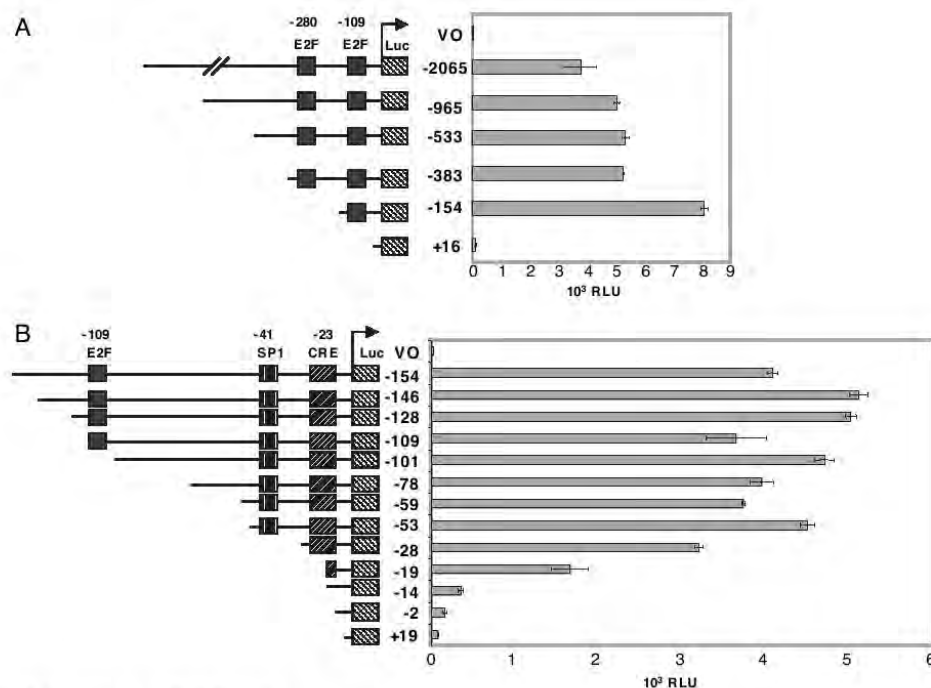


Fig. 2. Mapping the ART-27 Promoter

A. Large-scale deletion analysis of the ART-27 5'-regulatory region. HeLa cells were transfected with a series of human ART-27-luciferase reporter constructs or the empty pGL3 vector (VO), along with Lac-Z to control for transfection efficiency for 24 h. The locations of previously identified E2F-binding sites are indicated. Relative luciferase units (RLU) normalized to β -galactosidase activity are shown. **B.** Fine-scale analysis of the ART-27 -154/+19-bp region. Luciferase assay was performed as described in A. RLU normalized to β -galactosidase activity are shown. The locations of consensus Sp1-binding site and CRE are indicated, in addition to the E2F-binding site.

Upon incubation with HeLa cell nuclear extracts, we observed a shift in mobility of the labeled oligonucleotide probe spanning the CRE (-28/-7). This binding is competed by an excess of unlabeled probe (lane 2 vs. 3) and can also be competed with a consensus sequence for CREB binding (lane 6) but not consensus C/EBP α - and Sp1-binding sites (lanes 4–5) (Fig. 4C). Although probes containing substitutions S3 or S7, which fall outside the CRE, still compete, probes containing substitutions S4–S6 within the CRE fail to compete with the wild-type-labeled probe for protein binding (lanes 7–14), indicating sequence-dependent recognition of the CRE by the bound protein (Fig. 4C).

Upon incubation with CREB antibody, but not C/EBP α antibody, a supershift in probe mobility is also observed (lanes 15–17) (Fig. 4C). Because the CREB antibody used in this experiment also reacts with activating transcription factor-1 (ATF-1) and cAMP-responsive element modulator, we are unable to exclude association of these two factors with the CRE at this point. These results indicate that CREB or a related

family member associates with the ART-27 CRE in a sequence-specific manner.

CREB Is Recruited to the ART-27 Promoter

To determine whether CREB is specifically recruited to the CRE of the ART-27 gene, ChIP assays were performed (Fig. 5). LNCaP cells were cross-linked with formaldehyde, chromatin was prepared and sheared, and the cross-linked protein-DNA complexes were precipitated with antibodies against CREB or control IgG. PCR was then performed on the precipitated DNA fragments to amplify the CRE-binding site 23 bp upstream of the start site of transcription. A region approximately 5 kb upstream of the ART-27 promoter [upstream region (UPS)] was amplified as a control for ChIP specificity. We observed an 8-fold enrichment of CREB at the CRE relative to the UPS in LNCaP cells (Fig. 5B).

We also examined whether CREB could occupy the ART-27 regulatory region in 293 cells by ChIP. Recall

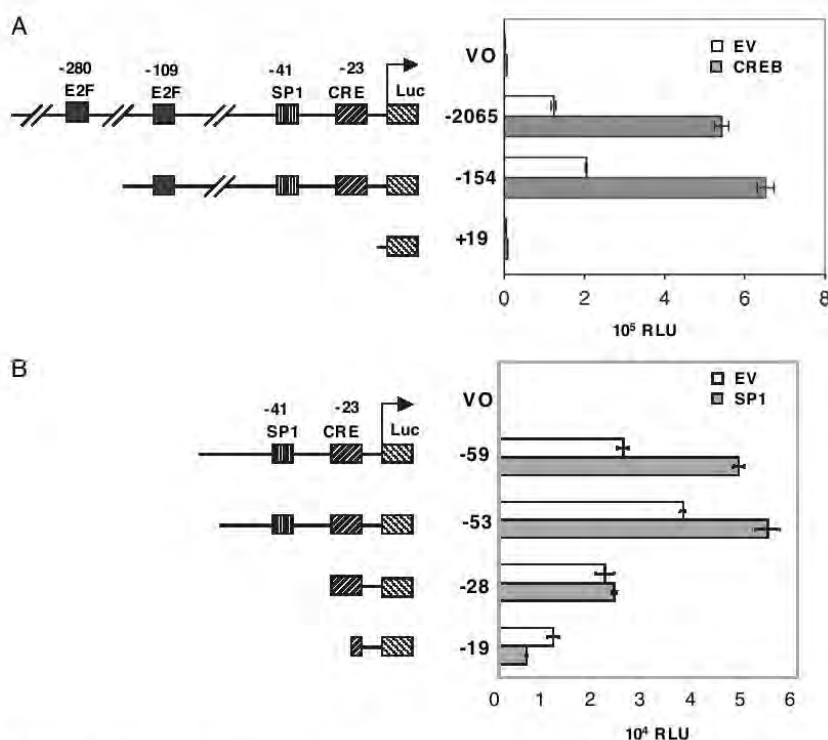


Fig. 3. CREB and Sp1 Enhance ART-27 Promoter Activity

A, CREB activates ART-27-luciferase reporter constructs. HeLa cells were seeded in 24-well plates and transfected for 24 h with either the 0.1 μ g empty pGL3 vector (VO) or ART-27-luciferase reporter, with 10 ng Lac-Z and 0.1 μ g empty vector (EV) or CREB. Relative luciferase units (RLU) normalized to β -galactosidase activity are shown. B, Sp1 also activates ART-27-luciferase reporter. Cells were seeded as above and transfected with 0.1 μ g empty pGL3 vector (VO) or ART-27-luciferase reporter, 10 ng of Lac-Z plasmids, and 0.1 μ g empty vector (EV) or Sp1 for 24 h. RLU were normalized to β -galactosidase activity from three independent experiments. Error bars represent sd.

that ART-27 displays basal mRNA expression in 293 cells that is not induced by serum but can be further elevated by TSA. We found a 3-fold enrichment of CREB at the CRE relative to the UPS in 293 cells (Fig. 5B). Thus, CREB binds the ART-27 CRE *in vitro*, activates the ART-27 promoter in reporter assays (Figs. 3A and 4C), and occupies the CRE region of the ART-27 promoter in LNCaP and, to a lesser extent, in 293 cells.

Histone Modifications at the ART-27 Promoter

To determine whether there are cell-specific differences in chromatin modification near the ART-27 CRE, we compared the levels of repressive and active histone marks, trimethylated (3Me)-H3K27 and acetylated (Ac)-H3K9/14, respectively, at the ART-27 promoter in 293 and LNCaP cells by ChIP. We detected higher levels of the active histone H3 modification Ac-H3K9/14 at the ART-27 regulatory region in LNCaP

than in 293 cells (Fig. 5C). This suggests that in LNCaP cells the ART-27 promoter is in a chromatin context permissive for activation and is consistent with the robust induction of ART-27 expression by CREB observed in LNCaP as compared with 293 cells.

By contrast, a greater amount of the repressive chromatin mark 3Me-H3K27 was observed at the ART-27 CRE in 293 relative to LNCaP cells (Fig. 5C), suggesting that the ART-27 promoter is in a repressive chromatin environment in 293. These results indicate that cell-specific regulation of ART-27 mRNA is associated with differences in histone modification at the ART-27 promoter.

Epidermal Growth Factor (EGF) Induces ART-27

To mediate transcription, CREB is activated by phosphorylation at serine 133 (S133). This phosphorylation is mediated by several kinases including protein kinase A in response to increased cAMP levels, protein

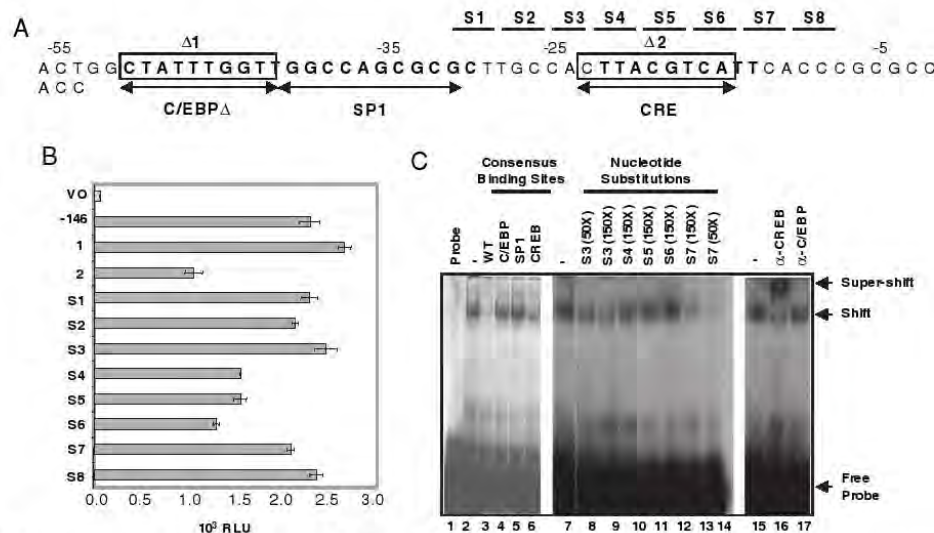


Fig. 4. Fine-Structure Analysis of the ART-27 Regulatory Region

A. Sequence of the ART-27 5'-regulatory regions responsible for mediating transcriptional activation. Below the sequence are the predicted transcription factors binding sites. The consensus C/EBP α -binding site (Δ 1) or CRE (Δ 2) within the -146/+19-Luc reporter were deleted. In addition, trinucleotide substitutions (S1-S8) were introduced at the indicated regions. **B.** Functional analysis of regulatory regions controlling ART-27 expression. HeLa cells were transfected with the indicated ART-27-luciferase reporter constructs as described in Fig. 2. **C.** A CREB family member binds the functional response element in the ART-27 gene regulatory region. EMSA was performed using an oligonucleotide containing sequences derived from -28/+7 of the wild type (WT) 5'-regulatory region of the ART-27 gene (probe) incubated with HeLa cell nuclear extracts. Binding to the probe was competed with the indicated molar excess of unlabeled WT, consensus transcription factor-binding sites (left), or mutant (S3-S7) sequences (middle). The binding mixtures were also incubated with the indicated antibodies before resolution on polyacrylamide gel (right).

kinase B/Akt upon activation of the phosphatidylinositol 3-kinase pathway, and the 90-kDa ribosomal protein S6 kinases (RSKs and MSKs) upon activation of the MAPK pathway (25–29). Although many peptide growth factors can activate these pathways, we examined the role of CREB activation in ART-27 gene expression by EGF, because EGF plays an important role in prostate development and cancer, and expression of its receptor, EGFR/ErbB1 is androgen sensitive in LNCaP cells (30–32).

EGF stimulation leads to S133 phosphorylation of CREB in both 293 and LNCaP cells (Fig. 6A), suggesting that EGF activates CREB in both cell types. However, EGF-dependent phosphorylation of CREB was much more robust in LNCaP compared with 293 cells. For example, after 10 min of EGF stimulation, CREB phosphorylation is enhanced only 2-fold in 293 cells but nearly 10-fold in LNCaP cell (Fig. 6A). In addition, 293 cells show constitutive phosphorylation of ATF-1 compared with LNCaP cells (Fig. 6A).

We next examined EGF-dependent recruitment of CREB to ART-27 and found that CREB and its coactivator p300 are recruited to the ART-27 promoter in an EGF-dependent manner in LNCaP but not 293 cells (Fig. 6B). Consistent with this finding, LNCaP cells up-regulate ART-27 mRNA when stimulated with EGF (Fig. 6C), whereas 293 cells do

not. And although TSA increases ART-27 mRNA level in 293 cells, treatment with EGF does not further enhance the expression of ART-27 (Fig. 6C).

The lack of EGF-dependent recruitment of CREB and regulation of ART-27 mRNA in 293 cells is consistent with repressive histone modifications detected at the ART-27 promoter (Figs. 5 and 6). TSA does not affect CREB recruitment or Ac-H3K9/14 levels at the ART-27 promoter in 293 cells (supplemental Fig. S2A, published as supplemental data on The Endocrine Society's Journals Online web site at <http://mend.endojournals.org>), suggesting that repression of ART-27 expression is dominant over activation.

Induction of ART-27 by EGF in LNCaP cells is also observed in DU145 cells, which like LNCaP cells show strong EGF-dependent phosphorylation of CREB (data not shown). These results indicate that EGF leads to robust S133 phosphorylation of CREB, enhances recruitment of CREB and p300 to the ART-27 promoter, and increases expression of ART-27 mRNA in LNCaP but not 293 cells.

CREB Mediates Induction of ART-27 by EGF

To determine whether CREB is required for EGF-dependent induction of ART-27, we depleted CREB ex-

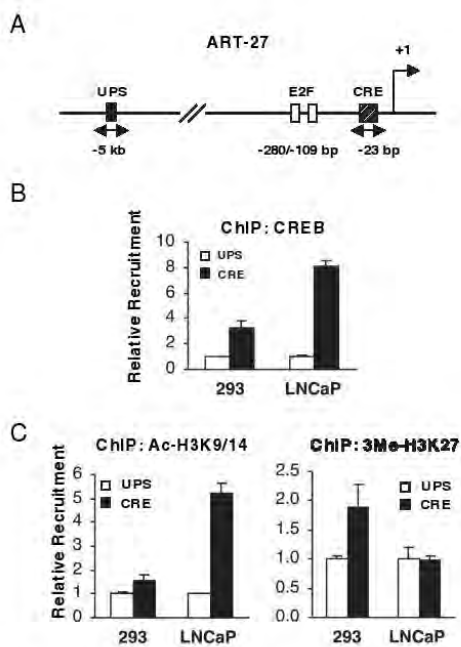


Fig. 5. CREB Recruitment and Chromatin Modification at the ART-27 Promoter

A, Schematic illustration of the ART-27 5'-regulatory region. CREB and E2F binding sites are shown as *hatched* and *white* boxes, respectively, whereas the *black* box upstream represents a region that serves as negative control for the ChIP assays. PCR primers (*arrows*) spanning a control UPS (–4966 bp) or the CRE (–23 bp) were used to amplify input and precipitated DNA. B, CREB recruitment to ART-27. ChIPs were performed on 293 and LNCaP cells with antibody against CREB. C, Histone H3 modifications at the ART-27. ChIP assays were performed on 293 and LNCaP cells with antibodies against Ac-H3K9/14 (activating mark) and 3Me-H3K27 (repressive mark). Recruitment was normalized to input and shown as fold recruitment relative to UPS, which was arbitrarily set as 1. Data were averaged from three independent experiments. The *error bars* represent *so*.

pression in LNCaP cells using RNA interference (RNA-i) and assessed the effect of EGF on ART-27 gene expression. LNCaP cells transfected with CREB siRNA express approximately 70% less CREB protein than control cells (Fig. 7A). Not only are CREB levels reduced, but the active S133 phosphorylated form of CREB is also decreased, whereas EGF-dependent phosphorylation of ATF-1 and ERK1/2 as well as total ERK1/2 protein levels are unaffected (Fig. 7A). Importantly, EGF fails to induce ART-27 expression if CREB expression is reduced in LNCaP cells (Fig. 7B), indicating that CREB is required for EGF-induction of ART-27.

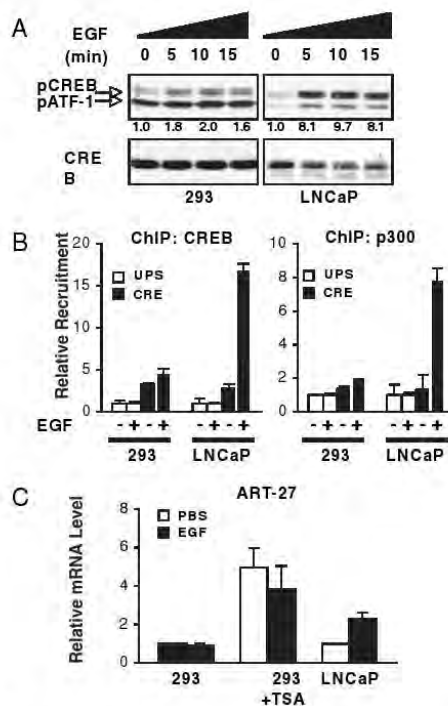


Fig. 6. EGF-Dependent Recruitment of CREB and Induction of ART-27

A, Activation of CREB by EGF. Western blot analysis for pCREB, pATF-1, and CREB in whole-cell lysates of 293 and LNCaP cells serum starved for 24 h and then treated with 100 ng/ml EGF for 0, 5, 10, and 15 min. The *numbers below the blots* represent the ratio of pCREB to total CREB levels. B, EGF-dependent recruitment of CREB to ART-27. 293 and LNCaP cells were serum starved for 24 h and stimulated with 100 ng/ml EGF for 20 min. ChIP assays were performed as described in Fig. 5 using CREB and p300 antibodies. C, Effect of EGF on ART-27 gene expression. Cells were deprived of serum for 24 h, treated with DMSO or 100 ng/ml TSA for 1 h, and then stimulated with PBS vehicle or 100 ng/ml EGF for 3 h. Total RNA was isolated and analyzed by RT-QPCR. Relative ART-27 mRNA levels normalized to RPL19 are shown.

To further investigate the function of CREB activation in ART-27 gene expression, 293 and LNCaP cells were treated with the MAPK kinase inhibitor U0126. In 293 cells, U0126 treatment abolished ERK1/2 phosphorylation but had no effect on CREB phosphorylation (supplemental Fig. S2B). Likewise, U0126 did not affect ART-27 mRNA expression or induction by TSA in 293 cells (data not shown; supplemental Fig. S2C). These results suggest that CREB phosphorylation and TSA induction of ART-27 occurs through an ERK-independent mechanism in 293 cells.

In contrast, U0126-treated LNCaP cells showed reduced CREB and ERK1/2 phosphorylation upon EGF

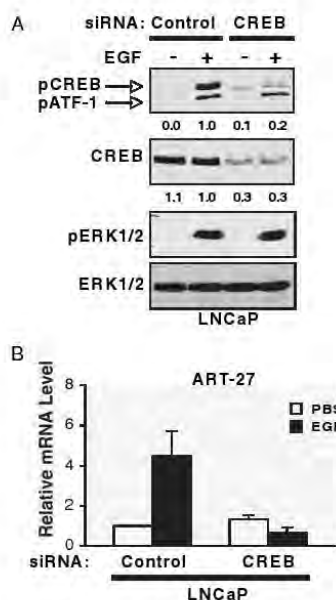


Fig. 7. CREB Mediates Induction of ART-27 by EGF

A, Transient depletion of CREB via RNA-i. LNCaP cells were transfected with nonsilencing siRNA (control) or siRNA against CREB. After 48 h, cells were serum starved for 24 h and then stimulated with 100 ng/ml EGF for 5 min. Whole-cell lysates were analyzed by Western blot. Relative pCREB and CREB levels are indicated as numbers below the blots. B, Effect of CREB depletion on ART-27 mRNA level. LNCaP cells were transfected and serum starved as described above and then stimulated with EGF for 3 h. Total RNA was analyzed by RT-QPCR. Relative ART-27 mRNA levels normalized to RPL19 are shown.

stimulation (Fig. 8A). Notably, LNCaP cells treated with U0126 fail to induce ART-27 mRNA in response to EGF (Fig. 8B). The effect of EGF activation on ART-27 expression is also observed at the protein level. LNCaP cells stimulated with EGF up-regulate ART-27 protein in a dose-dependent manner (Fig. 8C), and U0126 blocks this induction (Fig. 8D). These results indicate that ERK activation in LNCaP cells is associated with CREB phosphorylation and induction of ART-27 gene expression in response to EGF.

Expression and S133 Phosphorylation of CREB during Prostate Development

We have previously shown that ART-27 is regulated during human prostate development (15). Early in development, ART-27 is not detected in the undifferentiated prostatic buds that lack a defined lumen, whereas later in development, ART-27 is detected when the buds differentiate and contain a well-defined lumen (15). Moreover, in the developing urogenital sinus from which the prostate develops, and in adult

prostate, ART-27 protein is undetectable in smooth muscle and other stromal cells (15).

Because we have shown that CREB mediates induction of ART-27 in cultured prostate cells, we examined CREB and phospho-CREB (pCREB) expression in human prostate development. Sections of early (15-wk) and late (21 wk) urogenital sinus were stained using CREB and pCREB (S133) antibodies. Early in development, both the stromal and epithelial cells surrounding the urethra and prostatic buds stain for CREB (Fig. 9A). In contrast, stromal cells do not stain for pCREB, whereas pCREB antibody stains a majority of epithelial cells (Fig. 9B). Later in development, there is still virtually no pCREB immunoreactivity in stromal cells (Fig. 9D), but pCREB staining remains detectable in luminal epithelial cells (Fig. 9D). These results are consistent with CREB activation preceding epithelial cell-specific induction of ART-27 and suggest that activated CREB mediates ART-27 induction in prostate epithelial cells.

DISCUSSION

ART-27 is an epithelial cell-specific AR cofactor that is regulated in both prostate development and cancer (11, 15). In this study, we define the *cis*-acting DNA regulatory elements and *trans*-acting factors controlling ART-27 gene expression. We show that ART-27 expression is regulated through cell type-specific transcriptional mechanisms. E2F transcription factors have previously been shown to repress ART-27 mRNA expression in 293 cells (16, 17). E2Fs are not only transcriptional activators, but certain family members, such as E2F6 are also repressors that recruit EZH2-containing PRCs (19, 20, 33). PRC2 contains class I histone deacetylase (HDAC) activity and thus are sensitive to inhibition by TSA (34). TSA induces ART-27 mRNA in 293 but not LNCaP cells (Fig. 1A), suggesting that transcriptional repression of the ART-27 gene involves cell-specific factors such as E2F6 and EZH2. This effect is specific for type 1 but not type 3 HDAC because inhibition of type 3 NAD⁺-dependent HDACs by nicotinamide does not induce ART-27 expression (data not shown). EZH2 specifically trimethylates histone H3 on lysine 27 (3Me-H3K27), and this modification is associated with gene repression by EZH2/PRC2 (35, 36). Consistent with this idea, a 3Me-H3K27 mark is detected at the ART-27 promoter in 293 cells but not LNCaP cells (Fig. 5C), and E2F6 mRNA levels are higher in 293 and a prostate stromal cell line as compared with LNCaP cells (supplemental Fig. S1). Our results indicate that a type 1 HDAC is involved in repression of ART-27 expression.

CREB is constitutively active, relatively insensitive to EGF stimulation, and occupies the ART-27 regulatory region in 293 cells, and this likely results in the basal ART-27 mRNA expression and lack of EGF induction observed in this cell type. In contrast, in LNCaP cells, CREB is activated in response to EGF and is recruited to the ART-27 promoter to induce ART-27 gene expression

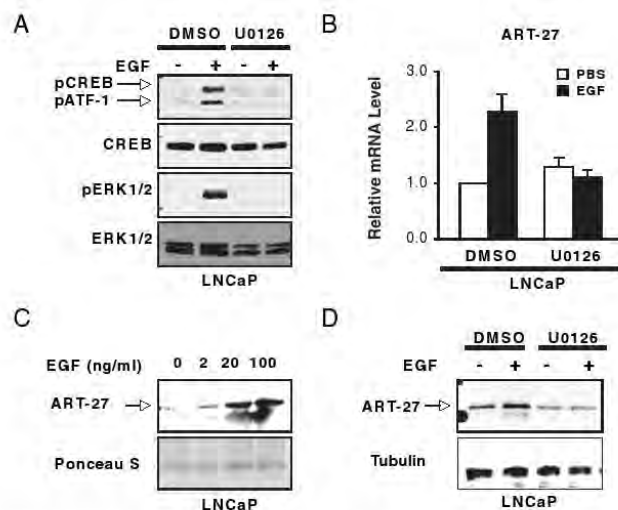


Fig. 8. The MAPK Kinase Inhibitor U0126 Blocks Induction of ART-27 by EGF

A, Effect of U0126 on CREB activation by EGF. LNCaP cells were serum starved for 24 h, pretreated with DMSO vehicle or 10 μ M U0126 for 1 h, and then stimulated with PBS vehicle (–) or 100 ng/ml EGF for 5 min. Whole-cell lysates were analyzed by Western blot. **B,** Effect of U0126 on ART-27 mRNA levels. LNCaP cells were deprived of serum for 24 h, pretreated with DMSO vehicle or 10 μ M U0126 for 1 h, and then stimulated with PBS vehicle or 100 ng/ml EGF for 3 h. Total RNA was analyzed by RT-QPCR. Relative ART-27 mRNA levels normalized to RPL19 are shown. **C,** LNCaP cells up-regulate ART-27 protein in response to EGF. LNCaP cells were seeded equally in a 24-well dish, deprived of serum for 24 h, and treated with PBS vehicle (0) or 2, 20, or 100 ng/ml EGF for 3 h. Whole-cell lysates were analyzed by Western blot. **D,** U0126 inhibits EGF induction of ART-27 protein. LNCaP cells were deprived of serum for 24 h, pretreated with DMSO vehicle or 10 μ M U0126 for 1 h, and then stimulated with PBS vehicle or EGF for 3 h. Whole-cell lysates were analyzed by Western blot.

(Figs. 5–7). Our data suggest that the pattern of ART-27 gene expression is a result of E2F/EZH2/PRC2-mediated repression in undifferentiated epithelial precursors and CREB-mediated activation of the ART-27 promoter in differentiated luminal cells (Fig. 10).

The activation of CREB and induction of ART-27 in epithelial cells during prostate development is likely mediated by EGFR signaling. It is likely that stromal cells secrete paracrine factors, such as EGF and keratinocyte growth factor, whose receptors (EGFR/ErbB1 and KGFR/FGFR2, respectively) are expressed exclusively by epithelial cells (37, 38) and induce ART-27 mRNA expression. Therefore, EGFR signaling is a good candidate pathway to govern CREB activation and ART-27 expression during prostate development.

What is the mechanism underlying diminished ART-27 expression in prostate cancer? Although up-regulation of the E2F/EZH2/PRC2 transcriptional repression complex or reduced phosphorylation and recruitment of CREB to the ART-27 promoter are attractive mechanisms for reduced ART-27 expression, it is not clear that the down-regulation of ART-27 protein observed in prostate cancer occurs at the level of transcription. It is conceivable that changes in ART-27 translation and/or degradation could also affect its expression. Indeed, expression profiling studies suggest that ART-27 mRNA is present at roughly

similar levels throughout the stages of prostate cancer (39, 40), despite clear indications that ART-27 protein levels are reduced in human prostate cancer (15). In addition, we have recently shown that a somatic alteration in AR associated with prostate cancer (AR-P340L) shows a diminished transcriptional response to ART-27 and may bypass the need for ART-27 in AR-dependent cell growth suppression (41). Therefore, it is likely that multiple mechanisms underlie reduced ART-27 function in prostate cancer.

Based on these findings, we propose that developmental regulation of ART-27 expression is important in restraining AR-mediated prostate epithelial cell proliferation by regulating a subset of AR-responsive genes important to prostate growth inhibition and differentiation. This implies that alterations in the level of ART-27 modulate AR target gene selectivity, which, in turn, affects AR-dependent cell growth regulation, a hypothesis we are currently testing.

MATERIALS AND METHODS

Cell Culture

LNCaP cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and 1% penicillin-streptomycin (PS) (Mediatech/Cellgro, Herndon, VA). HEK-293T (293) cells:

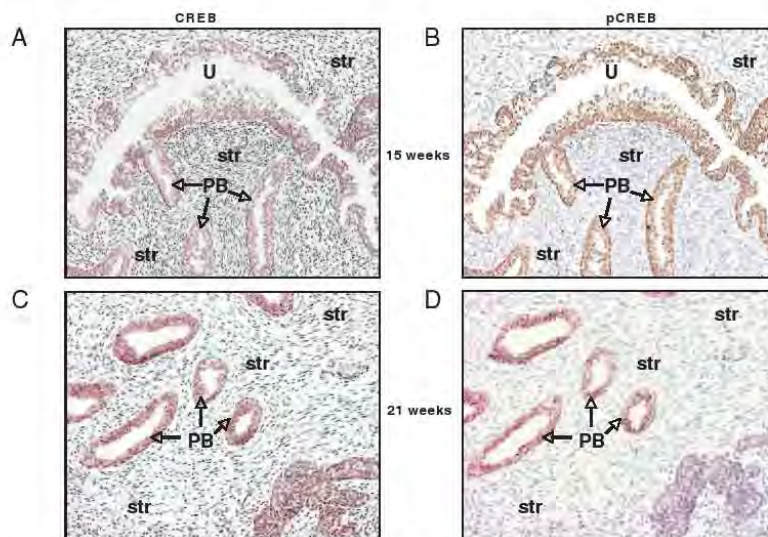


Fig. 9. Expression of CREB and pCREB in Human Prostate Development

Paraffin-embedded sections of 15-wk-old (A and B) and 21-wk-old (C and D) human fetal urogenital sinus were stained with CREB (A and C) and pCREB (S133) (B and D) antibodies. A and C show that cells in the stroma (str) and epithelial cells surrounding the lumen of the urethra (U) and prostatic buds (PB) express CREB. B and D show that CREB is phosphorylated at S133 in epithelial cells but not in most stromal cells.

were cultured in DMEM (Cellgro) supplemented with 10% FBS and 1% PS. HeLa cells were cultured in DMEM supplemented with 10% FBS and 2 mM L-glutamine (Invitrogen) and 1% PS. Cells were maintained at 5% CO₂ in a 37°C incubator. The prostate stromal cells immortalized with hTERT, were a kind gift from Dr. Peng Lee (NYU School of Medicine) and were maintained in RPMI 1640 medium plus 10% FBS and 1% PS.

Real-Time Quantitative PCR (QPCR)

Total RNA was isolated using the RNeasy kit with on-column DNase digest (QIAGEN Inc., Valencia, CA). Total RNA was

reverse transcribed at 55°C for 1 h, using Superscript III reverse transcriptase and oligo-(dT)₂₀ primers (Invitrogen). Real-time PCR was performed using specific primers to ART-27 (forward 5'-CAACAGCCTCACCAGGACT-3' and reverse 5'-TCTGCAGGCCTGTAGTCTC-3' or forward 5'-CTGGAGTTGACACTGGCAGA-3' and reverse 5'-AGTCCTTGGTGAGGCTGTTG-3') or ribosomal protein L19 (RPL19, forward 5'-CACAAGCTGAAGGCAGACAA-3' and reverse 5'-GGGTGCTTCCTTGGTCTTAG-3') and 2× SYBR green Taq-ready mix (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's directions. Amplifications were performed at 60°C in a Roche Lightcycler (Roche, Indianapolis, IN). No signal was detected in reactions performed without prior

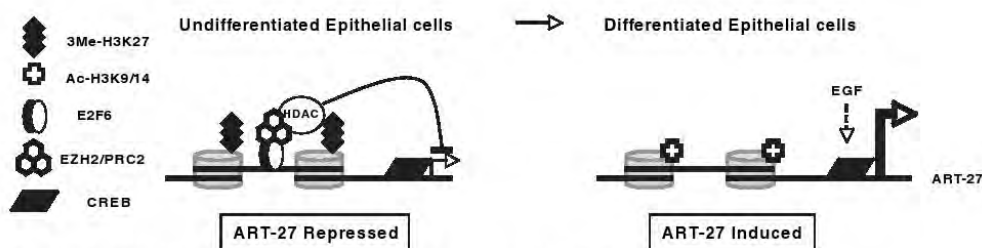


Fig. 10. Model for Regulation of ART-27 Gene Expression in Prostate Epithelial Cells

We propose that in undifferentiated cells (left), ART-27 is repressed by an E2F6/EZH2/PRC2 complex that trimethylates histone H3K27 (3Me-H3K27). PRC2 contains a type 1 HDAC that accounts for enhanced ART-27 gene expression in 293 cells upon TSA treatment. In differentiated cells (right), the level or activity of the E2F6/EZH2/PRC2 complex is reduced, and ART-27 gene expression is no longer repressed. TSA has no effect on ART-27 mRNA, and 3Me-H3K27 is not detected at the ART-27 promoter. In response to EGF or other growth factors, CREB increasingly occupies the ART-27 promoter, recruits coactivators (e.g. p300) that acetylates histone H3K9/14 (Ac-H3K9/14), and results in activation of ART-27 gene expression.

reverse transcription. Reactions with dissociation curves that do not show a single, sharp peak were excluded from analysis. Relative mRNA levels were determined as previously described, using RPL19 as control (42). Error bars represent sd between replicates.

Cloning and Construction of ART-27 Promoter Reporter Plasmids

Genomic sequences between –2060 and +346 bp from the ART-27 transcription-initiation site (+1 bp) were retrieved from the human genome database and amplified from normal human genomic DNA by PCR using oligonucleotides 5'-GCATGGT-(G)A(G)CCTCAGCGCTGTAATC-3' and 5'-GCAAGCT(G)CGA-GGTTCCAGCCTTC-3'. Bases in parentheses were changed to the underlined bases to generate *KpnI* restriction sites for subcloning. The resulting product was cloned into the *EcoRV* site of pBluescript SK+ (Stratagene, La Jolla, CA). ART-27-regulatory regions were cloned into a pGL3 basic plasmid (Promega, Madison, WI) to generate the reporter constructs. All constructs were verified by restriction digest and sequenced. Transcription factor analysis of the promoter was performed using MatInspector (22). Deletions $\Delta 1$ and $\Delta 2$ and mutations S1–S8 in the ART-27 regulatory region were generated using the QuikChange site-directed mutagenesis kit (Stratagene) and oligonucleotides listed in Supplemental Table 1 according to the manufacturer's recommendations.

Luciferase Assay

HeLa and 293 cells were seeded in a 24-well plate at a density of 3×10^4 or in a six-well plate at a density of 1.5×10^5 in phenol red-free DMEM supplemented with 10% charcoal-stripped FBS. Transfection was performed using Lipofectamine Plus (Invitrogen) reagent according to the manufacturer's instructions. For transfection of cells in 24-well plates, each well received 100 ng of the control pGL3 or ART-27 regulatory region-luciferase reporter plasmid, and 10 ng of CMV-LacZ. After 4 h, transfection mixtures were removed, and the cells were refed with phenol red-free medium plus 10% FBS. After 24 h, the transfectants were washed with PBS and lysed in 1× luciferase cell culture lysis reagent (Promega). The cell extracts were analyzed for luciferase activity, and the values were normalized to β -galactosidase activity, except where indicated. Luciferase activity was quantified in a reaction mixture containing 15 μ l lysate and 100 μ l luciferase assay reagent [25 mM glycylglycine (pH 7.8), 10 mM MgSO₄, 1 mM ATP, 0.1 mg/ml BSA, 1 mM dithiothreitol], using an LMax microplate reader luminometer and 1 mM D-luciferin as substrate.

EMSA

Double-stranded oligonucleotides were end-labeled with [³²P]_γATP by using T4-polynucleotide kinase. HeLa cell nuclear extracts (10 μ g) was added to the radiolabeled double-stranded oligonucleotides in a total volume of 20 μ l with 2.5 μ g poly (dl-dC) and 1 μ g herring sperm DNA in binding buffer [10 mM Tris (pH 8.0), 40 mM KCl, 0.05% Nonidet P-40, 6% glycerol, and 1 mM dithiothreitol] and incubated for 30 min at room temperature. C/EBP α consensus was 5'-TGCAGATTGCGCAATCT-GCA-3' (Santa Cruz sc-2525; Santa Cruz Biotechnology, Santa Cruz, CA); CREB consensus was 5'-AGAGATTGCTGACGT-CAGAGAGCTAG-3' (Santa Cruz sc-2504); and Sp1 consensus was 5'-ATTCGATCGGGGCGGGGCGAGC-3' (Santa Cruz sc-2502). Binding reactions were resolved on 6% nondenaturing polyacrylamide gels in 0.25× Tris-borate-EDTA buffer at room temperature. Gels were dried before autoradiography. Antibodies against CREB (Sc-186X) and C/EBP α (Sc-9314X) were purchased from Santa Cruz Biotechnology.

ChIP Assay

LNCaP and 293 cells cultured in medium supplemented with 10% FBS for 96 h were cross-linked in 50 mM HEPES (pH 8.0), 1 mM EDTA (pH 8.0), 100 mM NaCl, 11% formaldehyde at room temperature for 10 min. Cross-linking was stopped upon incubation in 0.1 M glycine for 5 min. The fixed cells were washed twice, lifted in cold PBS, and then centrifuged for 5 min. Chromatin was prepared as previously described (43), with some modifications. Nuclei were washed and resuspended in 3 ml modified RIPA buffer [1% Triton X-100, 0.1% deoxycholate, 1 mM EDTA, 0.5 mM EGTA, 10 mM Tris-HCl (pH 8.0), 140 mM NaCl, and 1× protease inhibitor cocktail (Sigma)], sonicated, and centrifuged for 10 min at 4°C. The supernatant was precleared with a protein A-agarose, sheared salmon sperm DNA slurry (Upstate USA, Inc., Lake Placid, NY), centrifuged, and incubated with CREB (48H2) (Cell Signaling Technology, Danvers, MA), p300 (C-20) (Santa Cruz), acetylated histone H3K9/14 (Abcam, Cambridge, MA), or trimethylated histone H3K27 (Abcam) antibodies overnight at 4°C. Protein A-agarose plus sheared salmon sperm DNA slurry was then added, and incubation was continued for 1–2 h. The beads were subject to three sequential 10-min washes with buffers I [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 150 mM NaCl], II [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 500 mM NaCl], and III [0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.1)], and rinsed twice in Tris-EDTA buffer. Samples were resuspended in 100 μ l proteinase K-SDS (0.5% SDS, 200 μ g/ml Proteinase K in Tris-EDTA buffer) and incubated at 55°C for 3 h and then at 65°C overnight to reverse the cross-link. DNA was purified using QIAquick PCR purification kit (QIAGEN). Real-time PCR was performed at 60°C using 2 μ l of the DNA. The PCR primers used are as follows: –5 kb UPS, forward 5'-CTTGAAAG-CAGGAGGAAACG-3' and reverse 5'-TTCTGGCTTCCAT-GTTTCC-3', and CRE, forward 5'-TGCCACTTACGTTCAT-TCACC-3' and reverse 5'-CCAGCAATAAGAAC GGTTGG-3'.

RNA-i

CREB-1 SMARTpool siRNA was purchased from Dharmacon (Lafayette, CO). LNCaP cells were transfected for 4 h with 100 nM nonsilencing or CREB-1 siRNA using Lipofectamine 2000 (Invitrogen). The cells were then allowed to recover for 48 h. Subsequently, the medium was changed to phenol red- and serum-free RPMI 1640 for an additional 24 h before EGF treatment.

Western Blot

Whole cells were lysed in the presence of 1% protease-inhibitors cocktail and 1 mM Na₂VO₄. Samples were subject to SDS-PAGE, transferred onto Immobilon (Millipore, Billerica, MA), and probed with rabbit affinity-purified ART-27 (15), tubulin (Covance, Princeton, NJ), and ERK1/2 (9102), phospho-ERK1/2 (Thr202/ Tyr204) (9101S), pCREB (S133) (9191), and CREB (48H2) antibodies (Cell Signaling Technology). Membranes were then washed and incubated with antimouse or antirabbit, horseradish peroxidase-conjugated secondary antibodies (KPL, Gaithersburg, MD) for 1 h. After washing, signals were detected on x-ray film using the ECL chemiluminescent detection kit (GE Health Sciences, Boston, MA). Quantitation was performed using a GS-800 calibrated imaging densitometer (Bio-Rad, Hercules, CA).

Immunohistochemistry

The NYU School of Medicine Institutional Review Board approved the use of all human samples. Paraffin-embedded human fetal prostate tissues and immunohistochemistry pro-

cedures used in this study have been previously reported (15). CREB and pCREB antibodies were purchased from Cell Signaling Technology.

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Address all correspondence and requests for reprints to: Michael J. Garabedian, Department of Microbiology, 550 First Avenue, New York, New York 10016. E-mail: garabm01@med.nyu.edu.

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Supplementary Figure Legends

Figure S1: Relative mRNA levels of E2F family members, EZH2 and SUZ12. Real time qPCR analysis of total RNA isolated from 293, LNCaP, and prostate stromal primary cells immortalized with hTERT, using the indicated gene-specific primers and normalized to RPL19. The mRNA level of each gene in LNCaP cells is arbitrarily set to 1.

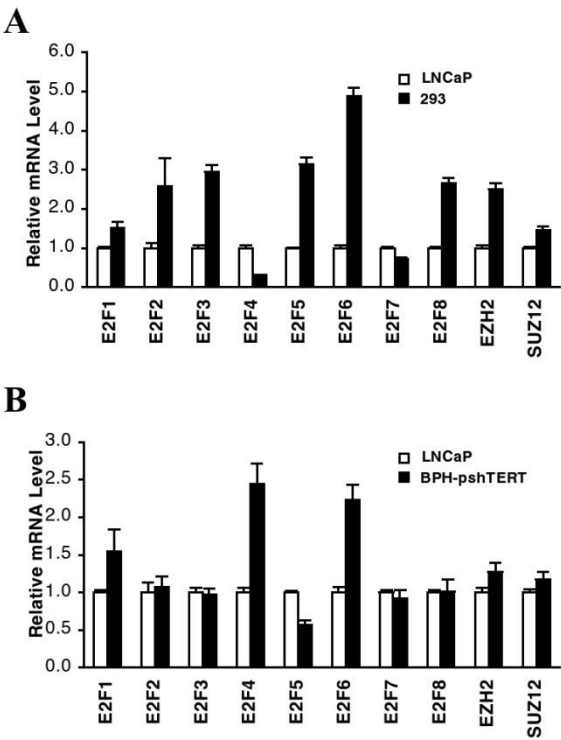
The PCR primers used are as follows:

	<u>Forward (5'-3')</u>	<u>Reverse(5'-3')</u>
E2F1	GGAAAAGGTGTGAAATCCC	CTTCTTGGCAATGAGCT
E2F2	ACAGTGATTGCCGTCAAGG	TGCAGGTTGTCTCAGTCCT
E2F3	ATATCCCTAAACCCGCTTCC	TGAGCAATCGCTATGTCCTG
E2F4	CCCACAGGTGTTTTGGAAC	CACTCTCGTGTGGGATCAAA
E2F5	TGTCTTCTGACGTGTTTCCTC	TGTAGTCATCTGCCGGGGTA
E2F6	GTGTCGGGACCTCTTCATCT	CTTCTTCCTCAGGGCCTTCT
E2F7	CCAGCCACTAAAAGGCAAAG	GAGGCAAGGTCTGTGGAATC
E2F8	ATGCTCGAGGACAGTGGTTC	CAAGGTTGCGGAGACATTTT
EZH2	GACTGGCGAAGAGCTGTTTT	TCGATGCCGACATACTTCAG
SUZ12	GCATGG GTTTATTGCTGACA	CAAACAGCATACAGGCATGA

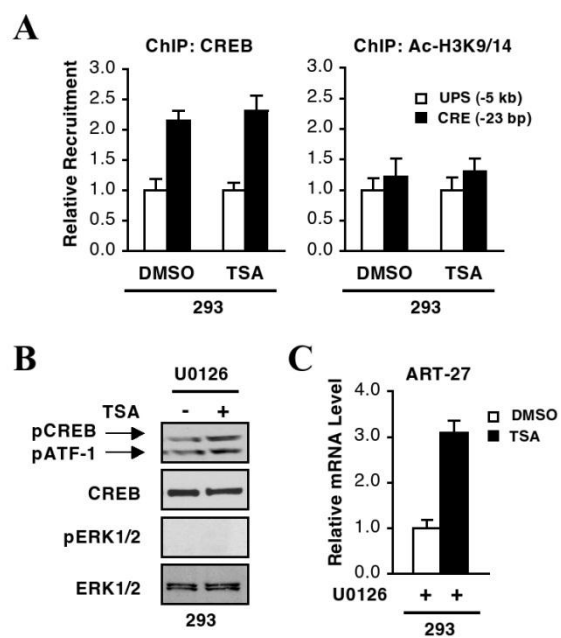
Figure S2: TSA increases ART-27 mRNA expression without changing CREB recruitment and Ac-H3K9/14 levels near the ART-27 promoter in 293 cells

A) ChIP assays using anti-CREB and anti-Ac-H3K9/14 antibodies, were performed on 293 cells serum-starved for 24 h and stimulated with DMSO or 100 ng/ml TSA for 2 hrs. B-C) Western blot using pCREB, CREB, ERK1/2 and pERK1/2 antibodies (B), and real time qPCR analysis for ART-27 mRNA levels (C) were performed on 293 cells serum-starved for 24 h, then treated with 10 mM U0126 and DMSO vehicle or 100 ng/ml TSA for 4 hrs.

Supplementary Figure: S1



Supplementary Figure S2



SUPPLEMENTARY TABLE 1:

MUTATION	FORWARD 5'→3'	REVERSE 5'→3'
Δ1	GCC CCT CCC CAA CTG GGG CCA GCG CGC TTG CCA C	CCA GTT GGG GAG GGG C
Δ2	CAG CGC GCT TGC CAC CAC CCG CGC CAC CCG G	GTG GCA AGC GCG CTG GCC
S1	GGC CAG CGC GAG GGC CAC TTA CG	CGT AAG TGG CCC TCG CGC TGG CC
S2	CCA GCG CGC TTT AAA CTT ACG TC	GAC GTA AGT TTA AAG CGC GCT GG
S3	CGCG CTT GCC CAG TAC GTC ATT C	GAAT GAC GTA CTG GGC AAG CGCG
S4	CG CTT GCC ACT GCA GTC ATT CACC	GG TGA ATG ACT GCA GTG GCA AG CG
S5	GCC ACT TAC TGA ATT CAC CCG CG	CGC GGG TGA ATT CAG TAA GTG GC
S6	CACT TAC GTC CGG CAC CCG CGCC	GGCG CGG GTG CCG GAC GTA AGTG
S7	CT TAC GTC ATT ACA CCG CGC CACC	GG TGG CGC GGT GTA ATG ACG TAAG
S8	CGT CAT TCA CAA TCG CCA CCC GG	CCG GGT GGC GAT TGT GAA TGA CG