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14. ABSTRACT

We propose that AR phosphorylation at serines 213 and 650 regulate differential target gene expression and recruitment to gene promoters via altered interaction with other cellular transcription factors. To test this hypothesis we have conducted yeast two-hybrid analysis with the N-terminus of wild type AR as well as AR S213A and AR S213E variants. Our preliminary analysis indicates that the screen is preferentially isolating proteins with a known role in gene transcription and we are currently assessing the phosphorylation-dependence of the putative AR interacting proteins. Additionally, we have generated PC3 cells stably transfected with wild type, S650A and S650E AR. We have shown that the cells activate endogenous target genes in response to androgens and are currently investigating classes of genes affected by differential AR phosphorylation. Further, we have optimized conditions to isolate pools of hyper- and hypo-phosphorylated AR in order to isolate proteins that interact with AR in a phosphorylation-dependent manner. These proteins were identified via mass spectrometry.

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Prostate Cell-Specific Regulation of Androgen Receptor Phosphorylation in Vivo

INTRODUCTION:

The androgen receptor regulates prostate cell growth and differentiation and plays a critical role in prostate cancer progression. Like other steroid hormone receptors, AR is a phospho-protein and phosphorylation is believed to regulate AR function. To provide insight into the regulation and function of AR phosphorylation, we generated novel antiserum that specifically recognizes AR phosphorylated on key serine residues. Utilization of these antibodies indicates that AR phosphorylation is tightly regulated in urogenital developmental and in differentiated adult prostate. Thus, the development of AR phosphorylation site-specific antibodies along with AR mutant molecules provides a unique opportunity to study the regulation of AR phosphorylation by cellular kinases as well as the impact of phosphorylation on AR function.

Using our novel anti-serum that specifically recognizes AR phospho-serine 213 (P-S213), a putative site of Akt phosphorylation, we demonstrated rapid phosphorylation at S213 in response to agonists R1881 and DHT, but not in response to antagonists bicalutamide or flutamide. By immunohistochemistry, the AR-P-S213 antigen was detected in prostate epithelial but not stromal cells despite the fact that an antibody recognizing both phosphorylated and non-phosphorylated forms of AR demonstrates that AR is present in both cell types as expected. In fetal tissue, the AR-P-S213 antigen was present in epithelial cells of the urogenital sinus when endogenous androgen levels are high, but absent at a later stage of development when endogenous androgen levels are low. Immunoreactivity is evident in differentiated cells lining the lumen of the urogenital sinus, but not in rapidly dividing, Ki67 positive cells within the developing prostate or stromal tissue, suggesting that site-specific phosphorylation of AR S213 by cellular kinases occurs in a non-proliferating cellular milieu. The exquisite cell type specificity of AR S213 phosphorylation suggests that phosphorylation is tightly regulated by cellular kinases and may function in AR-mediated transcription in a specified cellular context.

Examination of phosphorylation of AR at serine 650 (S650) was also conducted. Interestingly, phosphorylation of S650 is enhanced by treatment with forskolin (FSK), Epidermal Growth Factor (EGF) and phorbol-12-myristate-13-acetate (PMA)[Gioeli, D., J. Biol. Chem., 2002] suggesting that AR phosphorylation may be intricately linked to signal transduction processes regulating tumor promotion and cell growth. Consistent with the idea that multiple cell signaling pathways contribute to phosphorylation at AR S650, we find enhanced phosphorylation of AR S650 following R1881 treatment using antibody against AR phospho-serine 650. Characterization of signaling pathways that contribute to phosphorylation of AR S650 is in progress.

BODY

The original grant outlined two tasks in the statement of work. Each task is listed below followed by a description of the research progress relevant to the task.

Task 1. Determine the effect of phosphorylation on AR-mediated gene transcription. Transcription of AR target genes will be compared in HPr-1 prostate cell lines stably expressing wild type AR versus phosphorylation site mutants.

Although the goal of aim 1 is to learn more about AR signaling as it relates to prostate biology, our ultimate objective is to understand and circumvent prostate cancer signaling pathways.

Therefore, although the aim originally proposed to utilize an immortalized, non-transformed cell line, HPr-1, we decided that it would be better to use metastatic PC3 cells. Our initial reservation about using PC3 cells was that AR target genes might not be normally activated in these cells upon co-transfection with AR variants and androgen stimulation. Therefore wild type AR, AR S650A and AR S650E were inserted in the retrovirus vector and used to stably infect PC3 cells. GFP positive pools of cells were selected in the cell sorting facility. To determine if endogenous genes were activated in response to androgens, cells were steroid starved prior to treatment with the synthetic androgen, R1881, and real time PCR was conducted on selected AR targets, PSA, FKBP5 and F5.

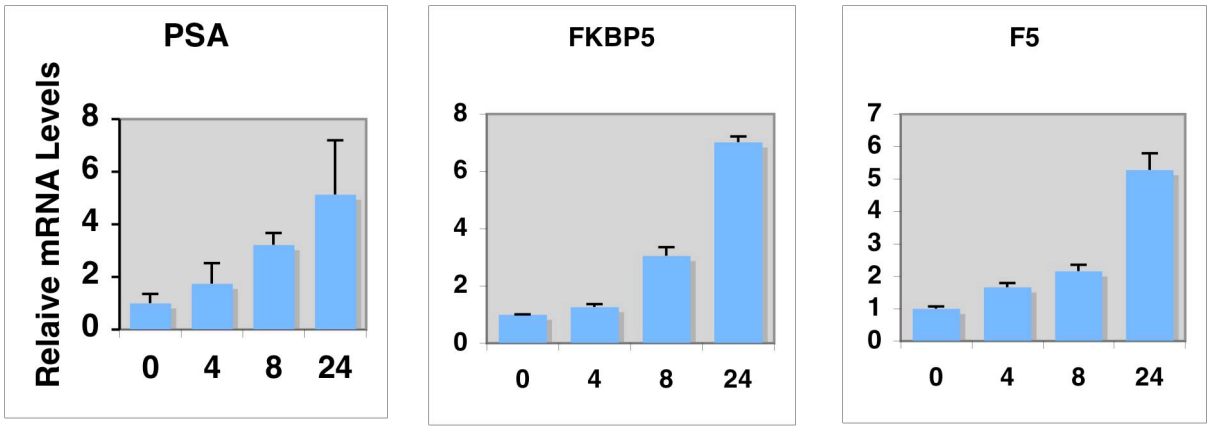

Figure 1. Analysis of AR target gene expression in stably transfected PC3 cells. The figure shows relative mRNA expression of PSA, FKBP5 and F5 as measured by real time PCR.

The results indicate that AR target genes PSA, FKBP5 and F5 are activated in a time dependent manner following androgen treatment, strongly suggesting that PC3 cells are suitable for the studies proposed in this aim (Figure 1).

Over time we have discovered that many investigators have trouble generating prostate cancer cell lines with altered levels of the androgen receptor, and we are no exception. Despite repeated attempts to generate PC3 cells with wild type and mutant AR, we find that with increased cell passage number, the cells stop expressing the receptor and this is especially true of the mutants. To circumvent this problem we have taken two approaches. We are currently analying Affymetrix expression array conducted with RNA made from PC3 cells transiently infected with the wild type AR and AR phosphorylation site mutants. Second, we treated LNCaP cells with agents that result in strong AR phosphorylation of serine 650 and determined the effect on endogenous target genes by Q-PCR. This was done in androgen independent prostate cancer cells because in androgen dependent cells, treatment results in transcriptional downregulation of the AR. Through this approach we have found that phosphorylation of serine 650 appears to inhibit genes involved in differentiation and enhance expression of genes invovled in cell growth. This may be important in AR regulation in prostate cancers, since serine 650 is frequently phosphorylated in human prostate cancer tissue specimens.

Task 2. Examine the effect of AR phosphorylation on recruitment of coactivators and corepressors to the androgen-regulated PSA promoter.

The previous report demonstrated that we could detect robust androgen-dependent AR recruitment to the PSA promoter via chromatin immunoprecipitation assays. Since then, we have conducted similar experiments with the phospho-antibodies. The results indicated that AR S-213 and AR-S-650 are not strongly recruited to the PSA promoter in an androgen-dependent manner in LAPC4 cells. We conducted these experiments in LAPC4 cells since our published findings showed that endogenous AR S213 is phosphorylated in LAPC4 cells. However, PSA may not be the optimal target on which to test the effects of phosphorylation since our results show that serine 650 phosphorylation has minmal effects on endogenous expression of PSA.

A candidate approach indicated that co-activators SRC-1 and GRIP-1 and the co-repressor NCoR, were not differentially recruited to hypophosphorylated versus hyperphosphorylated pools of receptor, however, we are testing other candidate proteins identified by IP/mass spec in task 3.

Task 3. Characterize proteins that interact with AR S213 and S650 in a phosphorylation dependent manner via yeast two-hybrid analysis.

We have undertaken several approaches to examine proteins that interact with the androgen receptor in a phosphorylation-dependent manner. Briefly, we have conducted yeast two hybrid to analyze proteins that interact with AR-P-S213 and we performed immunoprecipitation followed by mass spectrophotometry to analyze proteins that interact with AR-P-S650.Two different approaches were taken because the phosphorylation sites are in different regions of the AR and within different domains of the receptor. Serine 213 is within the 488 amino acid N-terminal region of the AR. This region has been used successfully for yeast two-hybrid screening previously so we felt that this approach will also be effective to identify proteins that bind to the N-terminus of AR in a phosphorylation-site specific manner. Approximately 45 clones have been isolated from yeast, transformed into bacteria and sequenced. Most of these contain identifiable proteins in BLAST searches. Clones that consisted of vector only or of short peptide sequences were discarded. All interesting clones have been re-transformed into yeast to verify the original result. Of the clones with which we have preceded the farthest, 8 out of 12 of the corresponding proteins have a documented role in transcription giving credence to the quality of the screen.

We have compared the b-gal expression induced by the interaction of library proteins with $AR_{1\text{-}488}$ S213E *vs.* $AR_{1\text{-}488}$ S213A to identify proteins that associate with AR in a manner dependent on phosphorylation and are currently testing isolated clones for effect on AR function and phosphorylation. Many of the isolated candidate proteins play a role in gene transcription.

Serine 650 is located in the hinge region, a small unstructured region that lies between the DNA binding domain and the ligand-binding domain. Since this region is only about 40 amino acids we were concerned that it might not fold properly in the yeast two-hybrid assay. We decided to perform immunoprecipitation followed by mass spectrophotometry to identify protein that bound to this region. Conditions were optimized to isolate pools of hyper and hypo-phosphorylated androgen receptor at serine 650 for analysis of associated proteins via IP/mass

spectrometry. IP/mass spec was conducted on cells treated with IL-1B in the presence and absence of R1881 and candidate proteins that preferentially bound to ligand activated AR in the presence of IL-1B were identified.

KEY RESEARCH ACCOMPLISHMENTS

- A reverse yeast two-hybrid screen has identified multiple proteins that interact with the AR phosphorylation-site mimetic S213E
- The identity of a dozen proteins has been confirmed so far and the majority play a documented role in gene transcription
- Stably infected PC3 cells have been made and we have shown that AR phosphorylation and target gene expression occurs in response to androgen in the cells.
- Initial experiments conducted with the PC3 cell lines indicates that AR target gene expression is altered in response to phosphorylation, however PC3 cells preferentially loss expression of the AR S605A and AR S650E mutations.
- An Affymetrix based gene expression array has been conducted to identify changes in gene transcription in PC3 cells infected with wild type or phosphorylation site mutant AR.
- Additional cytokines and stress pathways were shown to result in AR S650 phosphorylation in vivo. The results indicate that, consistent with up-regulation of stress pathways in prostate cancer, that these pathways also result in AR phosphorylation.
- Conditions were optimized to isolate pools of hyper and hypo-phosphorylated androgen receptor for analysis of associated proteins. A candidate approach indicated that SRC-1 and GRIP-1 were not differentially recruited to these pools of receptor; however, we have used IP/mass spec analysis to identify many proteins differentially recruited to the AR when serine 650 is in a hypo- versus hyper-phosphorylated form.

REPORTABLE OUTCOMES

Abstracts, 2006-2008

Jerome C. Nwachukwu, Rachel Ruoff, Susan Ha, Hong Ying Huang, Ellen Shapiro, Samir S. Taneja, Michael J. Garabedian, and **Susan K. Logan** ART-27, an epithelial cell specific co-factor of the androgen receptor Keystone Meeting, Steroid Sisters, 3/18/2006

Chen, W., Dang, T., Blind, R., Wang, Z., Cavasotto, C., Hittelman, A., Rogatsky, I., **Logan**, S.K., and Garabedian, M.J. Glucocorticoid receptor phosphorylation differentially affects target gene expression. Molecular Endocrinology. Aug;22(8):1754-66. Epub 2008 May 15

Jerome C. Nwachukwu, Rachel Ruoff, Susan Ha, Qianben Wang, Se-Te Joseph Huang Samir S. Taneja, Myles Brown, Michael J. Garabedian, and Susan K. **Logan** Gene-specific regulation by the androgen receptor cofactor ART-27 in prostate cancer cells. ENDO meeting, 2007, abstract

Paolo Mita, Jerome C. Nwachukwu, Susan Ha, Rachel Ruoff, Julia Staverosky, Michael J. Garabedian, Susan K. **Logan**. REGULATION OF ANDROGEN RECEPTOR MEDIATED TRANSCRIPTION BY RPB5 BINDING PROTEIN (RMP/URI), Cold Spring Harbor Laboratory Nuclear Receptors: Bench to Bedside, August 27, 08, Abstract

Julia Staverosky, Susan Ha, Rachel Ruoff, Michael J. Garabedian, Susan K. **Logan**. Stress Kinase Signaling and Androgen Receptor Phosphorylation Cold Spring Harbor Laboratory Nuclear Receptors: Bench to Bedside, August 27, 08, Abstract

Jerome C. Nwachukwu, Paolo Mita, Rachel Ruoff, Susan Ha, Qianben Wang, Se-Te Joseph Huang, Samir S. Taneja, Myles Brown, Michael J. Garabedian, Susan K. **Logan**
ROLES OF the androgen receptor cofactor, ART-27 in Prostate Cancer. Keystone Meeting, Steroid Sisters, 3/2008

Jerome C. Nwachukwu, Paolo Mita, Rachel Ruoff, Susan Ha, Qianben Wang, Se-Te Joseph Huang, Samir S. Taneja, Myles Brown, Michael J. Garabedian, Susan K. **Logan** Function of the Androgen Receptor Cofactor, ART-27 in prostate cancer. Cold Spring Harbor Laboratory Nuclear Receptors: Bench to Bedside, August 27, 08, Abstract

Publications, 2006-2008

Taneja, S.S., Ha, S., Swenson, N.K., Huang, H.Y., Lee, P., Melamed, J., Shapiro, E., Garabedian, M.J. and Logan, S.K. Cell specific regulation of androgen receptor phosphorylation *in vivo*. J Biol Chem. 2005 Oct 6; [Epub ahead of print]

Nwachukwu, J.C., Li, W., Huang, H.Y., Ruoff, R., Shapiro, E., Taneja, S., Logan, S.K. and Garabedian, M.J. Transcriptional regulation of the androgen receptor cofactor, ART-27. Mol Endocrinol. 2007 Aug 30; [Epub ahead of print]

Shapiro, E., Huang, HY., Ruoff, R., Lee, P., Tanese, N. and Logan, S.K. Regulation of the HP1 family in prostate development and cancer. J Urol. 2008 Apr 22;179(6):2435-2439 [Epub ahead of print]

Nwachukwu, J.C. Mita, P., Ruoff, R., Ha, S., Wang, Q., Huang, S.J., Taneja, S., Brown, M., Gerald, W.L., Garabedian, M.J. and Logan, S.K.. Androgen receptor trapped clone-27 modulates androgen receptor-mediated transcription in steroid-deprived prostate cancer cells. Cancer Res. 2009 Apr 1;69(7):3140-7.

Research training during the period of grant support (under my immediate supervision):

Raluca Pancratof	Sackler rotation student	1/15-06-4/14/06
Andrew Hanover	NYU undergraduate	1/30/06-5/29/06
Derick Mitchell	postdoctoral fellow	9/1/06-7/16/06

Shivani Garapaty	graduate student readings, weekly	3/19/07-5/31/07
Kyle Monk	SURP summer program	6/1/07-8/31/07
Neha Kaul	Sackler rotation student	4/11/07-7/10/07
Paolo Mita	Sackler student	4/11/07- presesnt
Lindsey DeCarlo	Sackler rotation student	9/18/07- 12/20/07
Julia Staverofsky	Sackler rotation student	5/11/07-present
Eric Schafler	Sackler graduate student	2009-present

In advisory function (thesis committee):

Jason Lieberthal	M.D./Ph.D. thesis defended 2007	Naoko Tanese, advisor
Jerome Nwachukwu	Ph.D. candidate-	Michael Garabedian, Susan Logan, advisors
Diah Douglas	MD/honors thesis, 2006	Iman Osman/Peng Lee, advisors
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Karen Imbert	Sackler student	Michael Garabedian, mentor
Garrett Daniels	Sackler student	Peng Lee, mentor
Haydeliz Martinez-Ruiz, Sackler student		Lili Yamasaki, mentor

CONCLUSION:

We propose that AR phosphorylation at serines 213 and 650 regulate differential target gene expression and recruitment to gene promoters via altered interaction with other cellular transcription factors. To test this hypothesis we have conducted yeast two-hybrid analysis with the N-terminus of wild type AR as well as AR S213A and AR S213E variants. Our preliminary analysis indicates that the screen is preferentially isolating proteins with a known role in gene transcription and we are currently assessing the phosphorylation-dependence of the putative AR interacting proteins. Additionally, we have generated PC3 cells stably transfected with wild type, S650A and S650E AR. We have shown that the cells activate endogenous target genes in response to androgens and are currently investigating classes of genes affected by differential AR phosphorylation.

Recently, we have also shown that AR is phosphorylated in response to stress activation pathways suggesting that inflammation and cellular stress modulate AR function. We have optimized conditions to isolate pools of hyper- and hypo-phosphorylated AR and identified numerous proteins that interact with the AR in a phosphorylation-dependent manner.

Genome-Wide Impact of Androgen Receptor Trapped clone-27 Loss on Androgen-Regulated Transcription in Prostate Cancer Cells

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Departments of [1]Pharmacology, [2]Microbiology, and [3]Urology, and NYU Cancer Institute, New York University School of Medicine, and [4]Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York, New York; [5]Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts; and [6]Department of Obstetrics, Gynecology, and Reproductive Sciences, Yale University School of Medicine, New Haven, Connecticut

Abstract

The androgen receptor (AR) directs diverse biological processes through interaction with coregulators such as AR trapped clone-27 (ART-27). Our results show that ART-27 is recruited to AR-binding sites by chromatin immunoprecipitation analysis. In addition, the effect of ART-27 on genome-wide transcription was examined. The studies indicate that loss of ART-27 enhances expression of many androgen-regulated genes, suggesting that ART-27 inhibits gene expression. Surprisingly, classes of genes that are up-regulated upon ART-27 depletion include regulators of DNA damage checkpoint and cell cycle progression, suggesting that ART-27 functions to keep expression levels of these genes low. Consistent with this idea, stable reduction of ART-27 by short-hairpin RNA enhances LNCaP cell proliferation compared with control cells. The effect of ART-27 loss was also examined in response to the antiandrogen bicalutamide. Unexpectedly, cells treated with ART-27 siRNA no longer exhibited gene repression in response to bicalutamide. To examine ART-27 loss in prostate cancer progression, immunohistochemistry was conducted on a tissue array containing samples from primary tumors of individuals who were clinically followed and later shown to have either recurrent or nonrecurrent disease. Comparison of ART-27 and AR staining indicated that nuclear ART-27 expression was lost in the majority of AR-positive recurrent prostate cancers. Our studies show that reduction of ART-27 protein levels in prostate cancer may facilitate antiandrogen-resistant disease. [Cancer Res 2009;69(7):3140–7]

Introduction

At its early stages, prostate cancer is an androgen-dependent disease. Hormone-based therapy, which involves the use of antiandrogens, induces tumor regression but fails to prevent biochemical recurrence (1). In fact, antiandrogen therapy is thought to trigger and/or select for cancer cells with unusual capabilities that enable cell survival and metastasis at subphysiologic androgen concentrations (1–5). Antiandrogen-resistant cancer cells often show increased expression of the androgen receptor (AR)—a nuclear receptor family member that functions as an androgen-sensitive transcription factor (3). Upon activation, AR binds androgen response elements (ARE) of its target genes and coordinates recruitment of its coregulators at these AREs (6). AR coregulators modulate physiologic androgen response, and select AR coregulators facilitate AR-mediated prostate cancer cell proliferation (7–11).

Our research group identified AR trapped clone-27 (ART-27/UXT), as a coregulator that binds the AR NH_2 terminus and enhances androgen-stimulated transcription (12, 13). The primary sequence of ART-27 is conserved throughout evolution from worms to humans and its predicted protein structure is homologous to the prefoldin-α family of chaperones. ART-27 also associates with at least one large, multiprotein complex whose constituents modulate transcription, genomic stability, apoptosis, and cell transformation (12, 14–20). ART-27 has also been described as a suppressor of cell transformation, and a nuclear factor-κB coregulator (21, 22).

In the prostate, ART-27 expression is restricted to epithelial cells (13). *In vivo*, its cell-specific expression pattern correlates with activation of cyclic AMP–response element binding protein, a transcription factor that is recruited to the ART-27 promoter and is required for epidermal growth factor-induced expression of ART-27 (23). However, ART-27 expression is often reduced in prostate cancer, and ART-27 overexpression suppresses cell proliferation in AR-dependent prostate cancer (LNCaP) cells, suggesting that ART-27 plays a tumor suppressor role in the prostate (13). Yet, the mechanism of ART-27 function in AR-mediated transcription is unclear. The effect of ART-27 on AR target gene expression has been previously examined in cell-based reporter gene assays. In this study, ART-27 recruitment to endogenous genes is examined, and the genome-wide effect of ART-27 on AR target gene expression is explored.

Materials and Methods

Cell culture. LNCaP cells were cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Hyclone) and 1% penicillin-streptomycin (Mediatech/Cellgro). The cells were maintained at 5% CO_2 in a 37°C incubator.

Antibodies. Anti–ART-27 antibody is previously described (13); anti-AR #441 (Santa Cruz Biotechnology); anti-Chk1 (G-4); anti-cyclin A (Santa Cruz Biotechnology); anti-Bub1 (Abcam); anti-tubulin (Covance); anti–extracellular signal-regulated kinase (ERK)1/2 (Cell Signaling Technology).

Chromatin immunoprecipitation assay. LNCaP cells were cultured in cs media (phenol red–free RPMI 1640 supplemented with 10% charcoal-stripped FBS) for 72 h, and stimulated with ethanol vehicle or 0.1 μmol/L

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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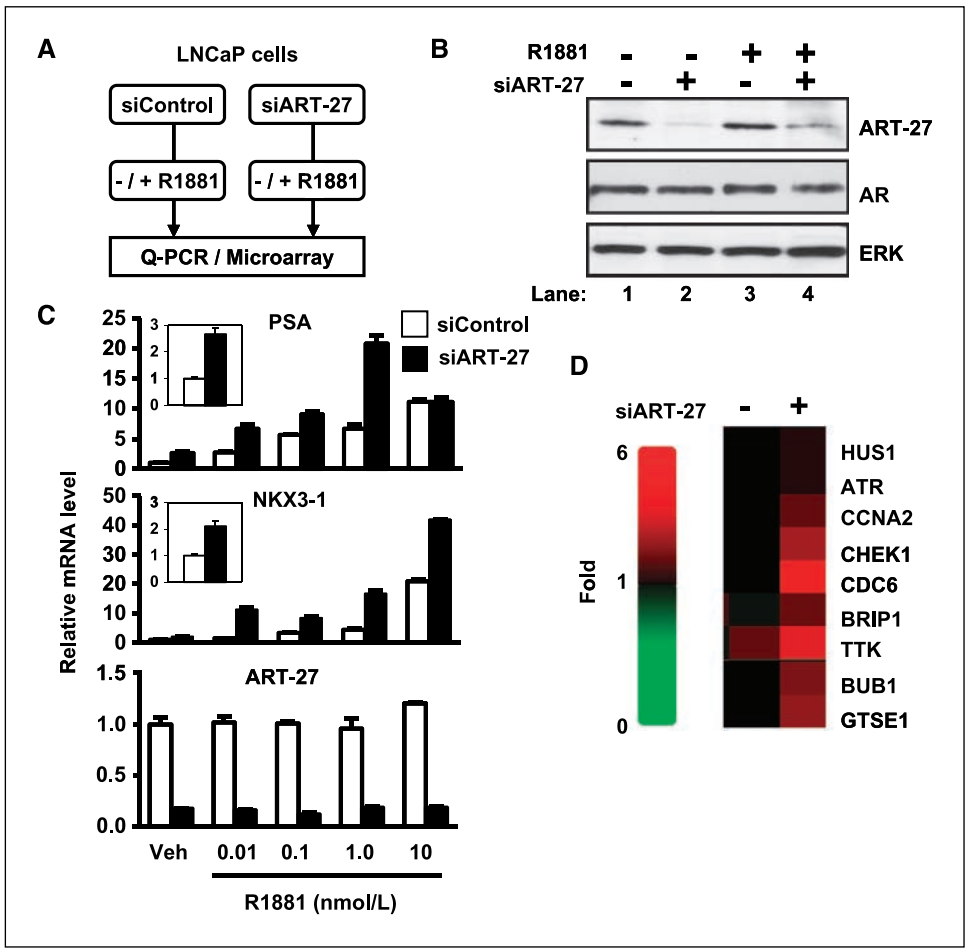

Figure 1. Effect of ART-27 depletion on expression of androgen-regulated genes. *A,* outline of the procedure used to examine androgen response in control and ART-27–depleted LNCaP cells. Briefly, LNCaP cells were steroid deprived for 72 h. During this period, the cells were transfected with control siRNA (*siControl*) or ART-27 siRNA (*siART-27*), and allowed to recover in steroid-deprived media. The cells were then stimulated with ethanol vehicle or R1881 for 18 h. Total RNA was isolated and analyzed by real-time, Q-PCR. For genome-wide studies, RNA was hybridized to Affymetrix Gene Chips and processed. *B,* AR and ART-27 protein expression in control and ART-27–depleted cells. Western blot showing AR and ART-27 protein levels in whole extracts obtained from siRNA transfectants treated with ethanol or 10 nmol/L R1881 for 16 h. Anti-ERK antibody was used as a loading control. *C,* Q-PCR results showing the relative levels of PSA, NKX3-1 and ART-27 mRNA in LNCaP cells treated as described in *A* with ethanol vehicle (*veh*), also shown in *insert*, or the indicated concentrations of R1881. *D,* microarray heat map comparing the expression of transcripts encoding the 9 DNA-integrity and cell cycle checkpoint regulators in steroid-deprived control (*siART-27–*) and ART-27–depleted (*siART-27+*) LNCaP cells.

R1881 for 17 h. The cells were fixed, and chromatin was prepared, sheared, and used in chromatin immunoprecipitation (ChIP) assay performed as previously described (23, 24), with some modifications. Precleared chromatin was incubated with anti–ART-27, or anti-AR antibodies overnight at 4°C. Quantitative PCR (Q-PCR) was performed on precipitated DNA, and relative enrichment is shown as a percentage of the input. The ChIP primers used for prostate-specific antigen (PSA) are previously described (25). The other primers used are provided in the Supplementary Materials and Methods section.

RNA-interference. Nonsilencing (control), ART-27 (SMARTpool), and AR siRNA were purchased from Dharmacon. LNCaP cells incubated overnight in cs media were transfected in Opti-MEM media (Invitrogen) using 100 nmol/L of each siRNA, and Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. After 4 h, the cells were allowed to recover overnight in cs media.

Q-PCR. Total RNA was isolated using the RNeasy kit (Qiagen, Inc.). 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 gene-specific primers (Supplementary Materials & Methods) and 2× SYBR green Taq-ready mix (Sigma-Aldrich) as previously described (23). Data were analyzed by the ΔΔCT method using RPL19 as a control gene, and calibrated to naive samples, which were arbitrarily set to 1 (26). The same results were obtained using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control gene.

cDNA microarray analysis. Total RNA was processed, hybridized, and analyzed at the Memorial Sloan-Kettering genomics core facility, using HG_U133A 2.0 gene chips (Affymetrix). Androgen-regulated genes in each condition were determined as previously described using Genespring software (Agilent Technologies) and a 2-fold change threshold (27). The *NetAffx* online tool[7] was used to sort androgen-regulated genes sets identified in each condition (28). *L2L* online microarray data analysis tool[8] was used to analyze the gene sets based on gene ontology classifications (29). The data discussed in this publication have been deposited in National Center for Biotechnology Information's Gene Expression Omnibus (Nwachukwu and colleagues, 2009) and are accessible through GEO Series accession number GSE14043.[9]

Thymidine-incorporation. After transfection, steroid-deprived LNCaP cells were seeded (4×10^4 cells per well) onto fibronectin-coated 24-well plates and incubated in cs media plus ethanol vehicle or 0.1 μmol/L bicalutamide at 37°C. The next day, 2 μCi of [^{3}H]-Thymidine (Sigma) were added to each well and incubated with the cells for 1 h at 37°C. The cells were subsequently placed on ice, washed with cold PBS, and fixed by incubation in cold methanol for 10 min on ice. The cells were then washed with 10% trichloroacetic acid, and solubilized in 0.5 mL of prewarmed 1% SDS in 0.3 N NaOH at 37°C for 2 h before scintillation counting.

Tissue array analysis. Arrays containing tissue specimens from individuals, who had undergone radical prostatectomy at Memorial Sloan Kettering Cancer Center between 1985 and 2003, were created with the approval of the Institutional Review Board of Memorial Sloan Kettering Cancer Center. Three representative tissue cores 0.6 mm in diameter were extracted from each specimen and mounted in paraffin blocks. Two individuals (J.C.N. and S.K.L.) blindly scored the tissue array based on

[7] http://www.affymetrix.com
[8] http://depts.washington.edu/l2l/
[9] http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE14043

staining intensity and proportion of positively stained cells. Each specimen was scored for intensity (0–3) and proportion of positively stained cells (0–5) in both the nucleus and cytoplasm, such that every sample has 4 scores. For intensity scores, negative staining was scored between 0 and 1. Weak but convincing stain was scored 1.5, moderate staining was typically scored 2, and strong staining was scored 3. The proportion of stained cells was scored as follows: 0, no positive cells; 1, 1 in 100 positive cells; 2, 1 in 10 positive cells; 3, one-third positive cells; 4, two-thirds positive cells; and 5, all positive cells. AR staining was used as a positive control for tissue integrity because AR staining is so robust in prostate. Thus, only AR-positive samples were included in the analysis. Samples that scored at or above 1.5 (intensity); 2 (proportion) were considered positive. The probability (P value) associated with a Student's t test was calculated by designating positive samples "1" and negative samples "0." Differences with P values of <0.05 were considered significant.

Results

ART-27 depletion alters androgen-regulated gene expression. To determine the role of ART-27 in AR-mediated transcription in LNCaP cells, the effect of ART-27 depletion on expression of the androgen-regulated genes *PSA* and *NK3* homeobox 1 (*NKX3-1*), was examined. Steroid-deprived LNCaP cells were transfected with nonsilencing (control) or ART-27–silencing (siART-27) small-interfering RNA. Cells were treated with ethanol vehicle or R1881 for 18 hours. ART-27 depletion was confirmed by Q-PCR (Fig. 1) and Western blot analysis (Fig. 1*B*). AR expression was largely unaffected (Fig. 1*B*). To show ART-27 siRNA specificity, cells were also treated with the four individual oligonucleotide duplexes that compose the SMARTpool. The results indicated that each separately diminished ART-27 expression, whereas nonspecific siRNA had no effect (data not shown).

After siRNA treatment, Q-PCR was conducted to compare PSA and NKX3-1 mRNA expression. ART-27 depletion increased PSA expression in steroid-deprived cells treated with ethanol vehicle or 0.01, 0.1, or 1 nmol/L of R1881 (Fig. 1*C*; compare *white* versus *black bars*), suggesting that ART-27 inhibits gene expression. Examination of NKX3-1 mRNA levels upon treatment with ART-27 siRNA indicates a modest increase in gene expression in the presence of vehicle alone (Fig. 1*C, inset*). However, ART-27 depletion increased expression of the NKX3-1 transcript at every concentration tested and almost 10-fold above cells treated with control siRNA at 0.01 nmol/L R1881 (Fig. 1*C*). These findings suggest that ART-27 inhibits AR-mediated gene expression over a wide range of androgen concentrations with more prevalent and uniform effects at low doses of androgen.

Whereas the effect of loss of ART-27 on PSA and NKX3-1 is shown above, the broader effect of ART-27 on AR-regulated genes is not known. To evaluate the genome-wide effect of ART-27 on androgen-regulated gene expression, steroid-deprived LNCaP cells were transfected with control or ART-27 siRNA and treated with ethanol or 10 nmol/L R1881 (Fig. 1*A*). RNA isolated from duplicate transfectants was then processed, hybridized to Affymetrix GeneChip microarrays (HG-U133A_2), and analyzed. The results from the duplicate samples were highly similar.

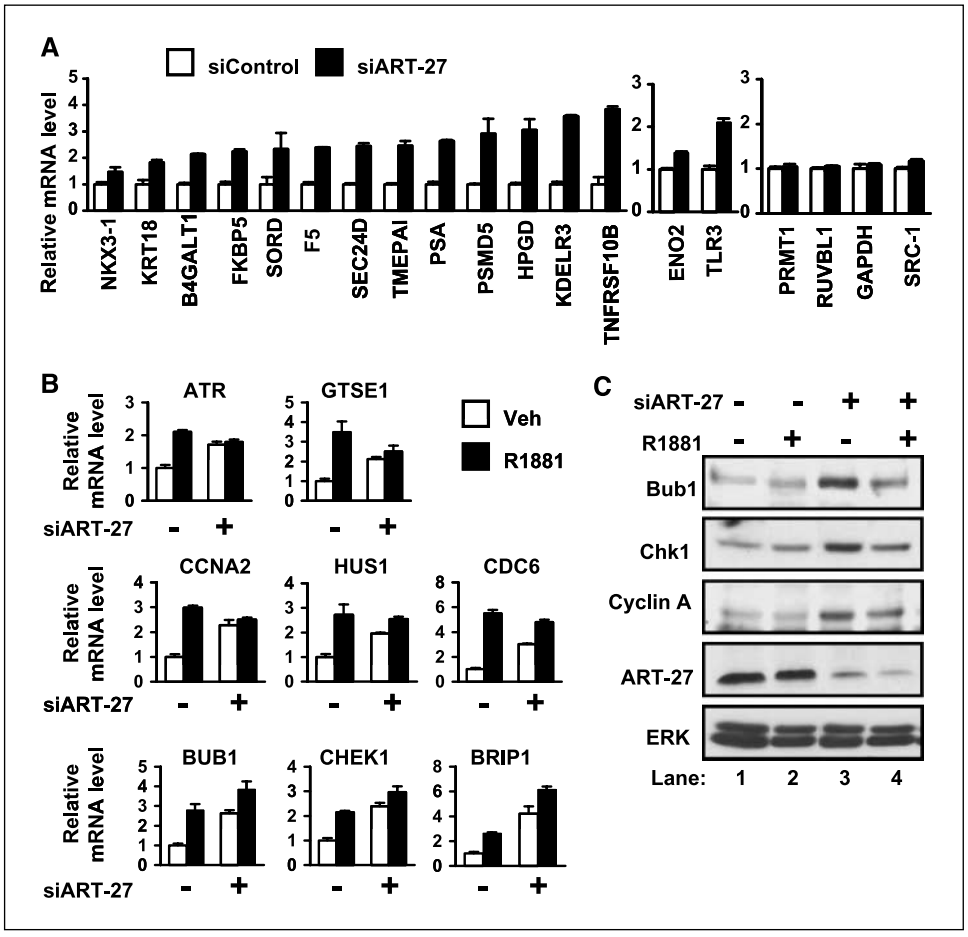

Figure 2. ART-27 inhibits expression of androgen-regulated genes. *A,* LNCaP cells transfected with control siRNA or ART-27 siRNA were steroid deprived for 72 h. Relative mRNA levels of the indicated androgen-induced, androgen-repressed, and androgen-insensitive genes were determined by Q-PCR (gene descriptions are provided in Supplementary Fig. S1). *B,* expression of the indicated transcripts was examined by Q-PCR in LNCaP cells treated as described in Fig. 1*A* with ethanol vehicle or 10 nmol/L R1881. *C,* expression of checkpoint proteins in whole cell extracts obtained from LNCaP cells treated as described in Fig. 1*A* was examined by Western blot.

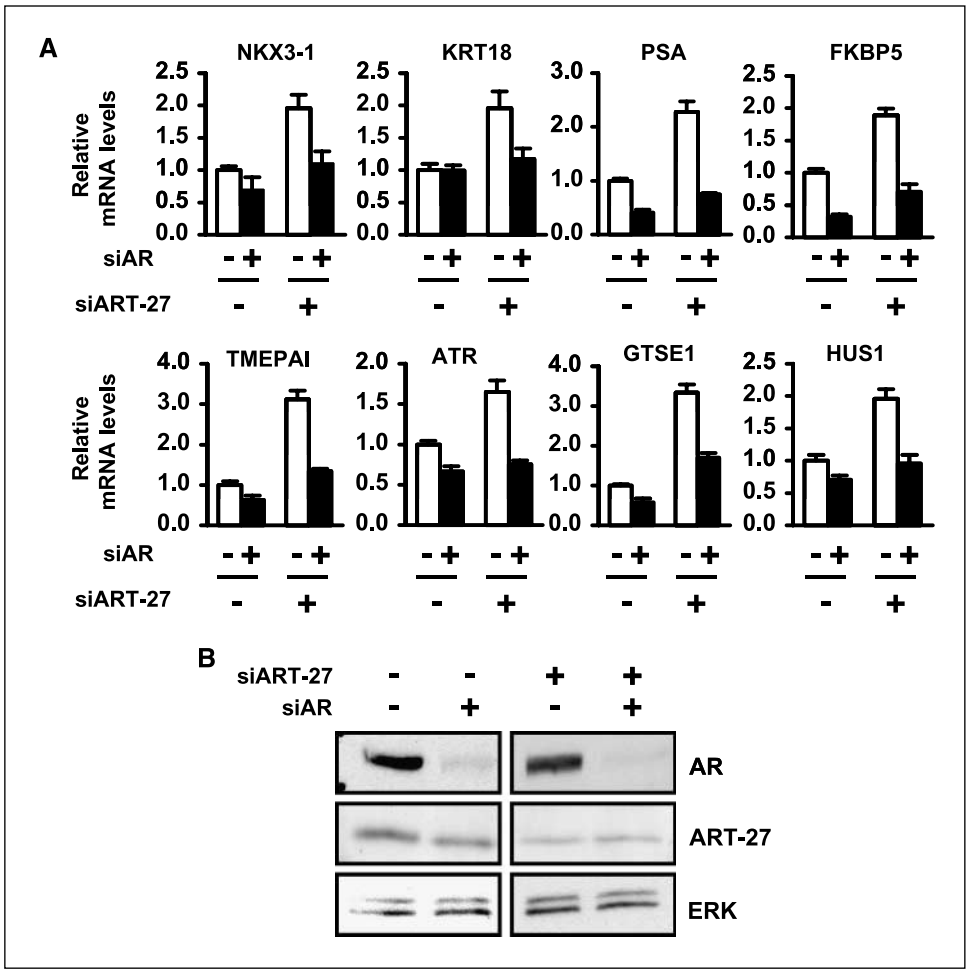

Figure 3. AR mediates the increase in gene expression observed upon ART-27 depletion. *A,* LNCaP cells were transfected with control siRNA or ART-27 siRNA and/or AR siRNA as indicated and steroid deprived for 72 h. Total RNA was isolated and analyzed by Q-PCR for the indicated genes. The mRNA levels are shown relative to the naive treatment, which were arbitrarily set to 1 for each gene. Data shown is the average of three independent experiments. *B,* LNCaP cells were transfected with control siRNA (−), siART-27, and/or AR siRNA as indicated and steroid deprived for 72 h. Whole cell extracts were analyzed by Western blot using anti-AR, and anti–ART-27 antibodies. Anti-ERK antibody was used as a loading control.

To identify androgen-regulated genes, comparisons between vehicle- and R1881-treated cells were performed separately for control and ART-27–depleted cells using GeneSpring software. A total of 651 androgen-induced (Supplementary Table S1*A*) and 517 androgen-repressed genes (Supplementary Table S1*B*) were detected. To identify genes that were dependent on ART-27 for androgen regulation, the set of androgen-regulated probes detected in ART-27–depleted cells was subtracted from those detected in control cells using *NetAffx*. This procedure revealed 271 androgen-induced and 230 androgen-repressed genes that were differentially regulated in control versus ART-27–depleted cells.

To estimate the effect of ART-27 loss on androgen-sensitive transcriptional programs, the *L2L* algorithm was used to identify cellular processes enriched by androgen in control and ART-27–depleted cells, based on gene ontology classification (29, 30). The greatest effect of ART-27 loss was observed in the set of 271 androgen-induced genes. *L2L* analysis identified 24 significantly enriched ($P < 0.01$) gene ontology biological processes (GOBP). Enrichment of 13 of these GOBPs was highly significant ($P < 0.001$). Surprisingly, all 13 GOBPs were associated with regulation of cell proliferation (Supplementary Table S2*A*). Two of these GOBPs showed remarkably high fold-enrichment and contained the DNA integrity/cell cycle checkpoint genes *ATR, BRIP1, CCNA2, GTSE1, CHEK1, HUS1, BUB1, CDC6*, and *TTK* protein kinase (full gene descriptions are provided in the legends, Supplementary Fig. S2). Under low steroid conditions, expression of these checkpoint genes was generally higher in ART-27–depleted than in control cells (Fig. 1*D*), consistent with the inhibitory effect of ART-27 on gene expression. Thus, in LNCaP cells, ART-27 unexpectedly seems to preferentially inhibit expression of genes that regulate DNA integrity and the cell cycle.

NetAffx analysis also identified 380 androgen-induced genes that were induced in both control siRNA and ART-27 siRNA-treated cells, indicating that they do not require ART-27 for induction by androgen. Groups of genes that were androgen induced in an ART-27–independent manner include many genes that play a role in normal metabolic processes of prostate epithelial cells (Supplementary Table S2*B*). This includes genes associated with production of prostatic secretory fluid and processes such as cholesterol and lipid metabolism, polyamine biosynthesis, and vesicle-mediated protein trafficking and secretion (31). These results suggest that ART-27 is not required for androgen regulation of a broad spectrum of transcriptional programs such as those that direct hemostasis and prostatic secretory fluid production.

The set of androgen-repressed genes detected in this study was diverse and did not easily stratify into functional groups. Within the set of 287 genes that were androgen repressed in an ART-27–insensitive manner, steroid metabolic process was the only GOBP significantly enriched ($P < 0.01$). *L2L* analysis of the set of 230 androgen-repressed genes that were ART-27 sensitive revealed significant enrichment ($P < 0.01$) of only two GOBPs: Cyclic nucleotide metabolism, which contained 4 genes (*NPR2, ADCY7,*

RORA, PDE4D), and Positive regulation of cell adhesion which contained 2 genes (*CX3CL1* and *VAV3*). Thus, ART-27 loss may also affect cellular processes controlled by androgen-repressed genes.

Loss of ART-27 enhances AR target gene expression. To verify the gene array results described above, regulation of genes selected from the microarray analysis was examined by Q-PCR (Supplementary Fig. S1). Genes identified on the array as being androgen responsive are indeed regulated by R1881, and this includes androgen-repressed genes such as *ENO2* and *TLR3* (Supplementary Fig. S1*A*), and the class of androgen-inducible DNA damage/cell cycle checkpoint genes identified in Fig. 2 (Supplementary Fig. S1*B*). Similar results were obtained using the natural androgen, dihydrotestosterone (data not shown). As a control, four genes that were unresponsive to androgen (*PRMT1, SRC-1, RUVBL1*, and *GAPDH*) are also shown (Supplementary Fig. S1*C*). Descriptions for genes that were not already discussed (Fig. 1) are provided in the Supplementary Figure Legends (Supplementary Fig. S1).

To validate the effect of ART-27 depletion on these genes, LNCaP cells were treated with control or ART-27 siRNA and androgen-regulated genes were examined by Q-PCR. Under low steroid conditions obtained after 72 hours of steroid deprivation, ART-27 depletion via siRNA increased mRNA expression of the androgen-regulated genes, including the androgen-repressed genes *TLR3* and *ENO2,* but not *PRMT1, SRC-1, RUVBL1,* and *GAPDH,* which were not induced by R1881 (Fig. 2*A*).

The effect of ART-27 ablation on the checkpoint genes identified above was also examined by Q-PCR. Similar to results in the array, the mRNA expression of the checkpoint genes was increased upon ART-27 depletion in steroid-deprived cells (Fig. 2*B*, compare *white bars* for each gene). Similar to PSA (Fig. 1*C*), expression of most of these transcripts was minimally affected in cells stimulated with 10 nmol/L R1881 (Fig. 2*B*, compare *black bars* for ATR, GSTE1, HUS1, CDC6, and CCNA2). However, similar to NKX3-1 (Fig. 1*C*), some genes (notably BRIP1) also showed increased expression in ART-27 siRNA-treated compared with control cells stimulated with 10 nmol/L R1881. Overall, the results suggest that ART-27 inhibits expression of androgen-regulated genes including the checkpoint genes.

To determine whether an increase in mRNA levels in ART-27–depleted cells affected checkpoint protein levels, protein expression was also examined in LNCaP cells. ART-27–depleted cells showed higher Chk1, Bub1, and Cyclin A protein expression, especially under low steroid conditions obtained after 72 hours of steroid deprivation (Fig. 2*C*, compare lanes *1* and *3*). This result is unexpected in that checkpoint proteins such as Chk1, Bub1, and Cyclin A are typically expressed in proliferating cells, whereas LNCaP cells generally do not proliferate under these conditions.

Up-regulation of cell cycle checkpoint genes observed in ART-27–depleted LNCaP cells suggests that the cells have acquired an antiandrogen-resistant–like phenotype and may proliferate faster under low androgen conditions. To determine the effect of ART-27 depletion on LNCaP cell proliferation, the proliferation rates of cells stably expressing a nonsilencing control short-hairpin RNA or an ART-27–silencing short-hairpin RNA were compared. Pools of LNCaP cells expressing an ART-27–silencing short-hairpin RNA showed a higher proliferation rate than control cells, upon treatment with either ethanol vehicle or 0.1 nmol/L R1881 (Supplementary Fig. S2).

Consistent with an inhibitory effect of ART-27 on transcription (Figs. 1 and 2), an ART-27–silencing short-hairpin RNA–expressing cells showed increased mRNA levels of AR target genes including *PSA, FKBP5*, and the checkpoint genes (Supplementary Fig. S2*C*). This increase was associated with decreased ART-27 protein expression but not changes in AR expression in vehicle-treated cells (Supplementary Fig. S2*D, AR*, compare *lane 1* versus *3*); interestingly, AR protein seemed to be up-regulated by an ART-27–silencing short-hairpin RNA in R1881-stimulated cells (Supplementary Fig. S2*D, AR*, compare *lane 2* versus *4*). Up-regulation of AR in response to ART-27 loss was not observed upon transient treatment with ART-27 siRNA in R1881-treated cells (Fig. 1*B, AR, lane 3* versus *4*), suggesting that effects on AR protein levels occur over a long period of time through an unknown mechanism that might be similar to up-regulation of AR that is characteristic of antiandrogen-resistant prostate cancer (3). This result is consistent with a tumor-suppressor role for ART-27 in the prostate (13, 21, 32), and supports the idea that ART-27 loss facilitates antiandrogen resistance in prostate cancer because antiandrogen-resistant prostate cancer cells proliferate faster than their

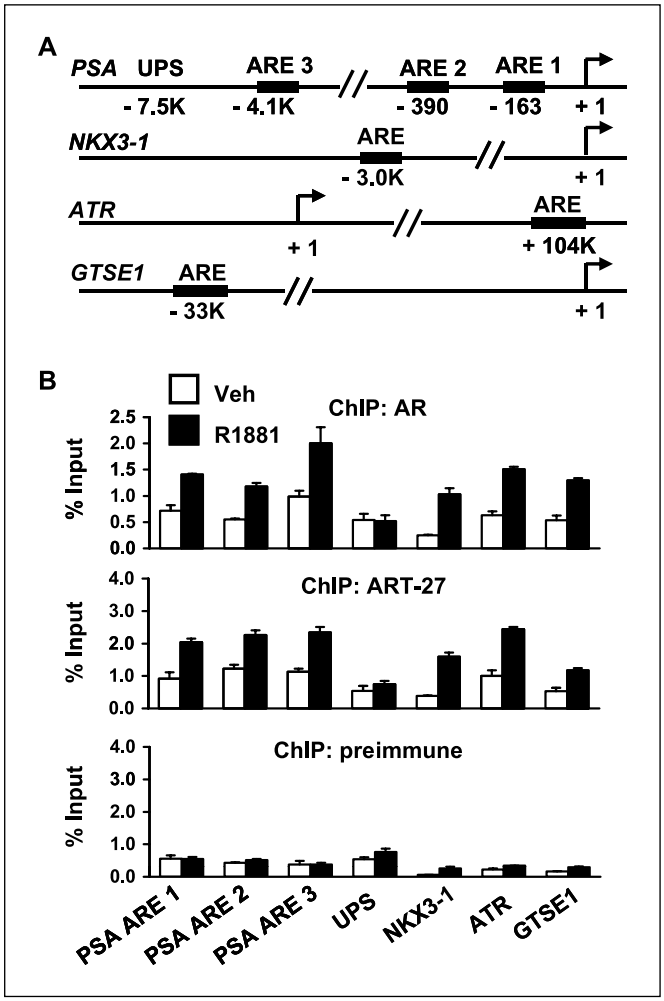

Figure 4. Recruitment of ART 27 to AR target genes. *A,* schematic illustration showing the positions of the well-characterized AREs of PSA and NKX3-1, the putative AREs of ATR and GTSE1, and the negative control upstream region (*UPS*), relative to transcription-start sites (+1 bp). *B,* LNCaP cells were steroid starved for 72 h and stimulated with ethanol vehicle or 0.1 μmol/L R1881 for 17 h. ChIP assay was performed using anti-AR or anti–ART-27 antibodies, or preimmune sera. Recruitment of AR and ART-27 to the indicated regions is shown as a percentage of the input. *Columns,* mean of three independent experiments; *error bars,* SE.

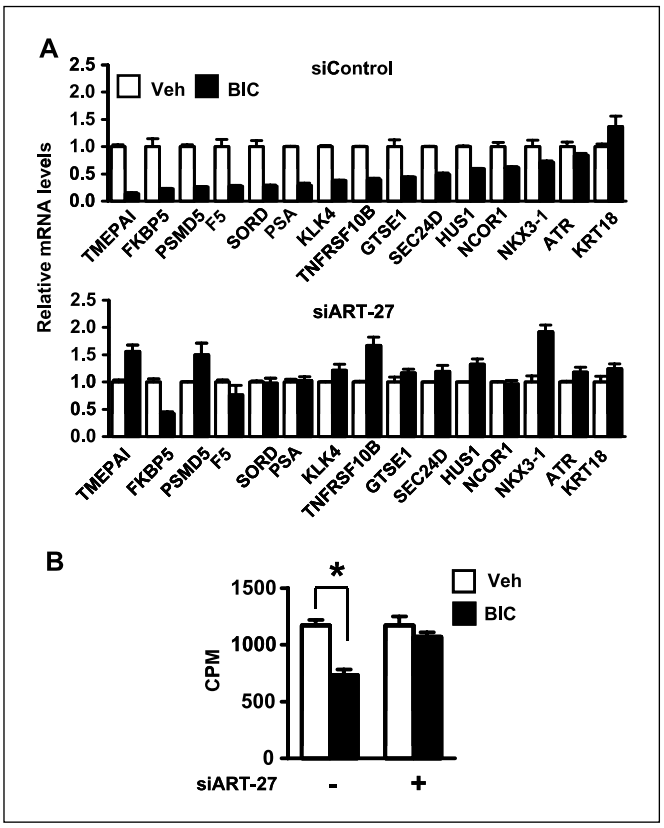

Figure 5. ART-27 loss facilitates resistance to the AR-antagonist bicalutamide. *A,* ART-27 depletion inhibits gene repression by bicalutamide. Q-PCR analysis showing relative mRNA levels of the indicated genes in LNCaP cells transfected with control siRNA or ART-27 siRNA and steroid deprived in the presence of ethanol vehicle or 0.1 μmol/L bicalutamide (*BIC*) for 72 h. The mRNA levels were normalized to ethanol-treated samples, which were arbitrarily set to 1. *B,* bicalutamide-resistant cell proliferation in ART-27–depleted cells. LNCaP cells transfected in duplicate with control or ART-27 siRNA were steroid deprived in the presence of ethanol vehicle or 0.1 μmol/L bicalutamide for 72 h. Cell proliferation rates were then estimated by [^{3}H]-thymidine incorporation assay and shown as average counts per minute (CPM). *Columns,* mean (*, $P = 0.006$); *error bars,* SE.

androgen-dependent counterparts, especially under low androgen conditions (3, 5, 33).

AR is required for increase in gene expression upon ART-27 depletion. To determine whether the increased expression of androgen-induced genes in ART-27–depleted cells is AR dependent, the effect of AR siRNA on gene expression in control or ART-27–depleted LNCaP cells was examined by Q-PCR, under low steroid conditions obtained after 72 hours of steroid deprivation (Fig. 3*A*). The extent of AR and ART-27 protein depletion is also shown (Fig. 3*B*). As expected, in the presence of AR, depletion of ART-27 results in enhanced levels of mRNA (Fig. 3*A*, compare *white bars*). However, upon AR depletion, most genes show little if any increase in gene expression upon ART-27 siRNA treatment (Fig. 3*A*, compare *black bars*). Thus, the increase in gene transcription exhibited upon ART-27 depletion requires the presence of AR. This implies that ART-27 functions as an AR corepressor.

ART-27 is recruited to AR target genes including checkpoint genes. Although previous studies have shown that ART-27 binds AR and effects gene transcription, its recruitment to endogenous AR target genes has not been shown. Therefore, to determine if the effect of ART-27 on gene expression could be direct, ChIP assays examining AR and ART-27 recruitment to established AR target genes and the checkpoint genes were performed. AR and ART-27 recruitment to the well-characterized AREs of PSA and NKX3-1 was examined (Fig. 4*A*; refs. 6, 25, 34). Recruitment to an upstream region previously described as a negative control for AR recruitment was also assessed (25). Using ChIP coupled with whole genome-tiling microarray (*ChIP-on-Chip*) procedure, AR-binding sites of ATR and GTSE1 were identified.[10] The positions of these putative AREs are illustrated (Fig. 4*A*). AR-binding sites for the remaining six checkpoint genes are unknown. Therefore, AR and ART-27 recruitment to ATR and GTSE1 were also examined by ChIP assay.

In response to R1881, both AR and ART-27 were recruited to PSA, NKX3-1, ATR, and GTSE1 but not to the upstream region (Fig. 4*B*). Under our experimental conditions, AR is rapidly recruited by 30 minutes, whereas robust recruitment of both AR and ART-27 does not occur until 4 to 17 hours after R1881 stimulation (Supplementary Fig. S3). Furthermore, LNCaP cells treated with AR siRNA show no recruitment of ART-27 at the PSA, NKX3.1, or ATR gene promoter/enhancer regions (data not shown), suggesting that the presence of ART-27 at these sites requires AR. ChIP assay using preimmune sera did not show recruitment at AREs relative to the upstream region (Fig. 4*B*). Therefore, these results indicate that AR and ART-27 are specifically recruited to target genes.

ART-27 depletion facilitates antiandrogen resistance. Antiandrogen-resistant prostate cancers are characterized by inability to respond to antiandrogens such as bicalutamide. Although its exact mechanism of action is still unclear, bicalutamide is thought to repress gene expression in an AR-dependent manner. Bicalutamide increases recruitment of AR and corepressors to the promoter-proximal AREs of PSA, inhibits AR-mediated transcription, and suppresses cell proliferation in androgen-dependent but not antiandrogen-resistant prostate cancer cells (3, 6, 34–36). If ART-27–depleted cells acquire antiandrogen-resistant status, they may also resist gene repression by bicalutamide. Therefore the effect of bicalutamide on gene expression in steroid-deprived control and ART-27–depleted cells was examined by Q-PCR.

Under low steroid conditions obtained after 72 hours of steroid deprivation, bicalutamide suppressed the expression of every androgen-inducible gene tested in cells treated with control siRNA (Fig. 5*A*), with the exception of KRT18, which is a luminal epithelial cell differentiation marker (2). (The fact that bicalutamide repression occurs in the charcoal stripped media used in these experiments likely indicates the presence of residual androgen in our cell culture conditions.) In contrast, bicalutamide repression was not observed in ART-27–depleted cells (Fig. 5*A*). In fact, bicalutamide increased expression of many of the genes indicating that bicalutamide is acting as a weak AR-agonist in the context of low ART-27 expression.

To determine if the observed transcriptional changes are linked to changes in cell proliferation rates, thymidine-incorporation assays were performed in vehicle- or bicalutamide-treated control and ART-27–depleted LNCaP cells (Fig. 5*B*). Bicalutamide suppressed the proliferation rate of control cells, indicative of their androgen-dependent status. However, the proliferation rate of ART-27–depleted cells was unaffected by bicalutamide, suggesting that ART-27–depleted cells are insensitive to bicalutamide treatment and that loss of ART-27 may facilitate antiandrogen resistance.

[10] Q. Wang and M. Brown, unpublished data.

Loss of ART-27 is prevalent in recurrent prostate cancer. The above experiments suggest that loss of ART-27 may play a role in the development of antiandrogen-resistant prostate cancer. To investigate the possible clinical relevance of these findings, we examined ART-27 protein expression in a tissue array, which enabled correlation with disease outcome. Samples were from radical prostatectomy of men not treated with antihormonal therapy at the time of tissue acquisition. Each sample was represented in triplicate. Because the AR antibody stains robustly, only those samples that stained positively for AR were scored, to ensure tissue integrity of each sample. A total of 58 AR-positive prostate cancer cases were identified, which includes 24 that later exhibited biochemical recurrence (as defined by 3 consecutive increases in PSA levels), 25 that did not reoccur after radical prostatectomy, and 9 high-grade prostatic intraepithelial neoplasia cases. Samples from all these cases showed an intensely nuclear AR staining pattern.

Recurrent cancers retain ART-27 cytoplasmic and perinuclear staining but tend to lose nuclear ART-27 staining relative to high grade prostatic intraepithelial neoplasia or nonrecurrent cancers (Fig. 6*A–B*). Only 33% (8 of 24) of recurrent cancers exhibited nuclear staining for ART-27, whereas 56% (14 of 25) of nonrecurring and 67% (6 of 9) of high-grade prostatic intraepithelial neoplasia stained positively (Fig. 6*B*). The remaining samples did not stain positively for nuclear expression of ART-27. Furthermore, among the recurrent cases, there was a significant difference between the PSA-doubling times (indicative of disease aggressiveness) of cases that exhibited loss of nuclear ART-27 versus those that did not. The continued presence of ART-27 in the nucleus correlated with lengthy PSA-doubling times, suggesting slow disease progression in recurrent cancers. Loss of nuclear ART-27 in recurrent cancers correlated with shorter PSA-doubling times indicative of faster disease progression (Fig. 6*C*). Perhaps this is not surprising given that loss of ART-27 enhances transcription of AR-regulated genes as shown above. Altogether, the loss of ART-27 may expedite cell proliferation and resistance to antiandrogen treatment of prostate cancer cells.

Discussion

Overall, the results in this study indicate that decreasing ART-27 protein expression enhances transcription of many androgen-responsive genes (Figs. 1–3). Thus, ART-27 functions primarily as an AR corepressor and in its normal capacity acts to repress transcription of androgen-regulated genes. Unlike conventional corepressors, such as Nuclear Receptor Corepressor (N-CoR), where the mechanism of repression via interaction with histone deacetylase is detailed, the mechanism of ART-27–mediated gene repression is unknown. However, ART-27 likely recruits other proteins to transcription complexes and is clearly part of a higher molecular weight complex that includes both Rpb5, a subunit shared by RNA polymerase (Pol) I, II, and III, and the corepressor, Unconventional prefoldin Rpb5-Interactor (URI/C19orf2; refs. 12, 14).

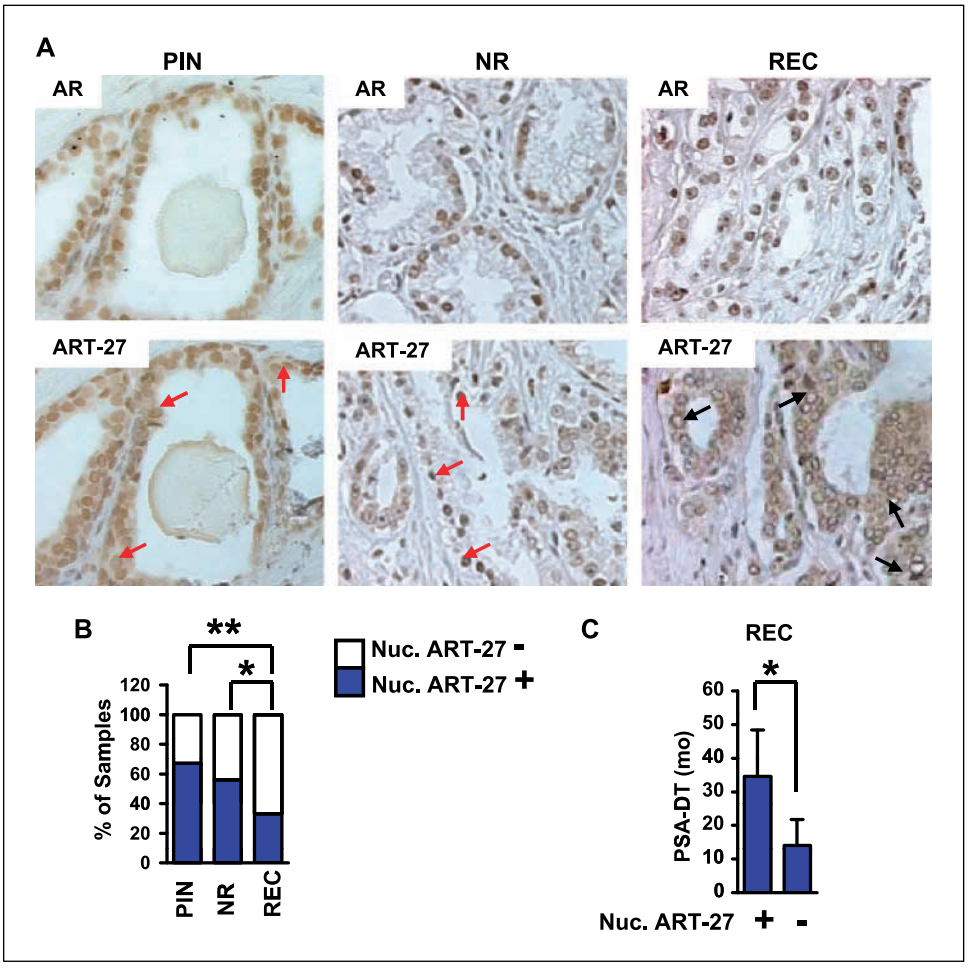

Figure 6. ART-27 expression and clinical outcome in AR-positive prostate cancer. *A,* ART-27 exhibits differential subcellular expression profiles in AR-positive prostate cancer. Prostate cancer tissue microarray containing 58 AR-positive cases [9 high-grade prostatic intraepithelial neoplasia (*PIN*); 25 nonrecurrent (*NR*), i.e., did not relapse after radical prostatectomy; and 24 recurrent (*REC*), i.e., relapsed after radical prostatectomy] were stained by immunohistochemistry using anti-AR (*top row*) and anti–ART-27 (*bottom row*) antibodies. *Columns,* representative samples from each group. *Red arrows,* cells showing nuclear (*nuc.*) ART-27 expression. *Black arrows,* cells showing largely cytoplasmic or perinuclear ART-27 expression. Magnification, ×40; *B,* summary of prostate cancer tissue microarray analysis. Nuclear ART-27 expression status is shown as a percentage of the total number of cases in each group. The *P* values for the indicated comparisons are also indicated (*, $P = 0.058$; **, $P = 0.054$). *C,* average PSA-doubling times of nuclear ART-27–positive and ART-27–negative recurrent cases (*, $P = 0.035$).

Prostate cancer cells can evade antiandrogen therapy by restoring AR expression and/or activity through several mechanisms including changes in AR coregulator function (1). The mechanism leading to reduction of nuclear ART-27 expression in prostate cancer is unknown; however, findings presented in this report indicate that cells with diminished ART-27 expression show elevated AR activity (Figs. 2–3) and resistance to the antiandrogen, bicalutamide (Fig. 5). Furthermore, loss of nuclear ART-27 correlates with prostate cancer disease recurrence (Fig. 6). In fact, the loss of ART-27 in recurring cancers presented in this report may represent an underestimate, in the sense that even cancer specimens scored as positive have areas that have lost nuclear ART-27 perhaps indicating foci of more and less aggressive disease within the same specimen. In addition, gene mutations may also play a role in ART-27 function; for example, we have identified somatic AR mutations that exhibit altered ART-27 interaction (32).

Results presented in Fig. 5 show that gene repression in response to the antiandrogen, bicalutamide, is relieved when ART-27 levels are diminished. This suggests that ART-27 plays an important role in bicalutamide-dependent repression of AR target genes, perhaps by affecting the recruitment of corepressors such as N-CoR and SMRT to the AR transcription complex. Alternatively, ART-27 or its binding partner, URI may interact with components of the chromatin modifying/remodeling machinery. The fact that URI interacts with the ATPases TIP48 and TIP49, and DNA methyltransferase 1–associated protein 1, supports this idea (14, 15). Overall, the results presented here suggest that prostate cancer cells can restore AR activity and evade antiandrogen therapy by reducing nuclear ART-27 expression, a hypothesis we are currently testing.

Disclosure of Potential Conflicts of Interest

No conflicts of interest were disclosed.

Acknowledgments

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References

1. Scher HI, Sawyers CL. Biology of progressive, castration-resistant prostate cancer: directed therapies targeting the androgen-receptor signaling axis. J Clin Oncol 2005;23:8253–61.
2. Long RM, Morrissey C, Fitzpatrick JM, Watson RW. Prostate epithelial cell differentiation and its relevance to the understanding of prostate cancer therapies. Clin Sci Lond 2005;108:1–11.
3. Chen CD, Welsbie DS, Tran C, et al. Molecular determinants of resistance to antiandrogen therapy. Nat Med 2004;10:33–9.
4. Arnold JT, Isaacs JT. Mechanisms involved in the progression of androgen-independent prostate cancers: It is not only the cancer cell's fault. Endocr Relat Cancer 2002;9:61–73.
5. Abate-Shen C, Shen MM. Molecular genetics of prostate cancer. Genes Dev 2000;14:2410–34.
6. Shang Y, Myers M, Brown M. Formation of the androgen receptor transcription complex. Mol Cell 2002;9:601–10.
7. Schuurmans AL, Bolt J, Veldscholte J, Mulder E. Regulation of growth of LNCaP human prostate tumor cells by growth factors and steroid hormones. J Steroid Biochem Mol Biol 1991;40:193–7.
8. Zhou HJ, Yan J, Luo W, et al. SRC-3 is required for prostate cancer cell proliferation and survival. Cancer Res 2005;65:7976–83.
9. Zheng Z, Cai C, Omwancha J, Chen SY, Baslan T, Shemshedini L. SUMO-3 enhances androgen receptor transcriptional activity through a sumoylation-independent mechanism in prostate cancer cells. J Biol Chem 2006;281:4002–12.
10. Link KA, Burd CJ, Williams E, et al. BAF57 governs androgen receptor action and androgen-dependent proliferation through SWI/SNF. Mol Cell Biol 2005;25:2200–15.
11. Chen SY, Cai C, Fisher CJ, et al. c-Jun enhancement of androgen receptor transactivation is associated with prostate cancer cell proliferation. Oncogene 2006;29:363–73.
12. Markus SM, Taneja SS, Logan SK, et al. Identification and characterization of ART-27, a novel coactivator for the androgen receptor N terminus. Mol Biol Cell 2002;13:670–82.
13. Taneja SS, Ha S, Swenson NK, et al. ART-27, an androgen receptor coactivator regulated in prostate development and cancer. J Biol Chem 2004;279:13944–52.
14. Gstaiger M, Luke B, Hess D, et al. Control of nutrient-sensitive transcription programs by the unconventional prefoldin URI. Science 2003;302:1208–12.
15. Delgermaa L, Hayashi N, Dorjsuren D, Nomura T, Thuy le TT, Murakami S. Subcellular localization of RPB5-mediating protein and its putative functional partner. Mol Cell Biol 2004;24:8556–66.
16. Dorjsuren D, Lin Y, Wei W, et al. RMP, a novel RNA polymerase II subunit 5-interacting protein, counteracts transactivation by hepatitis B virus X protein. Mol Cell Biol 1998;18:7546–55.
17. Parusel CT, Kritikou EA, Hengartner MO, Krek W, Gotta M. URI-1 is required for DNA stability in C. elegans. Development 2006;133:621–9.
18. Frank SR, Parisi T, Taubert S, et al. MYC recruits the TIP60 histone acetyltransferase complex to chromatin. EMBO Rep 2003;4:575–80.
19. Dugan KA, Wood MA, Cole MD. TIP49, but not TRRAP, modulates c-Myc and E2F1 dependent apoptosis. Oncogene 2002;21:5835–43.
20. Cho SG, Bhoumik A, Broday L, Ivanov V, Rosenstein B, Ronai Z. TIP49b, a regulator of activating transcription factor 2 response to stress and DNA damage. Mol Cell Biol 2001;21:8398–413.
21. McGilvray R, Walker M, Bartholomew C. UXT interacts with the transcriptional repressor protein EVI1 and suppresses cell transformation. FEBS J 2007;274:3690–71.
22. Sun S, Tang Y, Lou X, et al. UXT is a novel and essential cofactor in the NF-{κ}B transcriptional enhanceosome. J Cell Biol 2007;178:231–44.
23. Nwachukwu JC, Li W, Pineda-Torra I, et al. Transcriptional regulation of the androgen receptor cofactor androgen receptor trapped clone-27. Mol Endocrinol 2007;21:2864–76.
24. Oxelmark E, Roth JM, Brooks PC, Braunstein SE, Schneider RJ, Garabedian MJ. The cochaperone p23 differentially regulates estrogen receptor target genes and promotes tumor cell adhesion and invasion. Mol Cell Biol 2006;26:5205–13.
25. Wang Q, Carroll JS, Brown M. Spatial and temporal recruitment of androgen receptor and its coactivators involves chromosomal looping and polymerase tracking. Mol Cell 2005;19:631–42.
26. Bookout AL, Mangelsdorf DJ. Quantitative real-time PCR protocol for analysis of nuclear receptor signaling pathways. Nucl Recept Signal 2003;1:e012.
27. Koklanaris N, Nwachukwu JC, Huang SJ, et al. First-trimester trophoblast cell model gene response to hypoxia. Am J Obstet Gynecol 2006;194:687–93.
28. Cheng J, Sun S, Tracy A, et al. NetAffx Gene Ontology Mining Tool: a visual approach for microarray data analysis. Bioinformatics 2004;20:1462–3.
29. Newman JC, Weiner AM. L2L: a simple tool for discovering the hidden significance in microarray expression data. Genome Biol 2005;6:R81.
30. Ashburner M, Ball CA, Blake JA, et al. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet 2000;25:25–9.
31. DePrimo SE, Diehn M, Nelson JB, et al. Transcriptional programs activated by exposure of human prostate cancer cells to androgen. Genome Biol 2002;3:RESEARCH0032.
32. Li W, Cavasotto CN, Cardozo T, et al. Androgen receptor mutations identified in prostate cancer and androgen insensitivity syndrome display aberrant ART-27 coactivator function. Mol Endocrinol 2005;19:2273–82.
33. Gao M, Ossowski L, Ferrari AC. Activation of Rb and decline in androgen receptor protein precede retinoic acid-induced apoptosis in androgen-dependent LNCaP cells and their androgen-independent derivative. J Cell Physiol 1999;179:336–46.
34. Yoon HG, Wong J. The corepressors silencing mediator of retinoid and thyroid hormone receptor and nuclear receptor corepressor are involved in agonist- and antagonist-regulated transcription by androgen receptor. Mol Endocrinol 2006;20:1048–60.
35. Hodgson MC, Astapova I, Hollenberg AN, Balk SP. Activity of Androgen Receptor Antagonist Bicalutamide in Prostate Cancer Cells Is Independent of NCoR and SMRT Corepressors. Cancer Res 2007;67:8388–95.
36. Klokk TI, Kurys P, Elbi C, et al. Ligand-specific dynamics of the androgen receptor at its response element in living cells. Mol Cell Biol 2007;27:1823–43.

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

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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 coactivator 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-1α (PGC-1α) is a master regulator of energy metabolism and mitochondrial biogenesis and transducers of regulated CREB-binding proteins (TORCs) stimulate mitochon-

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Abbreviations: Ac, Acetylated; AR, androgen receptor; ART-27, androgen receptor trapped clone-27; ATF-1, activating transcription factor-1; C/EBPα, 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-1α, PRC, peroxisome proliferator-activated receptor-γ coactivator-1α; 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-1α (9). In addition, mutant huntingtin protein represses CREB-mediated transcription of PGC-1α 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-

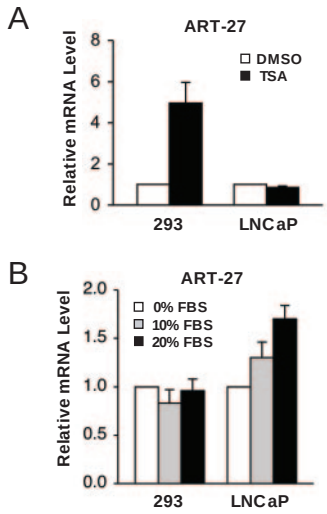

Fig. 1. Cell-Specific Regulation of ART-27 mRNA

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/EBPα)-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/EBPα-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/EBPα-binding site (Δ1) (Fig. 4B). In contrast, deletion of the CRE (Δ2) compromised ART-27 promoter activity by more than 50%, suggesting that the CRE but not the C/EBPα-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.

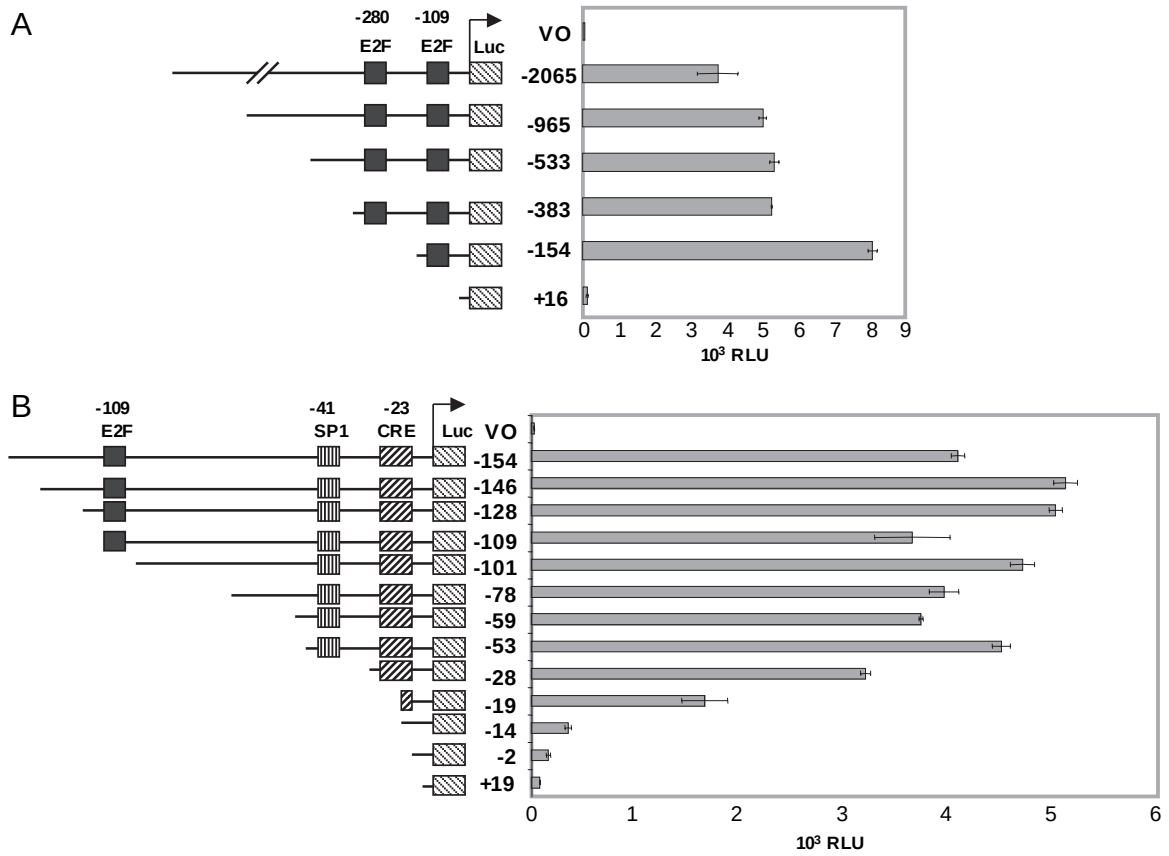

Fig. 2. Mapping the ART-27 Promoter

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/EBPα- 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/EBPα 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

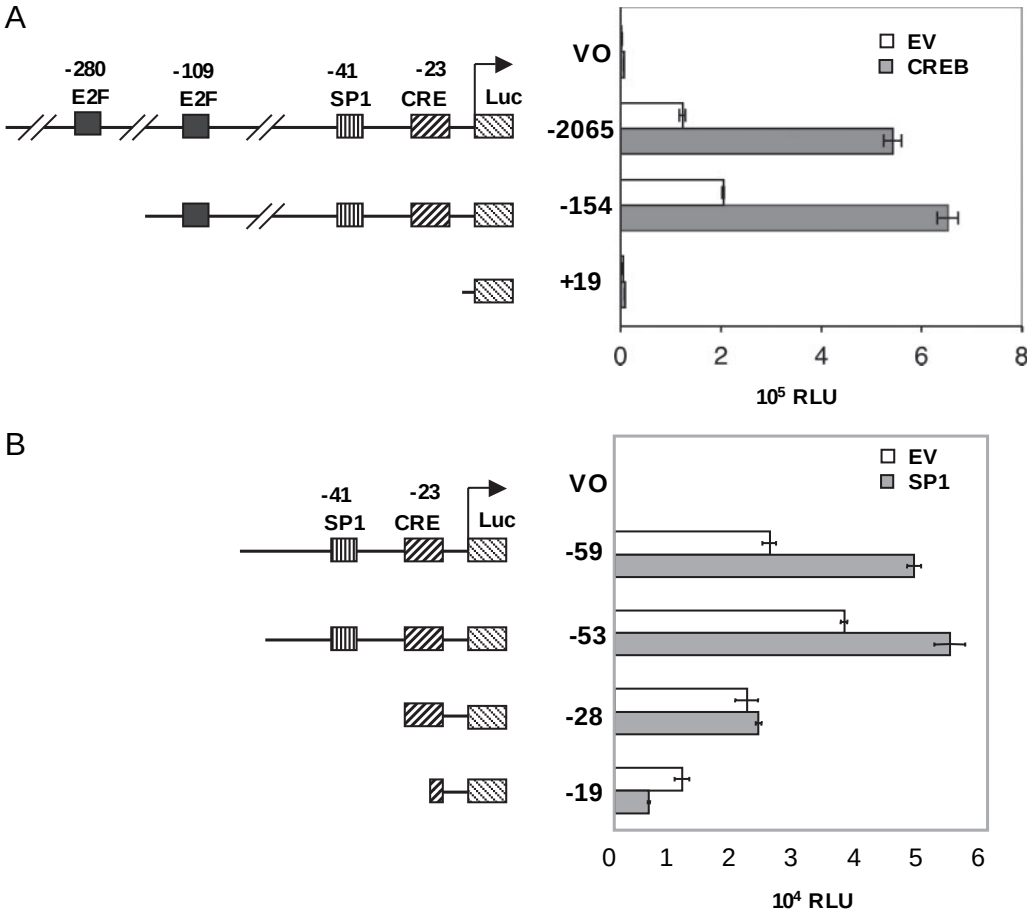

Fig. 3. CREB and Sp1 Enhance ART-27 Promoter Activity

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 μg empty pGL3 vector (VO) or ART-27-luciferase reporter, with 10 ng Lac-Z and 0.1 μg 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 μg empty pGL3 vector (VO) or ART-27-luciferase reporter, 10 ng of Lac-Z plasmids, and 0.1 μg 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

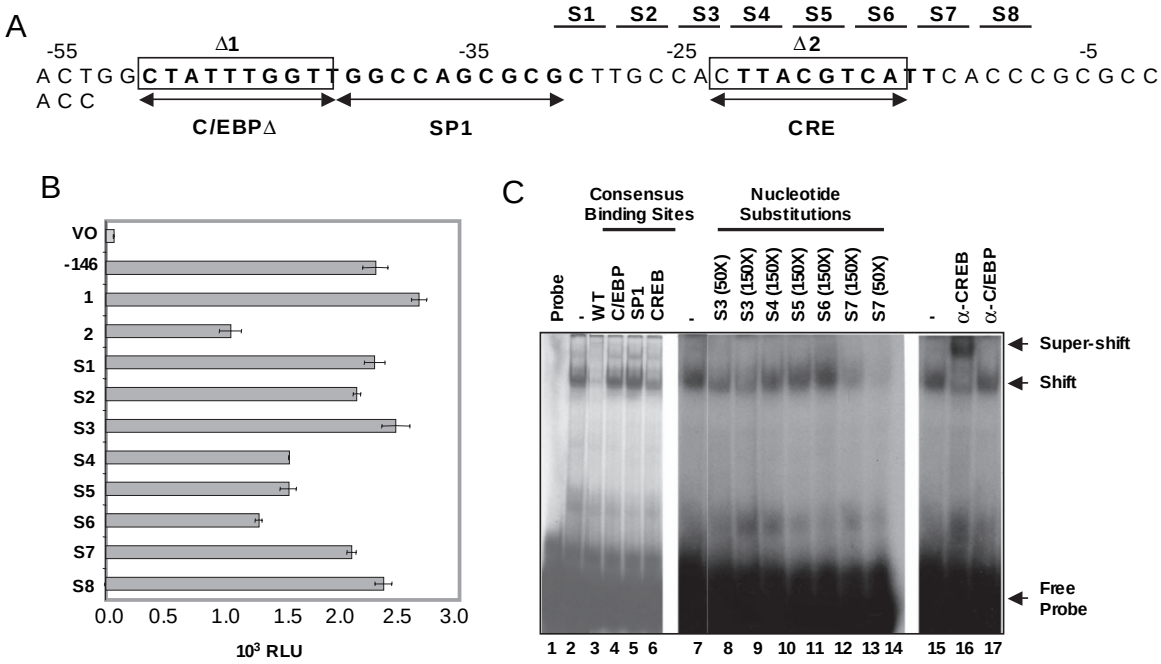

Fig. 4. Fine-Structure Analysis of the ART-27 Regulatory Region

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/EBPα-binding site (Δ1) or CRE (Δ2) 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-

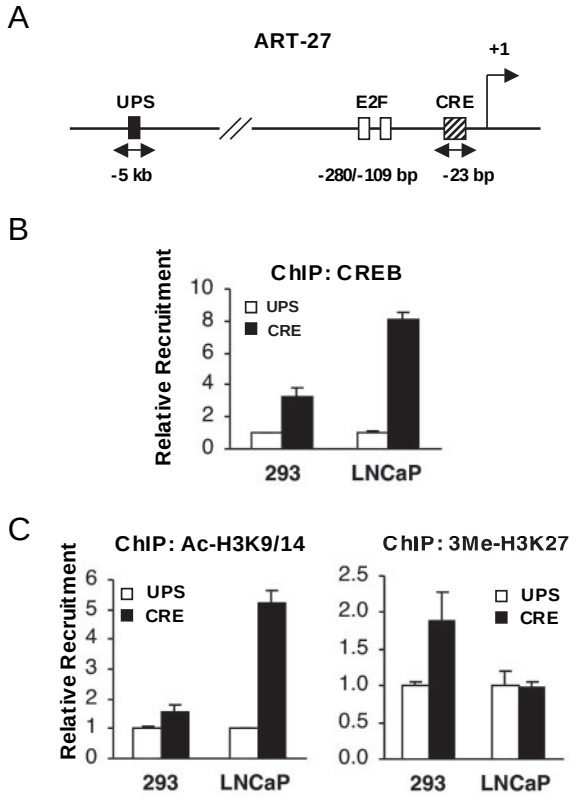

Fig. 5. CREB Recruitment and Chromatin Modification at the ART-27 Promoter

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 SD.

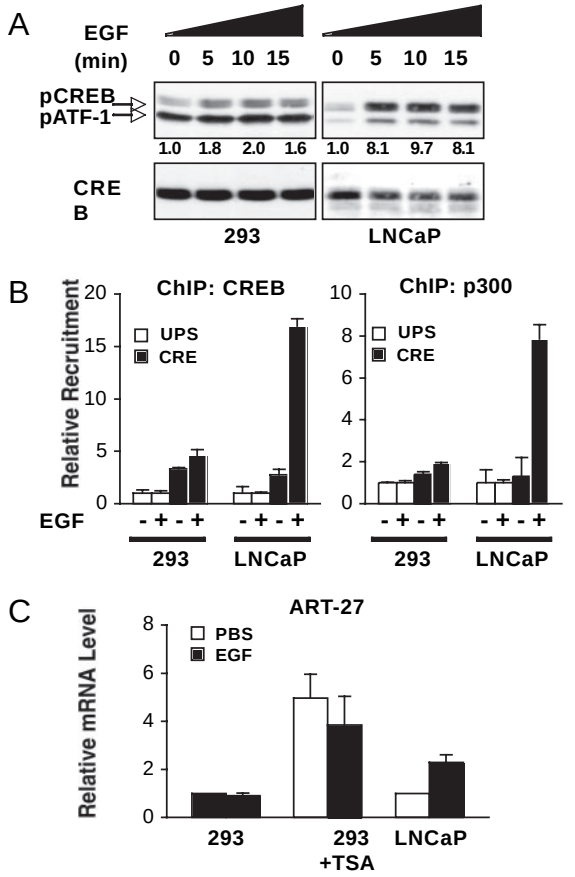

Fig. 6. EGF-Dependent Recruitment of CREB and Induction of ART-27

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.

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.

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

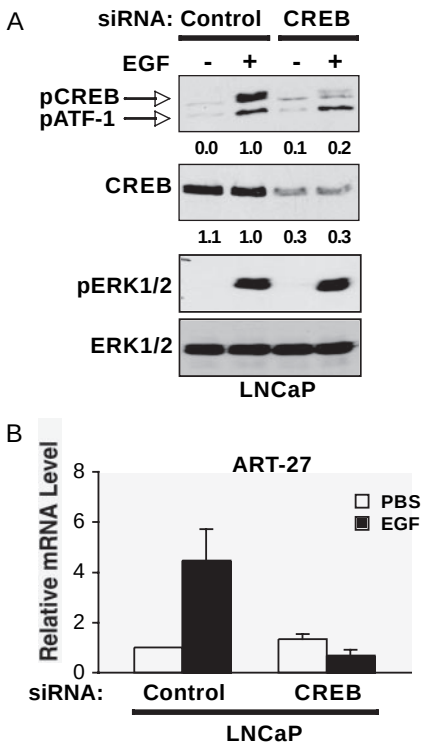

Fig. 7. CREB Mediates Induction of ART-27 by EGF

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

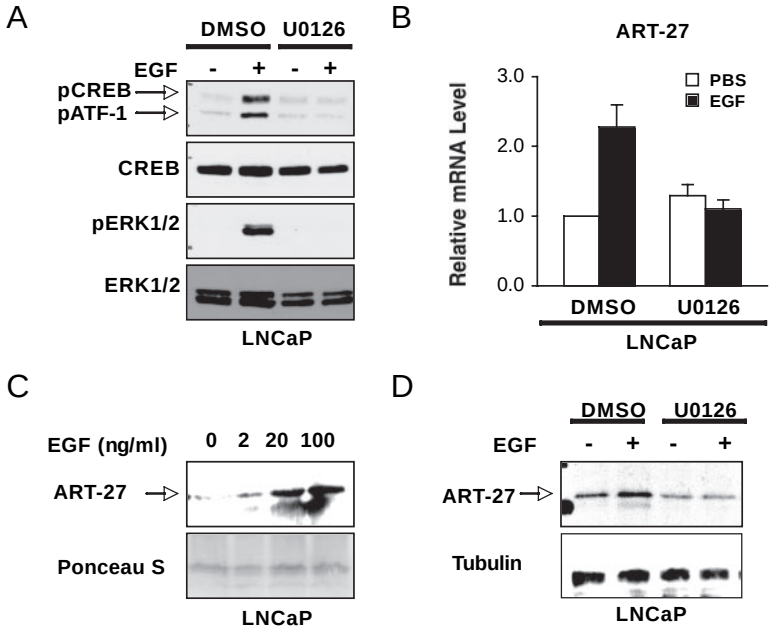

Fig. 8. The MAPK Kinase Inhibitor U0126 Blocks Induction of ART-27 by EGF

A, Effect of U0126 on CREB activation by EGF. LNCaP cells were serum starved for 24 h, pretreated with DMSO vehicle or 10 μM 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 μM 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 μM 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 of 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

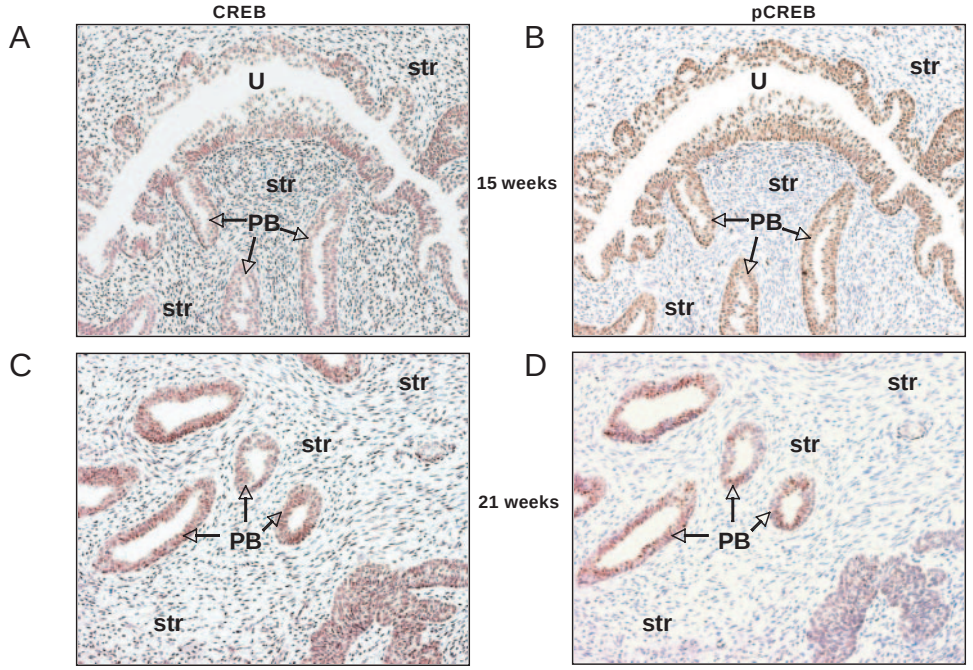

Fig. 9. Expression of CREB and pCREB in Human Prostate Development

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% CO_2 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′-CAACAGCCTCACCAAGGACT-3′ and reverse 5′-TCTGCAGGCCTTGTAGTTCTC-3′ or forward 5′-CTGGAGTTGACACTGGCAGA-3′ and reverse 5′-AGTCCTTGGTGAGGCTGTTG-3′) or ribosomal protein L19 (RPL19, forward 5′-CACAAGCTGAAGGCAGACAA-3′ and reverse 5′-GCGTGCTTCCTTGGTCTTAG-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

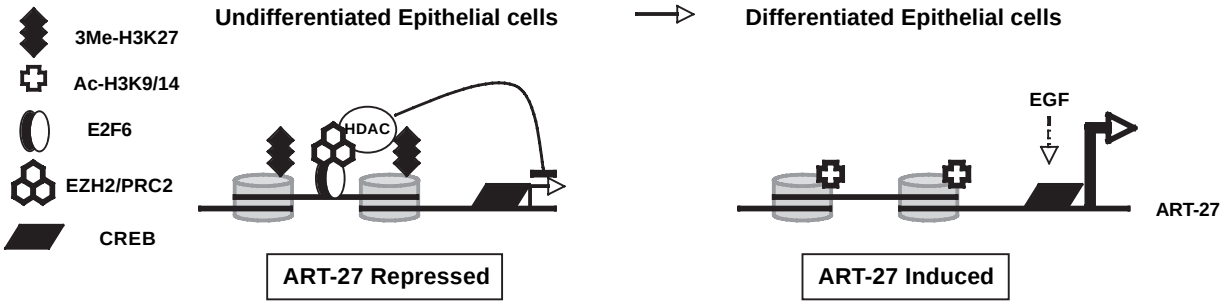

Fig. 10. Model for Regulation of ART-27 Gene Expression in Prostate Epithelial Cells

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)CCTCACGCCTGTAATC-3′ and 5′-GCAAGCT(G)CGA-GGTTCAGCCTTC-3′. Bases in parentheses were changed to the underlined bases to generate *Kpn*I restriction sites for subcloning. The resulting product was cloned into the *Eco*RV 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 Δ1 and Δ2 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 μl lysate and 100 μl luciferase assay reagent [25 mM glycylglycine (pH 7.8), 10 mM $MgSO_4$, 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 [^{32}Pγ]ATP by using T4-polynucleotide kinase. HeLa cell nuclear extracts (10 μg) was added to the radiolabeled double-stranded oligonucleotides in a total volume of 20 μl with 2.5 μg poly (dI-dC) and 1 μg 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/EBPα consensus was 5′-TGCAGATTGCGCAATCT-GCA-3′ (Santa Cruz sc-2525; Santa Cruz Biotechnology, Santa Cruz, CA); CREB consensus was 5′-AGAGATTGCCTGACGT-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/EBPα (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 μl proteinase K-SDS (0.5% SDS, 200 μg/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 μl of the DNA. The PCR primers used are as follows: −5 kb UPS, forward 5′-CTTGAAAG-CAGGAGGAAACG-3′ and reverse 5′-TTCTGGCTTCCAT-GTTTTCC-3′, and CRE, forward 5′-TGCCACTTACGTCAT-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 Na_3VO_4. 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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REFERENCES

1. Lonard DM, O'Malley BW 2006 The expanding cosmos of nuclear receptor coactivators. Cell 125:411–414
2. Shang Y, Myers M, Brown M 2002 Formation of the androgen receptor transcription complex. Mol Cell 9:601–610
3. Xu L, Glass CK, Rosenfeld MG 1999 Coactivator and corepressor complexes in nuclear receptor function. Curr Opin Genet Dev 9:140–147
4. Trimarchi JM, Lees JA 2002 Sibling rivalry in the E2F family. Nat Rev Mol Cell Biol 3:11–20
5. Mussi P, Yu C, O'Malley BW, Xu J 2006 Stimulation of steroid receptor coactivator-3 (SRC-3) gene overexpression by a positive regulatory loop of E2F1 and SRC-3. Mol Endocrinol 20:3105–3119
6. Masson N, Hurst HC, Lee KA 1993 Identification of proteins that interact with CREB during differentiation of F9 embryonal carcinoma cells. Nucleic Acids Res 21: 1163–1169
7. Shankar DB, Sakamoto KM 2004 The role of cyclic-AMP binding protein (CREB) in leukemia cell proliferation and acute leukemias. Leuk Lymphoma 45:265–270
8. Woloshin PI, Walton KM, Rehfuss RP, Goodman RH, Cone RD 1992 3′,5′-Cyclic adenosine monophosphate-regulated enhancer binding (CREB) activity is required for normal growth and differentiated phenotype in the FRTL5 thyroid follicular cell line. Mol Endocrinol 6:1725–1733
9. Wu Z, Huang X, Feng Y, Handschin C, Feng Y, Gullicksen PS, Bare O, Labow M, Spiegelman B, Stevenson SC 2006 Transducer of regulated CREB-binding proteins (TORCs) induce PGC-1α transcription and mitochondrial biogenesis in muscle cells. Proc Natl Acad Sci USA 103: 14379–14384
10. Cui L, Jeong H, Borovecki F, Parkhurst CN, Tanese N, Krainc D 2006 Transcriptional repression of PGC-1α by mutant huntingtin leads to mitochondrial dysfunction and neurodegeneration. Cell 127:59–69
11. Markus SM, Taneja SS, Logan SK, Li W, Ha S, Hittelman AB, Rogatsky I, Garabedian MJ 2002 Identification and characterization of ART-27, a novel coactivator for the androgen receptor N terminus. Mol Biol Cell 13:670–682
12. Gstaiger M, Luke B, Hess D, Oakeley EJ, Wirbelauer C, Blondel M, Vigneron M, Peter M, Krek W 2003 Control of nutrient-sensitive transcription programs by the unconventional prefoldin URI. Science 302:1208–1212
13. Zhao H, Wang Q, Zhang H, Liu Q, Du X, Richter M, Greene MI 2005 UXT is a novel centrosomal protein essential for cell viability. Mol Biol Cell 16:5857–5865
14. Parusel CT, Kritikou EA, Hengartner MO, Krek W, Gotta M 2006 URI-1 is required for DNA stability in *C. elegans*. Development 133:621–629
15. Taneja SS, Ha S, Swenson NK, Torra IP, Rome S, Walden PD, Huang HY, Shapiro E, Garabedian MJ, Logan SK 2004 ART-27, an androgen receptor coactivator regulated in prostate development and cancer. J Biol Chem 279:13944–13952
16. Oberley MJ, Inman DR, Farnham PJ 2003 E2F6 negatively regulates BRCA1 in human cancer cells without methylation of histone H3 on lysine 9. J Biol Chem 278: 42466–42476
17. Weinmann AS, Yan PS, Oberley MJ, Huang TH, Farnham PJ 2002 Isolating human transcription factor targets by coupling chromatin immunoprecipitation and CpG island microarray analysis. Genes Dev 16:235–244
18. Bracken AP, Ciro M, Cocito A, Helin K 2004 E2F target genes: unraveling the biology. Trends Biochem Sci 29: 409–417
19. Cartwright P, Muller H, Wagener C, Holm K, Helin K 1998 E2F-6: a novel member of the E2F family is an inhibitor of E2F-dependent transcription. Oncogene 17:611–623
20. Attwooll C, Oddi S, Cartwright P, Prosperini E, Agger K, Steensgaard P, Wagener C, Sardet C, Moroni MC, Helin K 2005 A novel repressive E2F6 complex containing the polycomb group protein, EPC1, that interacts with EZH2 in a proliferation-specific manner. J Biol Chem 280: 1199–1208
21. DePrimo SE, Diehn M, Nelson JB, Reiter RE, Matese J, Fero M, Tibshirani R, Brown PO, Brooks JD 2002 Transcriptional programs activated by exposure of human prostate cancer cells to androgen. Genome Biol 3:RESEARCH0032
22. Cartharius K, Frech K, Grote K, Klocke B, Haltmeier M, Klingenhoff A, Frisch M, Bayerlein M, Werner T 2005 MatInspector and beyond: promoter analysis based on transcription factor binding sites. Bioinformatics 21: 2933–2942
23. Quandt K, Frech K, Karas H, Wingender E, Werner T 1995 MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. Nucleic Acids Res 23:4878–4884
24. Quandt K, Grote K, Werner T 1996 GenomeInspector: a new approach to detect correlation patterns of elements on genomic sequences. Comput Appl Biosci 12:405–413
25. Cho KN, Choi JY, Kim CH, Baek SJ, Chung KC, Moon UY, Kim KS, Lee WJ, Koo JS, Yoon JH 2005 Prostaglandin E2 induces MUC8 gene expression via a mechanism involving ERK MAPK/RSK1/cAMP response element binding protein activation in human airway epithelial cells. J Biol Chem 280:6676–6681
26. Cox ME, Deeble PD, Bissonette EA, Parsons SJ 2000 Activated 3′,5′-cyclic AMP-dependent protein kinase is sufficient to induce neuroendocrine-like differentiation of the LNCaP prostate tumor cell line. J Biol Chem 275: 13812–13818
27. Gonzalez GA, Montminy MR 1989 Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell 59:675–680
28. Wiggin GR, Soloaga A, Foster JM, Murray-Tait V, Cohen P, Arthur JS 2002 MSK1 and MSK2 are required for the mitogen- and stress-induced phosphorylation of CREB and ATF1 in fibroblasts. Mol Cell Biol 22:2871–2881
29. Xing J, Ginty DD, Greenberg ME 1996 Coupling of the RAS-MAPK pathway to gene activation by RSK2, a

growth factor-regulated CREB kinase. Science 273: 959–963
30. Schuurmans AL, Bolt J, Mulder E 1988 Androgens and transforming growth factor β modulate the growth response to epidermal growth factor in human prostatic tumor cells (LNCaP). Mol Cell Endocrinol 60:101–104
31. Schuurmans AL, Bolt J, Mulder E 1988 Androgens stimulate both growth rate and epidermal growth factor receptor activity of the human prostate tumor cell LNCaP. Prostate 12:55–63
32. Schuurmans AL, Bolt J, Veldscholte J, Mulder E 1991 Regulation of growth of LNCaP human prostate tumor cells by growth factors and steroid hormones. J Steroid Biochem Mol Biol 40:193–197
33. Trimarchi JM, Fairchild B, Wen J, Lees JA 2001 The E2F6 transcription factor is a component of the mammalian Bmi1-containing polycomb complex. Proc Natl Acad Sci USA 98:1519–1524
34. van der Vlag J, Otte AP 1999 Transcriptional repression mediated by the human polycomb-group protein EED involves histone deacetylation. Nat Genet 23:474–478
35. Kirmizis A, Bartley SM, Kuzmichev A, Margueron R, Reinberg D, Green R, Farnham PJ 2004 Silencing of human polycomb target genes is associated with methylation of histone H3 Lys 27. Genes Dev 18:1592–1605
36. Kouzarides T 2007 Chromatin modifications and their function. Cell 128:693–705
37. Cohen DW, Simak R, Fair WR, Melamed J, Scher HI, Cordon-Cardo C 1994 Expression of transforming growth factor-α and the epidermal growth factor receptor in human prostate tissues. J Urol 152:2120–2124
38. Dahiya R, Lee C, Haughney PC, Chui R, Ho R, Deng G 1996 Differential gene expression of transforming growth factors α and β, epidermal growth factor, keratinocyte growth factor, and their receptors in fetal and adult human prostatic tissues and cancer cell lines. Urology 48: 963–970
39. Holzbeierlein J, Lal P, LaTulippe E, Smith A, Satagopan J, Zhang L, Ryan C, Smith S, Scher H, Scardino P, Reuter V, Gerald WL 2004 Gene expression analysis of human prostate carcinoma during hormonal therapy identifies androgen-responsive genes and mechanisms of therapy resistance. Am J Pathol 164:217–227
40. LaTulippe E, Satagopan J, Smith A, Scher H, Scardino P, Reuter V, Gerald WL 2002 Comprehensive gene expression analysis of prostate cancer reveals distinct transcriptional programs associated with metastatic disease. Cancer Res 62:4499–4506
41. Li W, Cavasotto CN, Cardozo T, Ha S, Dang T, Taneja SS, Logan SK, Garabedian MJ 2005 Androgen receptor mutations identified in prostate cancer and androgen insensitivity syndrome display aberrant ART-27 coactivator function. Mol Endocrinol 19:2273–2282
42. Bookout AL, Mangelsdorf DJ 2003 Quantitative real-time PCR protocol for analysis of nuclear receptor signaling pathways. Nucl Recept Signal 1:e012
43. Oxelmark E, Roth JM, Brooks PC, Braunstein SE, Schneider RJ, Garabedian MJ 2006 The cochaperone p23 differentially regulates estrogen receptor target genes and promotes tumor cell adhesion and invasion. Mol Cell Biol 26:5205–5213

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The Heterochromatin Protein 1 Family is Regulated in Prostate Development and Cancer

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Purpose: The HP1 family of evolutionarily conserved proteins regulates heterochromatin packaging, in addition to a less defined role in the regulation of euchromatic genes. To examine the possible role of HP1 proteins in fetal prostate development and prostate cancer the protein expression of HP1α, β and γ was evaluated in human archival tissue.

Materials and Methods: Tissue sections from human prostate cancer and fetal prostate were examined using antibodies against HP1 isoforms to evaluate HP1 modulation in cancer and development. Western blot analysis of HP1 proteins was also performed in extracts of cultured prostate cancer cells.

Results: HP1α, β and γ are differentially regulated in various cellular compartments in prostate development. HP1α is not expressed at 14 or 24 weeks of prostate development but it is expressed in adult prostate tissue. HP1β is highly expressed at 14 and 24 weeks, and it appears predominantly in epithelial cells compared to HP1γ, which is expressed at equal levels in epithelial and stromal cells. All 3 HP1 isoforms show altered expression in prostate cancer compared to that in normal adult prostate tissue.

Conclusions: HP1 proteins are tightly regulated during prostate development. In the adult prostate HP1α, β and γ antibodies detect high levels of HP1 antigen in a contiguous layer of epithelial cells. However, the detection of HP1 in prostate cancer ranges from undetectable to inconsistent staining of noncontiguous epithelial cells.

Key Words: prostate, prostatic neoplasms, heterochromatin-specific nonhistone chromosomal protein HP-1, fetal development

Heterochromatin protein 1 was originally characterized as an abundant protein that binds pericentric heterochromatin in Drosophila melanogaster.[1] HP1 isoforms are found in many organisms and human homologues, including HP1α, β and γ.[2] HP1 proteins comprise 3 protein domains, that is a chromo domain and a chromoshadow domain separated by a linker domain. The chromo domain at the N-terminus binds to dimethylated and trimethylated lysine 9 of histone H3 and methylation at this site is a mark of gene silencing.[2] While HP1 is generally thought to be involved in chromosomal and gene silencing, high resolution mapping in Drosophila cells indicates that HP1 forms large domains in pericentric chromatin but it is also localized to transcriptionally active single genes on the chromosome arms.[3] Although it is clear that HP1 proteins have a role in developmental processes, there is limited understanding of their function. Interestingly deletion of HP1 in Drosophila results in male specific lethality and expression analysis indicated that twice as many genes were regulated by HP1 in males vs females.[4]

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Study received New York University Institutional Review Board approval.

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To investigate whether HP1 proteins might also regulate aspects of male development in humans the expression patterns of HP1α, β and γ were examined in archival tissue encompassing the region of the urogenital sinus from which the prostate develops. The results of these studies indicated that HP1 isoforms are differentially regulated in prostate development. In addition to a likely role in developmental processes, it appears that HP1 proteins are likely misregulated in cancers. HP1α levels are decreased in breast cancer and over expression of HP1α inhibits breast cancer cell invasion in vitro.[5] To determine whether a loss of HP1 proteins might also have a role in prostate cancer we determined the protein expression of all 3 HP1 isoforms in archival tissue from benign human prostates compared to prostate cancer.

METHODS

Tissues

All human samples were used with approval of the New York University Institutional Review Board. Prostate specimens from human fetuses at gestational ages 14 and 24 weeks, respectively, were obtained following surgical abortion performed for reasons unrelated to this investigation. Informed consent was obtained by the consulting obstetrician for all specimens. Gestational age was estimated from the date of last menstrual period as well as from sonographic measurements of crown to rump and foot length. Prostates were formalin fixed and oriented in paraffin blocks. Each antibody was used to stain 2, 14-week specimens and 3, 24-week specimens. Figures 1 and 2 show representative staining.

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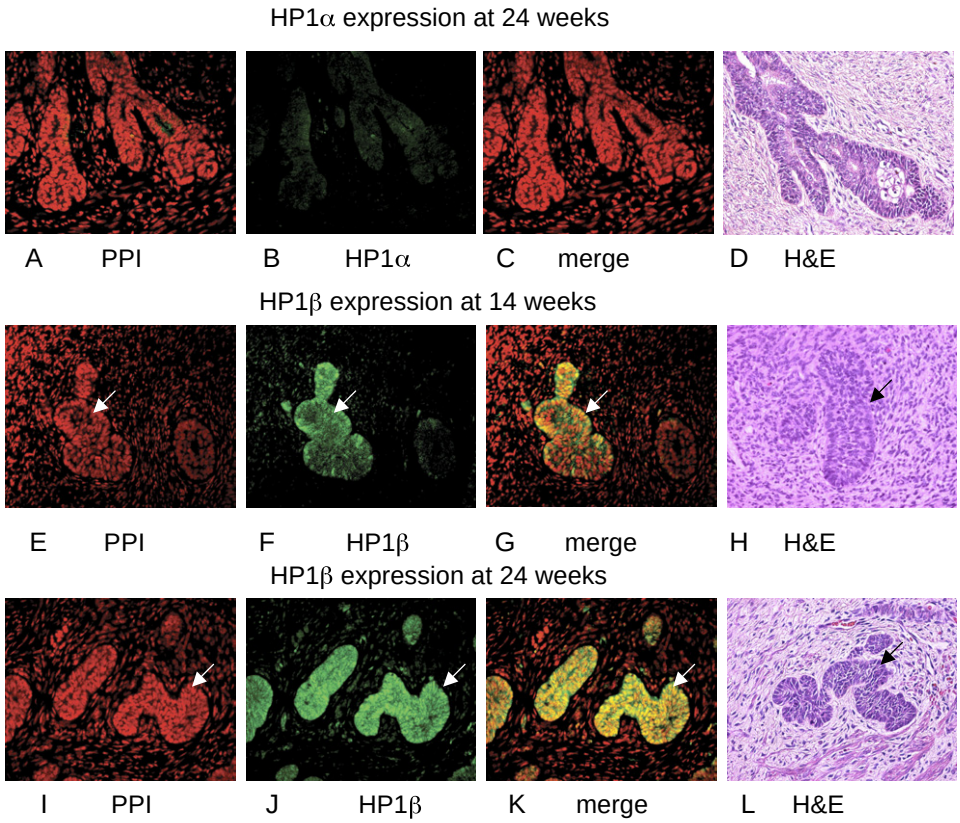

FIG. 1. Representative staining of 2 and 3 specimens per antibody shows HP1α and β expression in prostate development, including in sections of human fetal prostate at 14 (*E* to *H*) and 24 (*A* to *D* and *I* to *L*) weeks of gestation, respectively. Note merged images (*C*, *G* and *K*) and adjacent sections of same tissue blocks (*D*, *H* and *L*). PPI (*A*, *E* and *I*), antibody against HP1α (*B*) and antibody against HP1β (*F* and *J*) staining, and H & E (*D*, *H* and *L*), reduced from $\times$400.

Immunofluorescence and Antibodies

Tissue sections (5 μm) were cut and immunofluorescence was done within 1 week of sectioning. Tissue sections were dewaxed in xylene for 2 $\times$ 10 minutes and rehydrated in 100% ethanol for 2 $\times$ 5 minutes and in 95% ethanol 2 $\times$ 5 minutes. Antigen retrieval was performed by incubating paraffin sections with antigen unmasking solution (H-3300, Vector Laboratories, Burlingame, California) and microwave treatment at 900 W for 15 to 30 minutes, followed by treatment with 3% H_2O_2 and blocking with 20% normal goat serum. For immunofluorescence the samples were incubated with anti-HP1α (rabbit polyclonal, ab9057, Abcam®), anti-HP1β (rabbit polyclonal, 07-333) or anti-HP1γ (mouse monoclonal, clone 42s2, 05-690, Upstate Biotechnology, Waltham, Massachusetts), followed by FITC conjugated goat anti-rabbit (1:500) or FITC conjugated goat anti-mouse (1:500) (Molecular Probes) secondary antibody. Sections were mounted and immunofluorescence was detected using an Axioplan® 2 immunofluorescence microscope. For cancer studies 14 separate cases were examined for immunoreactivity with the HP1 antibodies (fig. 3). Four representative samples were chosen (fig. 3). In addition, 2 investigators scored 5 separate fields from 5 different cancers for HP1α, β and γ. The number of positive cells was assessed according to a scale of 0—0 cells, 1—1/100, 2—1/10, 3—1/3, 4—2/3 and 5—all cells. While benign appearing regions were scored as 5, in cancers the mean score $\pm$ SD was 1.3 $\pm$ 0.46, 1.36 $\pm$ 0.48 and 1.6 $\pm$ 0.66 for HP1α, β and γ, respectively.

Preparation of Nuclear and Cytoplasmic Lysates

Nuclear and cytoplasmic lysates were prepared according to Lee et al.[6] Cells were collected by centrifugation, swelled in hypotonic buffer and lysed by extruding them through a 25 gauge hypodermic needle. This homogenate was then centrifuged at 14,000 rpm for 5 minutes in a microcentrifuge to pellet the nuclei and the supernatant was saved as the cytoplasmic fraction. Proteins were extracted by resuspension of the nuclear pellet in high salt buffer and debris was separated by centrifugation. The resulting supernatant was retained as the nuclear fraction.

Immunoblots

Nuclear and cytoplasmic lysates were normalized by the Bradford assay (Bio-Rad®), separated on 10% polyacrylamide gel and transferred to Immobilon™ paper. Membranes were probed with HP1α or β antibody as described. Blots were developed using horseradish peroxidase coupled donkey anti-rabbit or sheep anti-mouse antibody and enhanced chemiluminescence.

RESULTS

HP1α, β and γ in Fetal Prostate Development

Immunohistochemistry was performed on archival tissue from 14 and 24-week old fetuses using antibodies specific for each HP1 isoform. Results showed that the expression of HP1α, β and γ differed dramatically from each other. HP1α

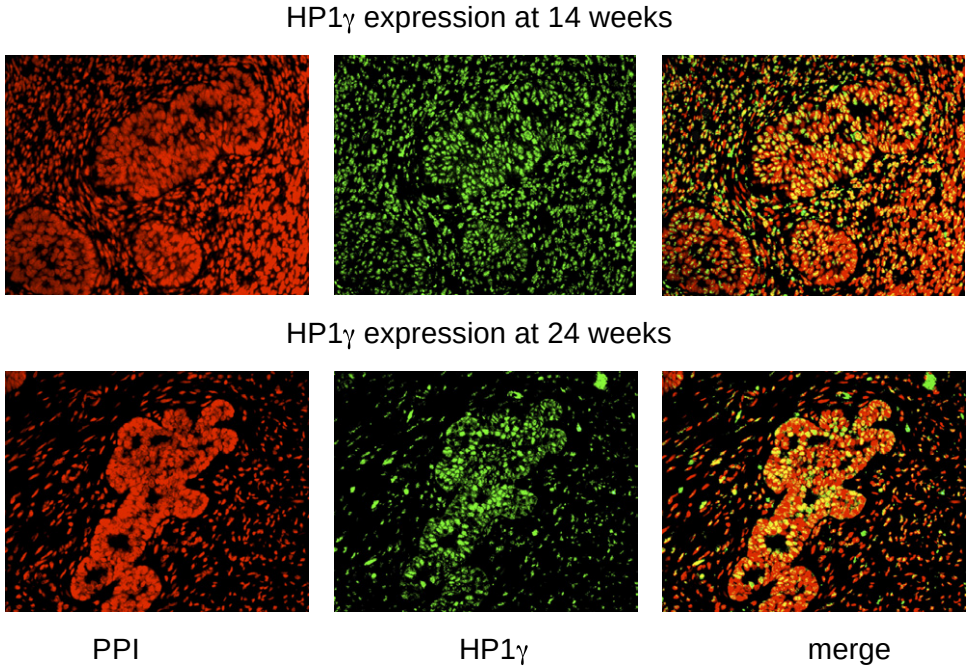

FIG. 2. Representative staining of 2 and 3 specimens demonstrates HP1γ expression in prostate development in human fetal prostate sections at 14 (top) and 24 (bottom) weeks of gestation, respectively. Note merged images (right). H & E staining of nearby tissue section at 14 and 24 weeks is same as in fig. 1, *H* and *L*, respectively. PPI (left) and antibody against HP1γ (middle) staining, reduced from ×400.

was completely undetectable at 14 weeks (data not shown) and it showed minimal if any expression at 24 weeks (fig. 1, *B*), although HP1α staining in adult prostate was evident (fig. 3). In contrast, HP1β showed robust expression at 14 weeks (fig. 1, *F*), continuing to 24 weeks (fig. 1, *J*). All samples were stained with HP1 antibody and FITC conjugated secondary for HP1α and β (fig. 1, *B*, *F* and *J*, green areas), in addition to PPI (fig. 1, *A*, *E* and *I*, red areas), to indicate nucleus localization. A merge of the HP1β stained image with the PPI image indicated that HP1β was predominantly nuclear, as expected for a chromatin binding factor. Using immunofluorescence, and hematoxylin and eosin the images showed a densely packed, epithelial cell laden structure (fig. 1, white and black arrows, respectively), which was predominantly yellow in merged images (fig. 1, *G* and *K*). They were the growing prostate buds, surrounded by more loosely packed stromal cells. While HP1β demonstrated some stromal cell expression (fig. 1, *J*), it was predominantly expressed in epithelial cells. HP1γ was also expressed at 14 and 24 weeks of prostate development (fig. 2), and it appeared more equally distributed between stromal and epithelial cells than HPIβ (figs. 1 and 2). These results indicate that the 3 HP1 isoforms are differentially regulated in human prostate development and suggest that HP1 proteins are an important component in genome-wide regulation of a developmental program.

Levels of HP1 Proteins Were Decreased in Prostate Cancers Compared to Those in Normal Prostate

To our knowledge the expression of HP1 isoforms has not been examined in prostate cancers, although breast cancers have decreased HP1α levels.[5] Figure 3 shows the expression of HP1 isoforms in normal adult prostate tissue compared to that in prostate cancer tissue. Figure 3, *A* to *C* shows HP1α, β and γ expression in benign tissue. Each sample was stained with PPI (fig. 3, red areas), in addition to incubation with antibody specific for HP1α, β or γ using FITC (fig. 1, green areas). However, figure 3 shows only the merged PPI and HP1 images, so that the stained cells (yellow areas) could be compared to the total number of cells (red areas). Results indicated robust staining of normal prostate tissue with antibodies against HP1α (fig. 3, *A*). HP1α staining was completely abolished by incubation with the immunizing peptide (data not shown). Recognition of antigen by HP1β and γ was also robust in normal tissue (fig. 3, *B* and *C*). Figure 3, *D* shows hematoxylin and eosin staining of a tissue section from the same sample to illustrate tissue morphology.

To determine whether HP1 protein levels are altered in prostate cancer we performed immunofluorescence in 4 separate prostate cancer samples (fig. 3, *E* to *T*, *1* to *4*). Figure 3 shows tissue morphology in hematoxylin and eosin stained panels as well as prostate cancers with a combined Gleason score of 6 (samples 1 and 2, fig. 3, *H* and *L*), 8 (cancer 3, fig. 3, *P*) and 7 (cancer 4, fig. 3, *T*). Again, to show the ratio of stained cells to the total cell number figure 3 shows images representing a merge between a PPI and an HP1 antibody treated sample with yellow areas indicating positive antigen reactivity. Staining patterns indicated that, while there was more immunoreactivity in some samples than in others (fig. 3, *F*, *G*, *M* and *Q*), relative to staining in normal tissue there were decreased levels and noncontinuous staining patterns in cancer containing tissue of HP1α (fig. 3, *E*, *I*, *M* and *Q*), HP1β (fig. 3, *F*, *J*, *N* and *R*) and HP1-γ (fig. 3, *G*, *K*, *O* and *S*). In samples showing positive reactivity not all epithelial cells stained and staining appeared noncontiguous, even in samples such as 1 and 2, which still appeared differentiated and glandular morphology was still evident (fig. 3, *E* to *G* and *I* to *K* vs *A* to *C*).

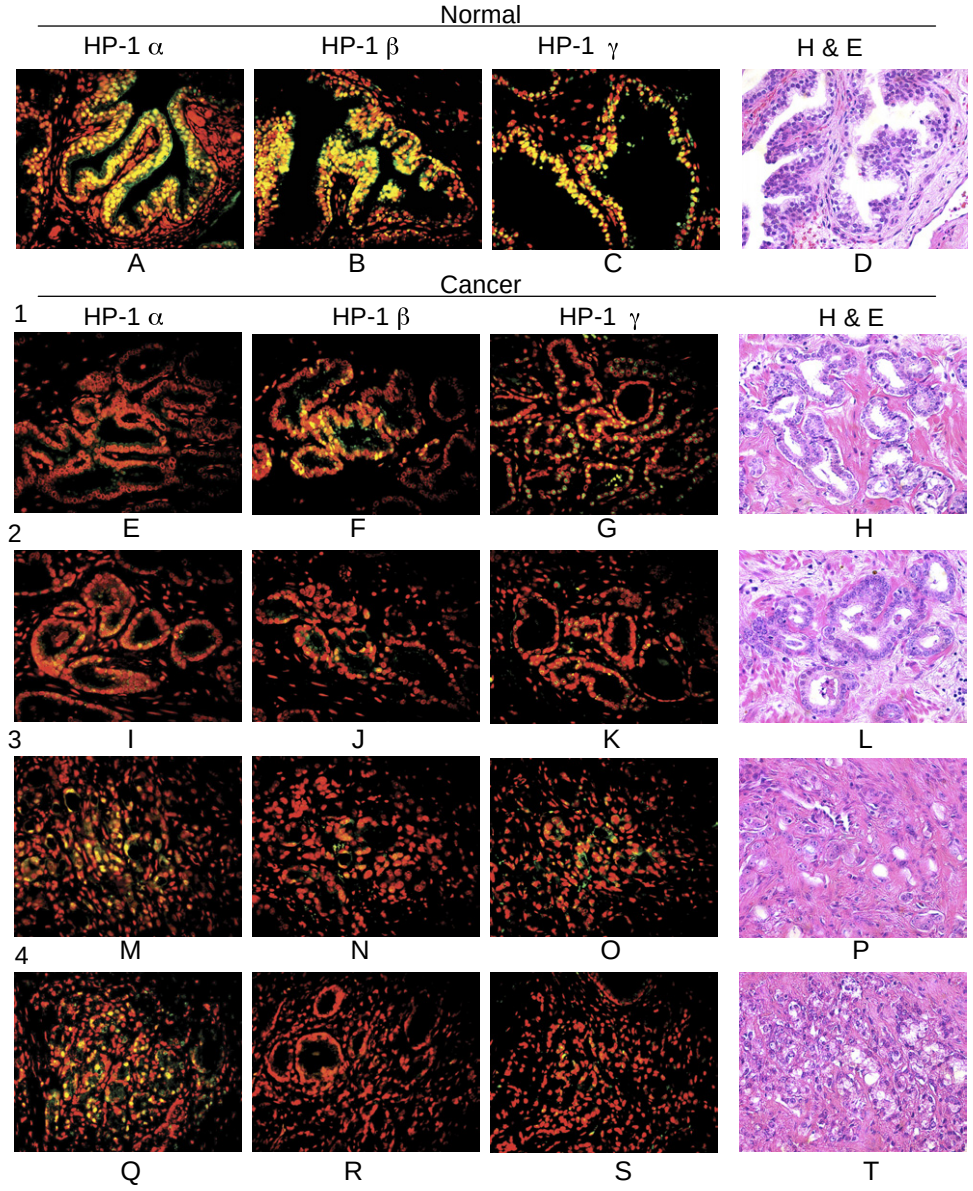

FIG. 3. Representative staining in 14 cancer cases per antibody reveals HP1 expression, including HP1α, β and γ in normal adult prostate tissue (*A* to *D*), and in 4 (*1* to *4*) prostate cancers (*E* to *T*). All samples were stained as in figure 1 but only merged images are shown. HP1α (*A*, *E*, *I*, *M* and *Q*), HP1β (*B*, *F*, *J*, *N* and *R*) and HP1γ (*C*, *G*, *K*, *O* and *S*) staining, and H & E (*D*, *H*, *L*, *P* and *T*), reduced from $\times$400.

To examine HP1 expression in cultured prostate cancer cells Western blots were performed in lysates from prostate cancer LNCaP cells. Nuclear and cytoplasmic lysates were prepared and used for immunoblot analysis using antibody against HP1α or β (fig. 4). LNCaP cells, which are nonmetastatic androgen dependent cells, are positive for HP1α. This may be have been similar to staining observed in lower Gleason score samples 1 and 2 (fig. 3). As expected, HP1α was predominantly nuclear (fig. 4, *A*). On the other hand, HP1β was not observed in LNCaP cells and antigen reactivity in HeLa cell lysates served as a positive control for immunoblotting conditions (fig. 4, *B*). HP1γ was not detected in HeLa or LNCaP cell lysates. However, we could not determine whether this was due to absent antigen or to poor antibody performance on Western blot analysis.

DISCUSSION

Little is understood about the role of HP1 proteins during development. However, what is known indicates that HP1 proteins have important roles. HP1 homologues in Caenorhabditis elegans are essential for vulval and germline development.[7] In Drosophila the deletion of HP1 results in male specific lethality and expression analysis indicated that twice as many genes are regulated in males as in females.[4] Consistent with these results, the discrepant pattern of HP1 staining in human prostate development in this study suggests that the 3 HP1 isoforms are tightly regulated during gestation and they have unique, nonoverlapping functions.

It is becoming increasingly clear that chromatin modification has a key role in cancer etiology.[8] The chromatin

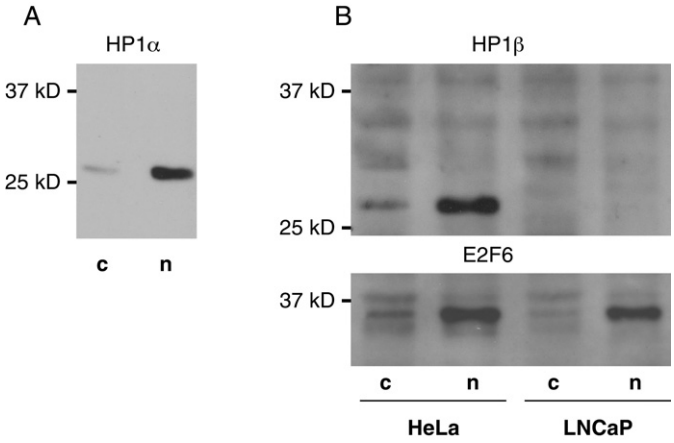

FIG. 4. Immunoblot analysis of HP1 shows cytoplasmic (*c*) and nuclear (*n*) lysates from LNCaP cells immunoblotted with antibody against HP1α (*A*), and from HeLa cells and LNCaP prostate cancer cells immunoblotted with antibody against HP1β and E2F6 (*B*). E2F6 immunoblot served as positive control for protein loading in LNCaP cell nuclear lysate lane. Values (left) indicate molecular weight markers. kDa, *kD*.

binding protein HP1α has already been implicated in cancer since breast cancers show decreased levels of HP1α protein. Furthermore, HP1α over expression results in the suppression of breast cancer cell invasion,[5] contingent on HP1α dimerization.[9] The fact that HP1 proteins may preferentially function in male specific transcription in some contexts coupled with the fact that prostate cancers show decreased levels of all 3 HP1 family proteins suggests that HP1 proteins may have an important function in prostate cancer etiology.

CONCLUSIONS

HP1 proteins are differentially regulated in human male prostate development. Furthermore, a comparison of benign prostate to prostate cancer tissue indicated that the expression of HP1α, β and γ is abundant in benign tissue, and highly variable in prostate cancer, suggesting an overall alteration of normal HP1 function in the chromatin regulation of cancer cells.

Abbreviations and Acronyms

FITC	=	fluorescein isothiocyanate
HP1	=	heterochromatin protein 1
PPI	=	propidium iodide

REFERENCES

1. James TC and Elgin SC: Identification of a nonhistone chromosomal protein associated with heterochromatin in Drosophila melanogaster and its gene. Mol Cell Biol 1986; **6:** 3862.
2. Lomberk G, Wallrath L and Urrutia R: The heterochromatin Protein 1 family. Genome Biol 2006; **7:** 228.
3. de Wit E, Greil F and van Steensel B: High-resolution mapping reveals links of HP1 with active and inactive chromatin components. PLoS Genet 2007; **3:** e38.
4. Liu LP, Ni JQ, Shi YD, Oakleley EJ and Sun FL: Sex-specific role of Drosophila melanogaster HP1 in regulating chromatin structure and gene transcription. Nat Genet 2005; **37:** 1361.
5. Kirschmann DA, Lininger RA, Gardner LM, Seftor EA, Odero VA, Ainsztein AM et al: Down-regulation of HP1Hsalpha expression is associated with the metastatic phenotype in breast cancer. Cancer Res 2000; **60:** 3359.
6. Lee KA, Bindereif A and Green MR: A small-scale procedure for preparation of nuclear extracts that support efficient transcription and pre-mRNA splicing. Gene Anal Tech 1988; **5:** 22.
7. Schott S, Coustham V, Simonet T, Bedet C and Palladino F: Unique and redundant functions of C. elegans HP1 proteins in post-embryonic development. Dev Biol 2006; **298:** 176.
8. Seligson DB, Horvath S, Shi T, Yu H, Tze S, Grunstein M et al: Global histone modification patterns predict risk of prostate cancer recurrence. Nature 2005; **435:** 1262.
9. Norwood LE, Moss TJ, Margaryan NV, Cook SL, Wright L, Seftor EA et al: A requirement for dimerization of HP1Hsalpha in suppression of breast cancer invasion. J Biol Chem 2006; **281:** 18668.