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Research Article

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

Jerome C. Nwachukwu,^{1,2} Paolo Mita,^{1,3} Rachel Ruoff,^{1,3} Susan Ha,^{1,3} Qianben Wang,⁵ S. Joseph Huang,⁶ Samir S. Taneja,³ Myles Brown,⁵ William L. Gerald,⁴ Michael J. Garabedian,^{2,3} and Susan K. Logan^{1,3}

Departments of ¹Pharmacology, ²Microbiology, and ³Urology, and NYU Cancer Institute, New York University School of Medicine, and ⁴Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York, New York; ⁵Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts; and ⁶Department 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 can-

cer 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 NH₂ 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- κ B 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% CO₂ 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).

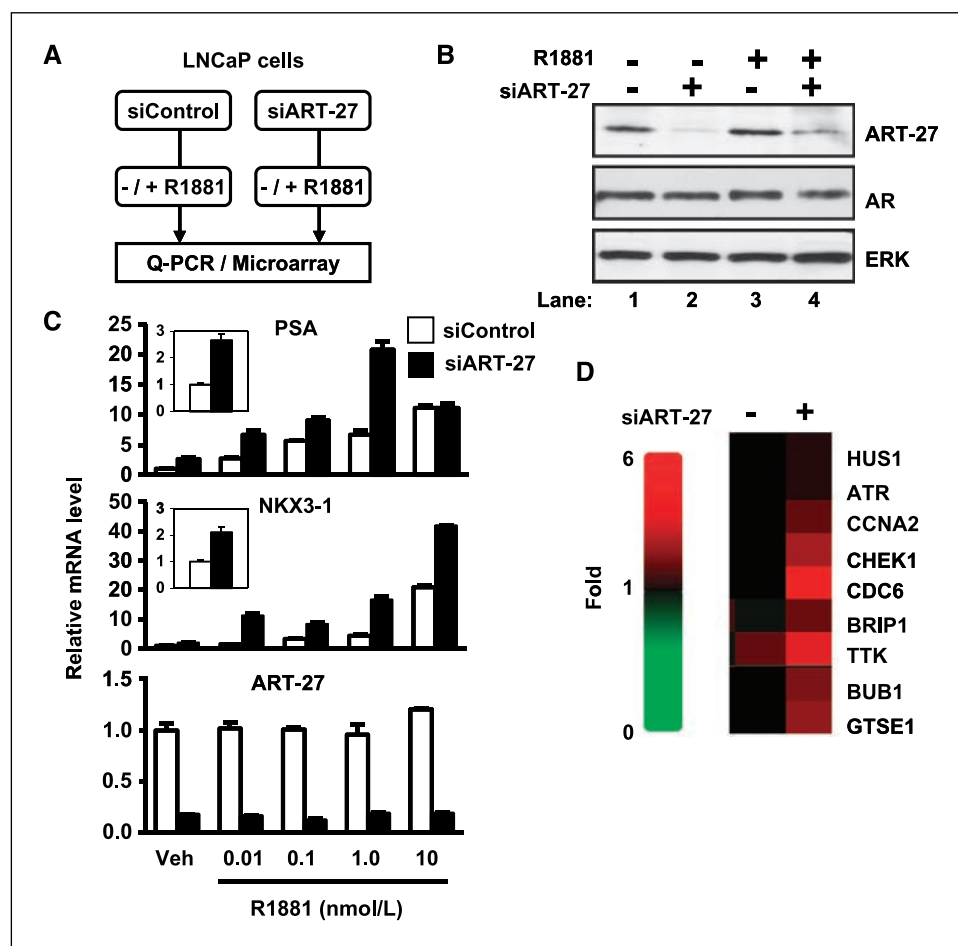
Chromatin immunoprecipitation assay. LNCaP cells were cultured in cs media (phenol red-free RPMI 1640 supplemented with 10% charcoal-stripped FBS) for 72 h, and stimulated with ethanol vehicle or 0.1 μ M/L

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

Requests for reprints: Susan K. Logan, Departments of Urology and Pharmacology, New York University School of Medicine, 550 First Avenue, MSB424, New York, NY 10016. Phone: 212-263-2921; Fax: 212-263-7133; E-mail: susan.logan@nyumc.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 $\Delta\Delta C_T$ 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 tool⁷ was used to sort androgen-regulated genes sets identified in each condition (28). *L2L* online microarray data analysis tool⁸ 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 μ mol/L bicalutamide at 37°C. The next day, 2 μ Ci of [³H]-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 *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. 1B). AR expression was largely unaffected (Fig. 1B). 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. 1C; 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. 1C, *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. 1C). 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. 1A). 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.

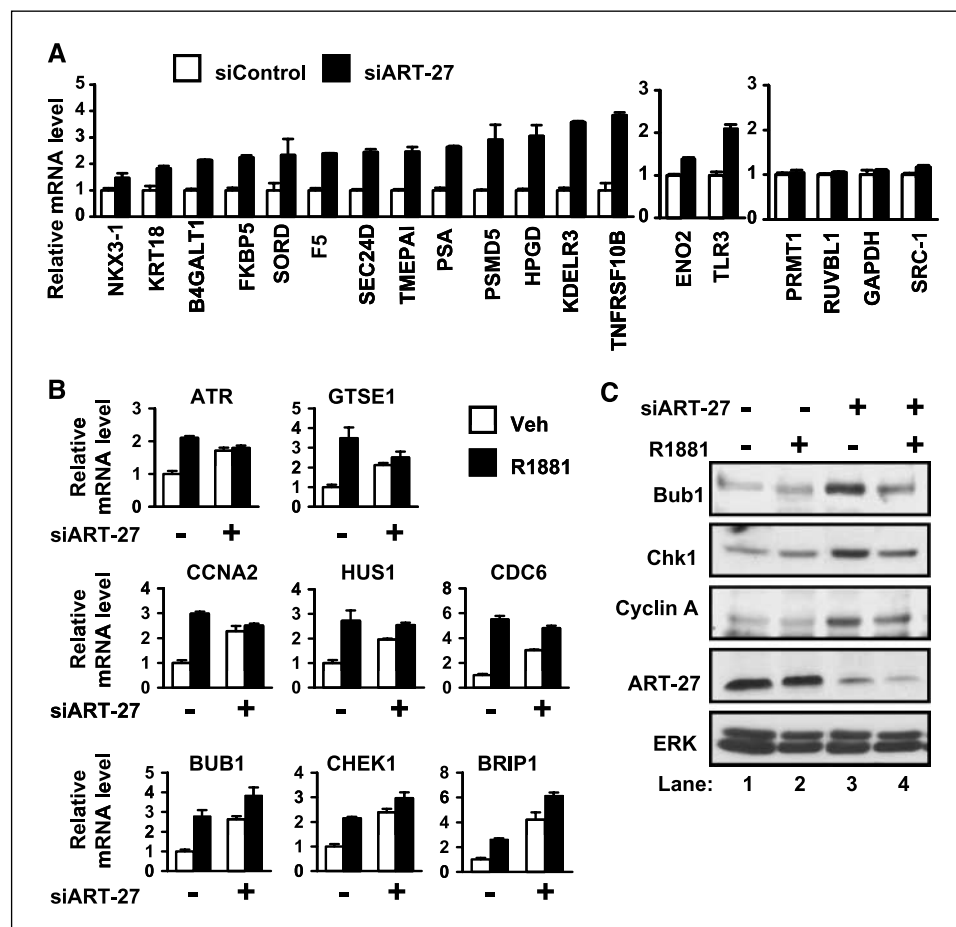
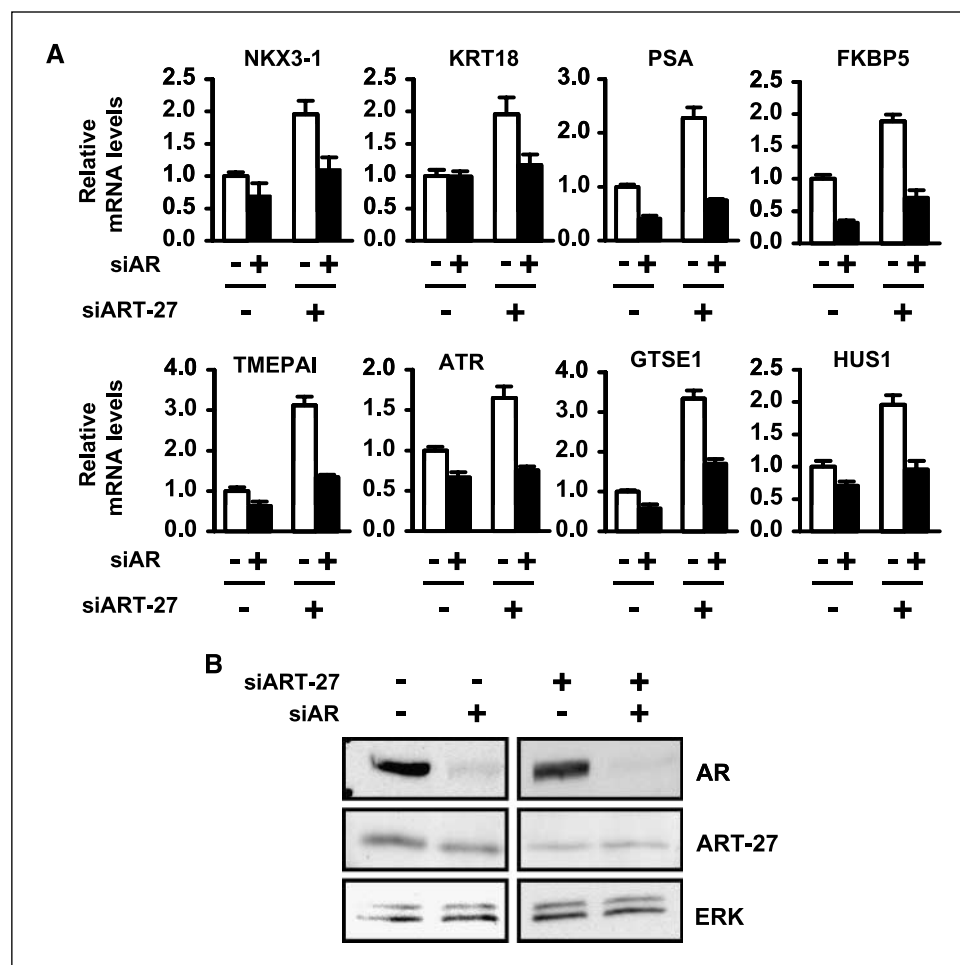


Figure 2. ART-27 inhibits expression of androgen-regulated genes. **A**, LNCaP cells transfected with control siRNA or ART-27 siRNA were steroid deprived for 72 h. Relative mRNA levels of the indicated androgen-induced, androgen-repressed, and androgen-insensitive genes were determined by Q-PCR (gene 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. 1A 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. 1A 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 S1A) and 517 androgen-repressed genes (Supplementary Table S1B) 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 S2A). 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. 1D), 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 S2B). 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 homeostasis 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. S1A), and the class of androgen-inducible DNA damage/cell cycle checkpoint genes identified in Fig. 2 (Supplementary Fig. S1B). 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. S1C). 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. 2A).

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. 2B, compare *white bars* for each gene). Similar to PSA (Fig. 1C), expression of most of these transcripts was minimally affected in cells stimulated with 10 nmol/L R1881 (Fig. 2B, compare *black bars* for ATR, GSTE1, HUS1, CDC6, and CCNA2). However, similar to NKX3-1 (Fig. 1C), 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. 2C, 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. S2C). This increase was associated with decreased ART-27 protein expression but not changes in AR expression in vehicle-treated cells (Supplementary Fig. S2D, 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. S2D, 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. 1B, 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

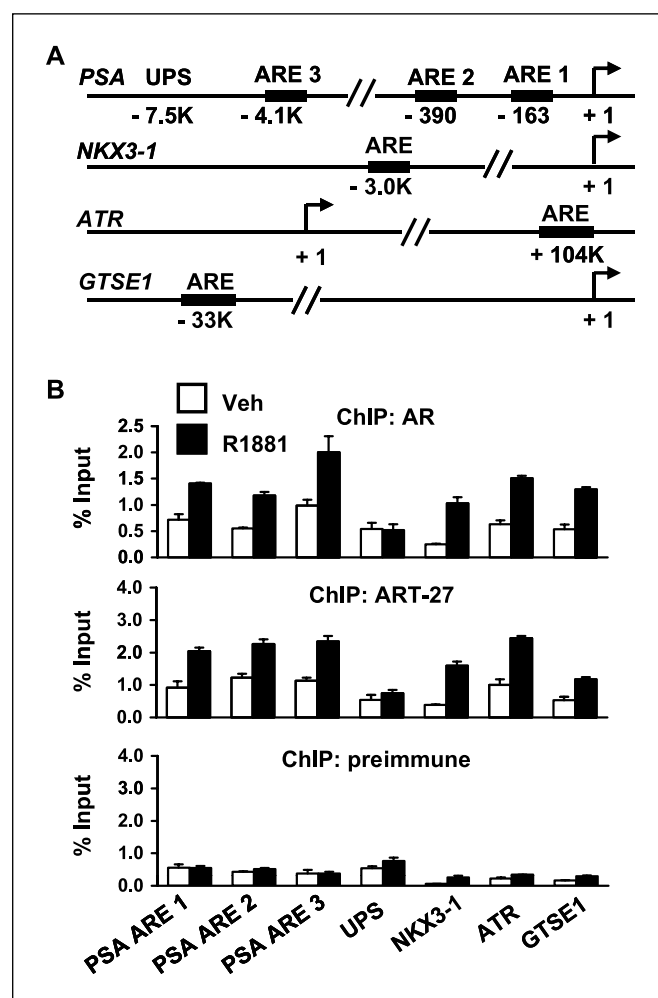


Figure 4. Recruitment of ART 27 to AR target genes. A, schematic illustration showing the positions of the well-characterized AREs of PSA and NKX3-1, the putative AREs of ATR and GTSE1, and the negative control upstream region (UPS), relative to transcription-start sites (+1 bp). B, LNCaP cells were steroid starved for 72 h and stimulated with ethanol vehicle or 0.1 μ M 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.

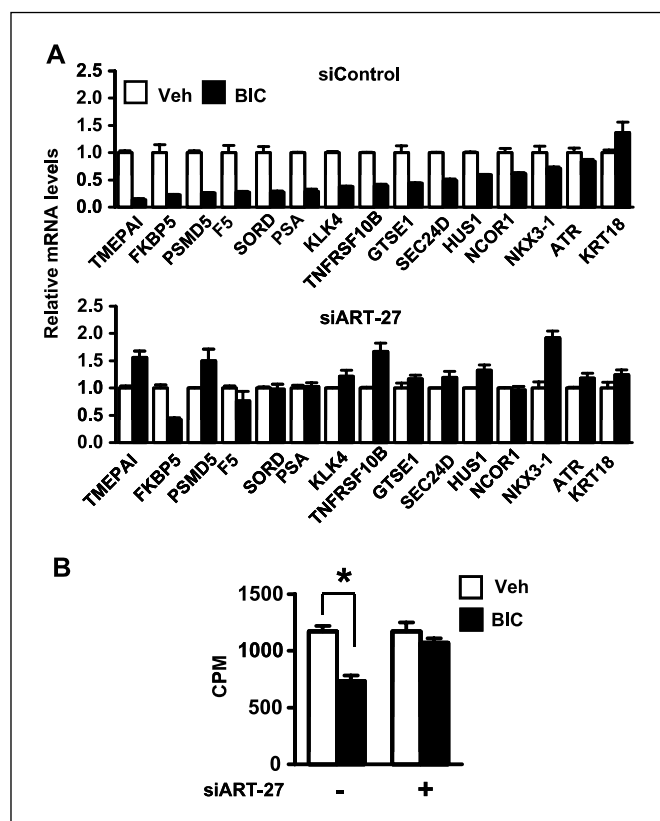


Figure 5. ART-27 loss facilitates resistance to the AR-antagonist bicalutamide. **A**, ART-27 depletion inhibits gene repression by bicalutamide. Q-PCR analysis showing relative mRNA levels of the indicated genes in LNCaP cells transfected with control siRNA or ART-27 siRNA and steroid deprived in the presence of ethanol vehicle or 0.1 μ M 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 μ M 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. 3A). The extent of AR and ART-27 protein depletion is also shown (Fig. 3B). As expected, in the presence of AR, depletion of ART-27 results in enhanced levels of mRNA (Fig. 3A, compare *white bars*). However, upon AR depletion, most genes show little if any increase in gene expression upon ART-27 siRNA treatment (Fig. 3A, 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. 4A; 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. 4A). 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. 4B). 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. 4B). 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. 5A), 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. 5A). 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. 5B). 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. 6A–B). Only 33% (8 of 24) of recurrent cancers exhibited nuclear staining for ART-27, whereas 56% (14 of 25) of nonrecurrent and 67% (6 of 9) of high-grade prostatic intraepithelial neoplasia stained positively (Fig. 6B). 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. 6C). 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).

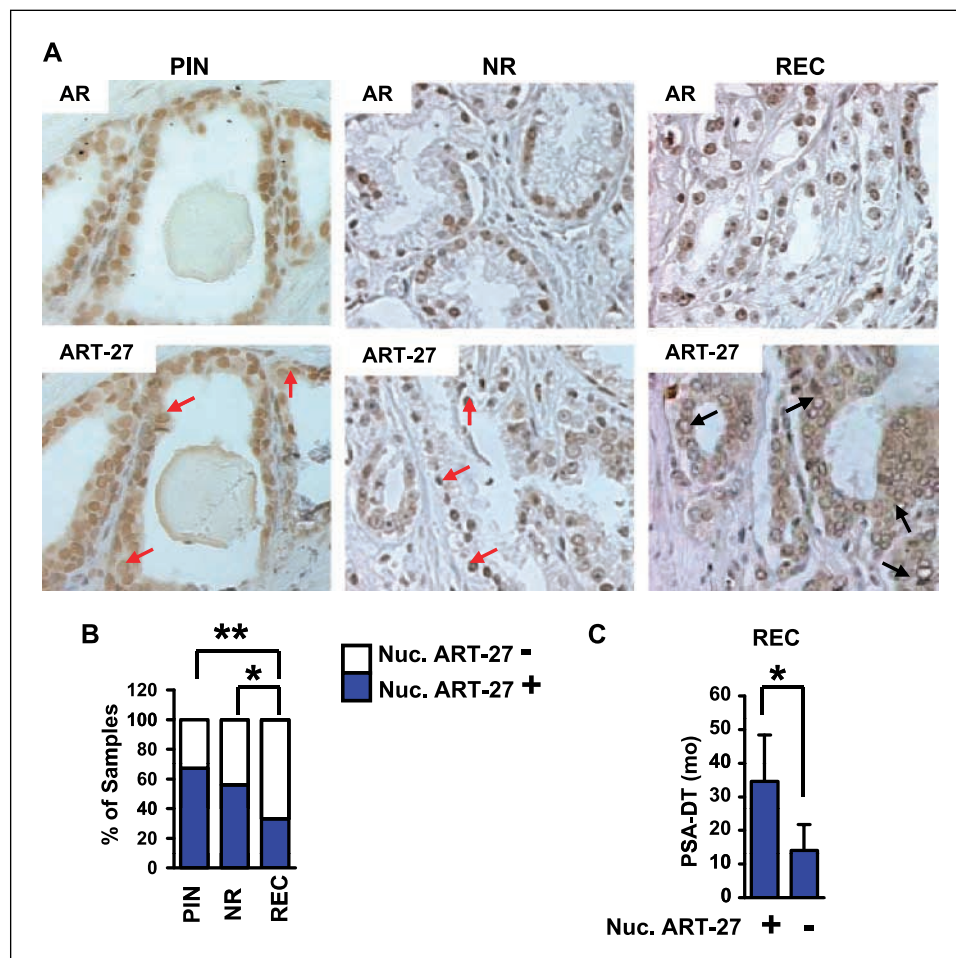


Figure 6. ART-27 expression and clinical outcome in AR-positive prostate cancer. **A**, ART-27 exhibits differential subcellular expression profiles in AR-positive prostate cancer. Prostate cancer tissue microarray containing 58 AR-positive cases [9 high-grade prostatic intraepithelial neoplasia (PIN); 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, $\times 40$; **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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