Nuclear Receptor Corepressor 1 Expression and Output Declines with Prostate Cancer Progression

Sandra M. Lopez1,2, Alexander I. Agoulnik3,4, Manqi Zhang1, Leif E. Peterson5, Eglu Suarez1, Gregory A. Gandarillas1, Anna Frolov6, Rile Li7, Kimal Rajapakse8, Christian Coarfa8, Michael M. Ittmann7,9, Nancy L. Weigel8, and Irina U. Agoulnik1,8,10

Abstract

Purpose: Castration therapy in advanced prostate cancer eventually fails and leads to the development of castration-resistant prostate cancer (CRPC), which has no cure. Characteristic features of CRPC can be increased androgen receptor (AR) expression and altered transcriptional output. We investigated the expression of nuclear receptor corepressor 1 (NCOR1) in human prostate and prostate cancer and the role of NCOR1 in response to antiandrogens.

Experimental Design: NCOR1 protein levels were compared between matched normal prostate and prostate cancer in 409 patient samples. NCOR1 knockdown was used to investigate its effect on bicalutamide response in androgen-dependent prostate cancer cell lines and transcriptional changes associated with the loss of NCOR1. NCOR1 transcriptional signature was also examined in prostate cancer gene expression datasets.

Results: NCOR1 protein was detected in cytoplasm and nuclei of secretory epithelial cells in normal prostate. Both cytoplasmic and nuclear NCOR1 protein levels were lower in prostate cancer than in normal prostate. Prostate cancer metastases show significant decrease in NCOR1 transcriptional output. Inhibition of LNCaP cellular proliferation by bicalutamide requires NCOR1. NCOR1-regulated genes suppress cellular proliferation and mediate bicalutamide resistance. In the mouse, NCOR1 is required for bicalutamide-dependent regulation of a subset of the AR target genes.

Conclusions: In summary, we demonstrated that NCOR1 function declines with prostate cancer progression. Reduction in NCOR1 levels causes bicalutamide resistance in LNCaP cells and compromises response to bicalutamide in mouse prostate in vivo.

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Introduction

Metastatic prostate cancer is treated with androgen ablation. Although it is initially successful, the resistance to the treatment almost invariably develops. Recurrent castration-resistant prostate cancer (CRPC) is the major cause of mortality of patients with prostate cancer, and better understanding of its biology is required for the development of novel therapies. Multiple studies show that the common mechanisms in prostate cancer progression are an increase in the androgen receptor (AR) expression and a reprogramming of AR transcriptional output (1–3); both are accelerated by androgen ablation. AR is a transcription factor that binds a broad range of androgen response elements (ARE) in regulatory regions of genes and intergenic loci. It assembles promoter-specific complexes composed of coactivators, corepressors, and general transcription factors and regulates gene expression (4). Inhibition of AR transcriptional activity eventually leads to an activation of prosurvival Akt signaling both in patients with prostate cancer and AR-dependent cell lines, caused largely by downregulation of PHLP (5) and INPP4B (6). Castration triggers increased expression of AR coactivators, such as TIF2 and CBP, that stimulate AR activity at low androgen levels (7). Another adaptation of prostate cancer to castration can be an increased synthesis and/or retention of androgens in tumor cells (8, 9).

NCOR1 and its close homolog, NCOR2, were first discovered as corepressors of unliganded thyroid receptor. NCOR1 has both nuclear and cytoplasmic fractions that have distinct cellular functions. Nuclear–cytoplasmic shuttling is regulated in part by NCOR1 phosphorylation. In HeLa cells, NCOR1 is distributed between cytoplasm and the nucleus; PKA activation causes NCOR1 phosphorylation and its complete nuclear translocation (10). In thyroid tumor cells, cytoplasmic NCOR1 interacts with...
**Translational Relevance**

Castration therapies are the standard-of-care treatment for men with advanced prostate cancer. The short- and long-term morbidities of this treatment are substantial. In addition, time lost for ineffective treatment can lead to the progression of the disease. With the advances of personalized medicine, it will be possible to more precisely characterize the tumor type for individual patients. Thus, markers that would predict therapeutic response are important. We examined the role of androgen receptor (AR) coregulator NCOR1 in response to antiandrogen treatments. In multiple datasets, NCOR1 mutations and loss of expression have been reported and, in this article, we show decline in NCOR1 protein and transcriptional output in prostate cancer tissues. We present data that suggest that patients with the loss of NCOR1 function due to mutation or loss of protein expression may be predictive of resistance to castration therapy in AR-expressing tumors.

PI3K regulatory subunit p85α and downregulates PI3K signaling (11). In the nucleus, NCOR1 binds various transcription factors and modulates their activity. Both NCOR1 and NCOR2 are recruited to PSA promoter in an AR-dependent manner (12) and can inhibit both agonist and partial antagonist signals were captured on a Gel Logic 2000 imaging system with conjugated secondary antibodies (Promega) for 1 hour, and NCOR1 modulates bicalutamide-dependent transcriptome. We show that response of prostate cancer cell lines LNCaP and LAPC4 to the AR antagonist bicalutamide (Casodex) requires expression of NCOR1. We demonstrate that NCOR1 modulates bicalutamide-dependent transcriptome. We show that the loss of NCOR1 changes the expression of a number of genes strongly implicated in prostate cancer progression. In agreement with previous reports, we discovered the AR and SIAH2 gene expression signatures among NCOR1 regulated genes (1, 13). We show that the NCOR1 protein levels are significantly lower in prostate cancer than in normal prostate with the corresponding increase in activated Akt. In advanced prostate cancer, NCOR1 signatures are significantly diminished in multiple cohorts. Our data indicate that NCOR1 is required for optimal bicalutamide response in mouse prostate, and the loss of its function is associated with prostate cancer progression.

**Materials and Methods**

Cell culture and reagents

LNCaP, LAPC4, PC3, VCaP, DU145, RWPE, PNT1A, and HeLa cell lines were purchased from ATCC and maintained in the recommended media. LNCaP AR-V7/pLumi was described previously (17). Media was purchased from Life Technologies. FBS and steroid-depleted charcoal-stripped serum (CSS) were purchased from Sigma. Bicalutamide (Casodex) and MDV3100 (enzalutamide) were purchased from Selleckchem.

**siRNA transfections**

siRNAs were transfected using Lipofectamine 2000 (Life Technologies) or electroporated in R buffer (Lonza) with the Nucleofector Device (Lonza). TARP was downregulated using siRNAs s226672 and s54578 (Life Technologies). NCOR1 was targeted with s201 and s203 (Life Technologies) and on-target SMARTpool L-003518-00-0050 (Thermo Fisher Scientific). Checkpoint kinase 1 (CHEK1)–specific siRNAs were s504 and s503 (Life Technologies). T-cell receptor γ chain alternative reading frame protein (TARP) was downregulated using s226672 and s54578 (Life Technologies). NCOR2, UGT2B15, and UGT2B17 were downregulated using SMARTpools (Thermo Fisher Scientific) and prevalidated siRNA from Life Technologies (s18467). Control noncoding siRNAs were purchased from Life Technologies and Thermo Fisher Scientific.

**Western blotting**

For AR, total Akt, pAkt (S473), actin, and tubulin, 30 μg of protein was resolved on SDS-PAGE and transferred to nitrocellulose membrane. For NCOR1 and NCOR2, 50 μg of protein was similarly processed. Membranes were blocked with 2% milk in TBST and incubated with 1:1,000 dilution of NCOR1 (Bethyl Laboratories), 1:1,000 dilution of NCOR2, 1:1,000 of total Akt (Cell Signaling Technology), 1:1,000 pAkt (S473), 1:2,000 dilution of N20 AR (Santa Cruz Biotechnology), 1:2,000 tubulin (Millipore), and 1:1,000 actin (Sigma) primary antibodies over-night at 4°C. Membranes were washed and incubated with HRP-conjugated secondary antibodies (Promega) for 1 hour, and signals were captured on a Gel Logic 2000 imaging system with Carestream Molecular Imaging Software (Carestream).

**Chromatin immunoprecipitation assay**

Chromatin immunoprecipitation assay was performed as described previously (6) using AR antibody N-20 (Santa Cruz Biotechnology) and NCOR1 antibody (Bethyl Laboratories).

**DNA synthesis and proliferation assays**

The [3H]thymidine incorporation was performed exactly as described previously (7). Cellular proliferation and motility was compared using Roche xCelligence RTCA (Roche Diagnostics) as described previously (6, 18). Cellular impedance in these assays is proportionate to the number of cells covering the E-plate and CIM-plate membrane.

Cell motility assays were performed using xCelligence RTCA (Roche Diagnostics) as described before (18) and Cellomics Cell Motility Kit as recommended by the manufacturer (Thermo Fisher Scientific).

**Gene expression array analysis**

To determine NCOR1-regulated genes, 2 × 10⁶ LNCaP cells were transfected with 800 pmol of either noncoding control or NCOR1 SMARTpool siRNA (Thermo Fisher Scientific) using R Buffer (Lonza) and Nucleofector Device (Lonza) exactly as recommended by the manufacturer. After 24 hours, cells were treated with either ethanol or 1 μmol/L bicalutamide for 48 hours, and RNA was purified using TRizol reagent (Life Technologies). For DHT-regulated gene expression, LNCaP cells were plated in RPMI medium supplemented with 10% CSS. Twenty-four hours later, cells were treated with 10 nmol/L DHT or ethanol vehicle. Cells were harvested 24 or 48 hours after treatment. RNAs were used for
expression analysis with Affymetrix 133A 2.0 Arrays at Genomic & RNA Profiling Core, Baylor College of Medicine (Houston, TX). Gene expression was marked as changed if the difference was statistically significant ($P < 0.01$) and the change was twofold or more. Gene expression data were deposited into GEO repository, series numbers GSE60721 and GSE60722.

**Gene set enrichment analysis**

Gene set enrichment analysis (GSEA) was performed using JAVA program (http://www.broadinstitute.org/gsea) as described previously (19). The AR gene signature was generated from genes upregulated in LNCaP after 48-hour DHT treatment ($P < 0.01$; Supplementary Table S3). The SIAH2 gene signature was generated by extracting genes changed more than twofold ($P < 0.01$) in LNCaP cell following SIAH2 knockdown (ref. 1; Supplementary Table S4).

**RNA extraction and quantitative PCR analysis**

RNA was extracted using TRI Reagent (Thermo Fisher Scientific) as recommended by the manufacturer. RNA was used to prepare cDNA using Verso cDNA Expression Kit (Thermo Fisher Scientific). Primers and probes are listed in Supplementary Table S5.

**Animal studies**

The mice were maintained under standard conditions at Florida International University (FIU; Miami, FL) animal facilities. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee at FIU and conducted in accordance with the National Academy of Science Guide for Care and Use of Laboratory Animals. Mice with Ncor1 floxed allele (Ncor1fl/fl, ref. 16) were kindly provided by Dr. Johan Auwerx (Switzerland); B6.Cg-Tg(Pbsn-cre)4Prb/Nci transgenic mice (ref. 2) were bred at our facility for the present study. Care and Use of Laboratory Animals. Mice with either oil alone or bicalutamide in sesame oil at 50 mg/kg. The mice were maintained under standard conditions at Florida International University (FIU; Miami, FL) animal facilities. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee at FIU and conducted in accordance with the National Academy of Science Guide for Care and Use of Laboratory Animals. Mice with Ncor1 floxed allele (Ncor1fl/fl, ref. 16) were kindly provided by Dr. Johan Auwerx (Switzerland); B6.Cg-Tg(Pbsn-cre)4Prb/Nci transgenic mice (ref. 2) were bred at our facility for the present study.

**Results**

**NCOR1 expression modulates response to bicalutamide in androgen-dependent prostate cancer cell lines**

Clearance of AR/NCOR1 complexes from a subset of promoters by SIAH2 interferes with androgen ablation (1). We asked whether direct NCOR1 depletion would similarly alter cellular responses to androgen ablation. First, we compared NCOR1 expression in several AR-positive and AR-negative cell lines. As shown in Fig. 1A and Supplementary Fig. S1A, all cell lines expressed NCOR1 protein, with somewhat higher expression in VCaP and LAPC4 prostate cancer cell lines. Next, we evaluated whether the loss of NCOR1 altered AR protein levels in androgen-responsive cell lines, LNCaP and LAPC4. No change of AR expression was observed following NCOR1 loss in both LNCaP (Fig. 1B) and LAPC4 cells (Supplementary Fig. S1B and S1C). In AR-negative cell lines, HeLa and DU145, knockdown of NCOR1 did not affect proliferation of cell lines grown in complete media (Fig. 1C and D). Depletion of NCOR1 in LNCaP cells increased proliferation in medium supplemented with CSS (Fig. 1E) but, similar to HeLa and DU145, had no effect on cells' growth in complete medium. A striking increase in proliferation following NCOR1 loss was observed in LNCaP cells treated with bicalutamide (Fig. 1E). We next tested whether NCOR1 mediates...
Figure 1.
NCOR1 suppresses proliferation of LNCaP cells in CSS-supplemented medium and in the presence of bicalutamide (Bic). A, fifty micrograms of protein extracted from LAPC4, LNCaP, VCaP, DU145, PC-3, and HeLa cells was resolved on SDS-PAGE and analyzed for NCOR1 and tubulin expression. B, LNCaP cells were transfected with control (C) or two independent NCOR1-specific siRNAs (s201 and s203). Cells were harvested 48 hours later and analyzed for NCOR1, AR, and tubulin expression by Western blot analysis. C, HeLa cells were transfected with either control or NCOR1-specific siRNA, and cells were grown for 48 hours in complete medium. DNA synthesis was compared by measuring rates of [3H] thymidine incorporation. Cells transfected in parallel were analyzed for levels of NCOR1 by Western blotting. D, DU145 cells were transfected and analyzed exactly as in C. E, LNCaP cells were transfected with control or NCOR1-specific siRNA and grown for 24 hours in medium supplemented with CSS or FBS as indicated. Cells were then treated for 24 hours with either vehicle (ethanol) or 1 μmol/L bicalutamide and [3H] thymidine incorporation measured. Cells transfected in parallel and treated with vehicle were analyzed for NCOR1 and tubulin expression by Western blot analysis. F, LNCaP cells were transfected with either control or NCOR1-specific siRNA and 24 hours later treated with either vehicle (ethanol), 1 μmol/L bicalutamide, 10 μmol/L bicalutamide, or 10 μmol/L MDV3100 for an additional 24 hours. B–F, experiments were repeated at least three times with two different NCOR1-specific siRNAs. Each point in DNA synthesis assay was done in triplicates. Average and SDs are shown. Unless the exact value is shown, asterisk (*) denotes differences between control and NCOR1 siRNA transfected cell with \( P < 0.05 \).
response to pure AR antagonist MDV3100 (enzalutamide). Both bicalutamide and MDV3100 at 10 μmol/L similarly suppressed DNA synthesis in LNCaP cells transfected with control siRNA (no statistically significant difference). However, MDV3100 inhibited much more then bicalutamide after NCOR1 knockdown (Fig. 1F). NCOR1 knockdown stimulated the proliferation of LAPC4 cells under all treatment conditions (Supplementary Fig. S1D). Opposite to NCOR1, NCOR2 knockdown did not stimulate LNCaP cell growth under any conditions (Supplementary Fig. S1E).

Bicalutamide also reduced LNCaP motility, and NCOR1 knockdown reversed this effect (Supplementary Fig S2). LNCaP remained noninvasive after NCOR1 knockdown (not shown). In PC-3 and DU145 cell lines, which express NCOR1 but not AR, NCOR1 knockdown did not alter cell motility and invasion (not shown).

Loss of NCOR1 alters bicalutamide-regulated gene expression profile

To understand how NCOR1 mediates bicalutamide resistance, we explored changes in gene expression caused by bicalutamide treatment and knockdown of NCOR1 using Affymetrix microarray. LNCaP cells were transfected with either control- or NCOR1-specific siRNAs and treated with either ethanol or 1 μmol/L bicalutamide generating four groups: Control siRNA_Vehicle (control siRNA transfected, treated with ethanol), Control siRNA_Bic (control siRNA transfected, treated with bicalutamide), NCOR1 siRNA_Vehicle (NCOR1 siRNA transfected, treated with ethanol), and NCOR1 siRNA_Bic (NCOR1 siRNA transfected, treated with bicalutamide; Fig. 2A). Gene expression analysis revealed pathways significantly affected by NCOR1 loss with or without bicalutamide treatment (Supplementary Table S1); we observed significant alteration in metabolic, cell signaling, and prostate cancer–associated pathways (Supplementary Table S1). We next examined whether NCOR1 regulated AR transcriptional activity using GSEA analysis. To determine DHT-regulated genes, we treated the LNCaP cell line with DHT or vehicle and performed microarray analysis. DHT treatment caused significant changes in genes associated with cell cycle, prostate cancer, and biosynthesis of steroids (Supplementary Table S2). A list of genes that were significantly increased by DHT treatment (P < 0.01; Supplementary Table S3) was used to analyze changes in gene expression in Control siRNA_Bic and NCOR1 siRNA_Bic pair (Fig. 2B). As expected, AR signature was significantly represented among bicalutamide-regulated genes with or without NCOR1 expression (Supplementary Fig. S3A and S3B). As seen from Fig. 2B, the AR signature was represented among NCOR1-regulated genes [normalized enrichment score (NES) = 1.4378, FDRq value = 0.1126, P < 0.0001]. Using a published list of SIAH2-regulated genes in LNCaP cells (1), we created the SIAH2 signature comprised of top SIAH2 up- and downregulated genes (Supplementary Table S4). As shown in Supplementary Fig. S3C, SIAH2 signature is present among NCOR1-regulated genes in bicalutamide-treated LNCaP cells (NES = 1.2258, FDRq value = 0.2101, P = 0.046).

NCOR1-regulated genes modulate cellular proliferation and response to castration and bicalutamide treatment

UDP glucuronosyltransferases 2B17 and 2B15 are expressed in luminal epithelium of human prostate and in LNCaP cells (26). They stimulate androgen removal and are implicated in prostate cancer (27). UGT2B15 and UGT2B17 are directly repressed by AR in an agonist-dependent manner (28). Loss of NCOR1 caused decrease in UGT2B15 expression under all treatment conditions (Fig. 3A and B). Expression of UGT2B17 declined in parallel with NCOR1 loss only in FBS or in CSS and R1881-supplemented media, suggesting that agonist-bound AR is needed for this regulation (Fig. 3C and D). Interestingly, the UGT2B17 and UGT2B15 expression was significantly lower in C4-2 cells, an LNCaP-derived cell line that proliferates and produces PSA in steroid-depleted medium (Supplementary Fig. S4). Consistent with previous reports (28), concomitant knockdown of both UGT2B15 and UGT2B17 resulted in increased AR transcriptional activity (Supplementary Fig. S5).

The most upregulated gene upon NCOR1 depletion was protecadherin 11 Y-linked (PCDH11Y). Previous reports showed that PCDH11Y overexpression allowed LNCaP cells proliferate in medium supplemented with CSS and made them resistant to TPA and serum starvation–induced apoptosis (29–31). We showed that PCDH11Y was suppressed by androgens in LNCaP cells at androgen concentrations as low as 0.1 nmol/L (Fig. 3E and F). Androgen depletion, bicalutamide treatment, and NCOR1 knockdown stimulated PCDH11Y expression (Fig. 3G, H, and I).

Following NCOR1 knockdown, the expression of TARP was significantly reduced. As previously reported, we show that TARP expression is induced by R1881 (32) almost 60-fold and suppressed by bicalutamide in medium with FBS (Fig. 4A). Intriguingly, TARP expression was consistently lower in medium supplemented with FBS than CSS plus R1881, suggesting additional modes of regulation by signaling pathways (Fig. 4A). NCOR1 knockdown significantly reduced TARP expression both in medium supplemented with CSS plus R1881 and with FBS. TARP is regulated similarly by NCOR1 and AR in LAPC4 cells (Supplementary Fig. S6A). As seen from Fig. 4B, TARP depletion increased LNCaP cell proliferation. Moreover, loss of TARP expression reduced LNCaP cells’ sensitivity to bicalutamide treatment (Fig. 4B).

We next tested whether the AR splice variant V7 (AR-V7) can induce TARP expression and whether NCOR1 is required for TARP optimal induction using previously described LNCaPAR-V7/plus LNCaP cell line inducibly expressing AR-V7 (17). As seen from Fig. 4C, doxycycline induction of AR-V7 expression increased TARP expression levels, and NCOR1 knockdown reduced AR and AR–V7–dependent induction of TARP. To analyze whether NCOR1 regulates AR-V7–specific transcription, we measured EDN2 gene expression. As previously reported (17), EDN2 was induced specifically by AR-V7, and optimal induction required NCOR1 expression (Fig. 4D).

NCOR1 knockdown resulted in significant loss of expression of CHEK1. CHEK1 kinase was not regulated by androgens in our LNCaP and LAPC4 cell lines (Fig. 4E and Supplementary Fig. S6B), and no recruitment sites were reported within 30 kb upstream or downstream of its gene locus (3). Depletion of NCOR1 (Fig. 4F) significantly reduced CHEK1 expression in LNCaP cells (Fig. 4G). In LAPC4 cells, NCOR1 depletion reduced CHEK1 expression in CSS (Supplementary Fig. S6B). As seen from Fig. 4H, loss of CHEK1 in LNCaP cells stimulated proliferation but did not abolish responsiveness to bicalutamide.

We next tested whether NCOR1 modulates AR activity by changing its recruitment to PSA and INPP4B promoters, genes whose expression is regulated by both AR and NCOR1 (6, 12). We observed NCOR1 recruitment to PSA and INPP4B promoters...
However, NCOR1 knockdown did not significantly alter AR recruitment to these loci (Supplementary Fig S7). NCOR1 mediates bicalutamide response in mouse prostate

To test whether NCOR1 is required for response to bicalutamide in mouse prostate, we obtained Ncor1<sup>fl/fl</sup>, Prbn-cre male mice with conditional deletion of Ncor1 gene in prostate epithelium and control Ncor1<sup>fl/fl</sup> littermate males with functional Ncor1 gene. Mutant males did not display any overt abnormality: total body weight and the weights of testes or seminal vesicles did not differ between two genotypes. Males with both genotypes were fertile and had normal prostates. Levels of Ncor1 expression evaluated by qRT-PCR were significantly lower in males with conditional deletion of Ncor1 (Fig. 5A), whereas Ar expression did not change when compared with the control group (Fig. 5B). After 48-hour treatment with bicalutamide at 50 mg/kg, no change was detected in Tmprss2 gene expression (Fig. 5C), confirming hypothesis that SIAH2/NCOR1/AR complexes do not regulate its expression in mouse prostate (1). Another three SIAH2/AR target genes in mouse prostate are ApoF, Nkx3.1, and Spink1 (1). In control
animals, the expression of AR target gene, ApoF, was reduced after bicalutamide treatment; downregulation of Ncor1 in mutant prostates abolished bicalutamide-dependent repression of ApoF. In control animals, we observed statistically significant downregulation of Nkx3.1 and Spink1 expression after treatment with bicalutamide. In mutant prostates, changes in expression of these genes were not statistically significant. Total reduction in Ncor1 was modest in prostate, which contains multiple cell types, but there is a substantial change in the regulation of epithelial-specific genes. Thus, we observed that the loss of Ncor1 compromised response to bicalutamide for a subset of AR target genes (Fig. 5D–F).

NCOR1 protein levels decline with prostate cancer progression in patients with prostate cancer

We next analyzed NCOR1 protein levels in normal prostate and prostate cancer samples from radical prostatectomy specimens using TMAs. Consistent with previous observations, strong staining was detected both in cytoplasm and nuclei of prostate luminal epithelium (Supplementary Fig. S8). In primary prostate tumors,
Figure 4.
NCOR1 is required for optimal expression of TARP and CHEK1. A, LNCaP cells transfected with either control or NCOR1-specific siRNAs were placed in medium supplemented with either CSS or FBS. LNCaP cells grown in CSS-supplemented medium were treated with ethanol vehicle (Veh) or 1 nmol/L R1881. Cells grown in FBS-supplemented medium were treated with ethanol vehicle or 1 µmol/L bicalutamide (bic). RNA was extracted and levels of TARP mRNA compared by qRT-PCR. Level of TARP expression in cells grown in CSS-supplemented medium transfected with control siRNA and treated with vehicle was assigned a value of 1 and all other values adjusted accordingly. B, cells transfected as in A were plated into E-plate of xCelligence analyzer at 10,000 cells per well 12 hours after transfection and treated with vehicle or 1 µmol/L bicalutamide. Cellular impedance was measured every 30 minutes and average and SE calculated for each time point. Statistical significance was calculated for the last time point. C–D, parental LNCaP and LNCaP\textsuperscript{R1881V} cells were transfected with control or NCOR1 siRNA and grown in CSS-supplemented medium. Cells were treated with either vehicle (V), 1 nmol/L R1881, or doxycycline (DOX) and RNA evaluated for TARP (C) and EDN2 (D) expression. E, LNCaP cells were treated with 1 nmol/L R1881 for 24 hours and RNA extracted and examined for CHEK1 expression. F–G, LNCaP cells grown in complete medium were transfected with either control, CHEK1, or NCOR1 siRNAs and expression of NCOR1 (F) and CHEK1 (G) compared 48 hours after transfection. H, LNCaP cells were transfected with CHEK1 or control siRNA. Twelve hours later, 10,000 cells were plated into each well of E-plate of xCelligence analyzer. Ethanol vehicle or 1 µmol/L bicalutamide were added and changes in cellular impedance measured every 30 minutes during 50 hours. An average and SE from four biologic replicates were calculated for each time point. The statistical significance was calculated for the last cellular impedance measurement between cells transfected with control and NCOR1 siRNAs. *, P < 0.05.
both cytoplasmic and nuclear levels of NCOR1 were significantly reduced (Fig. 6A). We correlated previously reported pAkt levels determined using the same array (23) with NCOR1 levels. In agreement with the observation that NCOR1 inhibits Akt pathway in thyroid cancer cells (11), we detected statistically significant negative correlation between the levels of pAkt (S473) and NCOR1 proteins in prostate cancer tissues (Fig. 6B). Consistent with this finding, NCOR1 knockdown in LNCaP and LAPC4 cells increased levels of pAkt (S473; Fig. 6C). On the other hand, NCOR1 knockdown did not change NCOR2 protein levels in LNCaP cells (Fig. 6C). In addition, no correlation between NCOR1 and NCOR2 was detected in patients with prostate cancer using Grasso (primary and CRPC; ref. 33), Stanborough (primary and metastatic; ref. 34), and Taylor (2) cohorts (Supplementary Table S7).

To determine whether NCOR1-regulated genes change with prostate cancer progression, we inferred transcriptome signature of NCOR1 silencing. Using previously employed methodology (2), we evaluated the NCOR1 silencing signature activity in three cohorts containing both primary and metastatic prostate cancer patients: Taylor and colleagues (2), Varambally and colleagues (24), and Cai and colleagues (25). In each cohort, the metastatic patients exhibited significantly higher activity scores for the NCOR1 silencing gene signature (P < 0.005 for each cohort), as presented in Fig. 6D–F, indicating a diminished activity of NCOR1 in metastatic prostate cancer.

Discussion
NCOR1 is a steroid receptor coregulatory protein, which was first described as a repressor of steroid receptor activity (13, 35). Unique to AR, NCOR1 was shown to repress both agonist- and antagonist-dependent transcriptional activity on ARE-driven reporters (13). We investigated whether NCOR1 modulates endogenous AR transcriptional activity and plays a role in response to bicalutamide treatment. Using independently
derived androgen-dependent cell lines LNCaP and LAPC4, we determined that NCOR1 suppresses cellular proliferation. Interestingly, in the LNCaP cells, which express moderate levels of NCOR1, the loss of NCOR1 stimulated proliferation in both CSS-supplemented medium and in full medium treated with bicalutamide. In the high NCOR1–expressing LAPC4 cells, depletion increased proliferation under all conditions (Supplementary Fig. S1D). Importantly, the loss of NCOR1 did not alter AR protein levels in either cell line, suggesting NCOR1-dependent changes in AR activity rather than AR level. Depletion of NCOR2 had the opposite effect on the proliferation of LNCaP cells (Supplementary Fig. S1E), confirming previous reports of nonredundant roles for these corepressors. Bicalutamide and the pure AR inhibitor MDV3100 suppressed LNCaP proliferation to a similar degree. However, in the absence of NCOR1, bicalutamide was much less effective than MDV3100 in inhibiting proliferation (Fig. 1E and F). This difference may be due to the different mechanisms of action of the two compounds. Bicalutamide allows some AR DNA binding and recruitment of protein complexes that include NCOR1. Depletion of NCOR1 reduces the inhibitory complexes. In contrast, MDV3100-bound AR does not bind to the DNA, and thus, the elimination of NCOR1 should have little or no effect on AR-dependent activity under these conditions, whereas AR-independent actions would be retained. Recently, MDV3100-regulated changes in gene expression have been reported for LNCaP (36). A comparison of MDV3100 transcriptional regulation (GSE44905 and GSE44924) in LNCaP and bicalutamide-dependent changes in our experiments showed that approximately 20% of the genes were regulated by both ligands.
To identify NCOR1-regulated genes and cellular pathways that mediate resistance to bicalutamide, we performed whole-genome gene expression analysis in LNCaP cells. As seen from Fig. 2A, NCOR1 regulates both bicalutamide-dependent and -independent transcription. In a previous report, loss of the ubiquitin ligase, SIAH2, reduced LNCaP proliferation, growth of colonies in soft agar, and sphere formation both in the presence and absence of androgens (1). This was presumably mediated by an increase in NCOR1–AR complexes on selected promoters. Indeed, the DHT-upregulated gene signature was highly significantly represented among NCOR1 target genes, suggesting a functional interaction between AR and NCOR1. This conclusion is supported by previous reports of physical and functional AR–NCOR1 interactions in the presence of androgens (1, 13) and bicalutamide (12). Significantly, the SIAH2 target gene signature was also significantly represented among NCOR1-regulated genes, confirming the finding that SIAH2 targets NCOR1–AR complexes for degradation in the presence of both androgen and bicalutamide (1).

Depletion of NCOR1 in control- and bicalutamide-treated LNCaP cells led to changes in the expression of genes that play significant roles in prostate cancer progression. The most downregulated gene was NCOR1, confirming successful knockdown. Many, but not all, of the genes identified are also AR regulated. The second most downregulated gene was an androgen-induced tumor suppressor, INPP4B, encoding inositol polyphosphate 4-phosphatase, type II. We have previously reported concordant induction of INPP4B by AR and NCOR1 (6). Consistent with this, we found that NCOR1 protein levels decline with prostate cancer progression. We also noted that INPP4B levels decreased with prostate cancer progression both in primary and especially in metastatic prostate cancer (2, 6), consistent with the positive correlation between NCOR1 and INPP4B expression observed in prostate cancer cell lines (6). NCOR1 loss also caused a significant induction of the PCDH11Y transcript, an androgen-repressed target. Androgen deprivation increases PCDH11Y expression in LNCaP cells. High levels of endogenously or exogenously expressed PCDH11Y stimulate androgen-independent LNCaP cell growth (29), confer resistance to apoptosis (30), and activate oncogenic Wnt signaling (30). PCDH11Y mRNA in normal prostates, primary prostate tumors, and untreated prostate cancer is low but increases significantly in CRPC (29), consistent with a negative correlation between NCOR1 and PCDH11Y observed in LNCaP cells. AR-repressed genes, UGT2B15 and UGT2B17, cause DHT conjugation and excretion (28, 37) and are associated with prostate cancer risk (38, 39). We show that NCOR1 is required for optimal UGT2B15 expression under all conditions, whereas for UGT2B17, expression of NCOR1 was required only in the presence of androgens. Both increased and decreased expression of these enzymes have been reported in prostate cancer (34, 40) potentially due to using samples from prostate tumors at different stages and treatments. It is also possible that levels of UGT2B15 and UGT2B17 enzymes decline only in a subset of prostate cancers as a mechanism to adapt to castration therapy by raising intracellular levels of androgens.

One of the most strongly downregulated transcripts when NCOR1 is depleted was TARP mRNA, a mitochondrial protein expressed specifically in normal prostate, prostate cancer, and breast cancer. At least four distinct protein-coding TARP variants are produced from this locus in response to androgens (32, 41). Splice variant, NM_001003799.1, changed expression with NCOR1 knockdown. TARP was strongly induced by androgens and by NCOR1 expression in LNCaP and LAPC4 cells. We found that TARP depletion significantly increased LNCaP cellular proliferation (Fig. 4B) and contributed to bicalutamide resistance. Our finding that AR-V7 also upregulates TARP and that NCOR1 is required for optimal induction suggests that in prostate cancers driven by various AR splice variants, NCOR1 is an important coregulator. This is confirmed by our observation that the induction of the AR-V7–specific target gene EDN2 requires NCOR1 expression (Fig. 5G and H). In an earlier report, exogenous expression of TARP increased cellular proliferation in an AR-negative prostate cancer cell line by increasing CAV1, AREG, and CXCL1 and suppressing IL1β expression (42). In LNCaP cells, levels of IL1β, AREG, and CAV1 are low to undetectable, whereas the loss of CXCL1 causes the opposite effect, increasing cell proliferation and anchorage-independent growth (43). TARP may also affect AR signaling, contributing to differences in its effect between AR-negative and AR-positive cells.

As shown in Fig. 4, NCOR1 also regulates CHEK1, and this regulation is androgen independent. CHEK1 is required for cell-cycle arrest in response to DNA damage and may also regulate cell cycle (44). A decline in CHEK1 expression was reported in prostate cancer (45), and compound loss of CHEK1 and PTEN triggers progression from high-grade prostatic intraepithelial neoplasia to invasive prostate carcinoma (46). CHEK1 knockdown increased LNCaP cellular proliferation but bicalutamide was still able to suppress proliferation (Fig. 4H). It is possible that in the NCOR1-negative prostate tumors, a decline in CHEK1 might contribute to more aggressive proliferative phenotype.

Additional confirmation of a role for NCOR1 in bicalutamide action was obtained using mutant mice with conditional prostate-specific Ncor1 gene deletion. We chose 48-hour time point for bicalutamide treatment because it would inhibit AR action without significant cell death in prostate epithelial compartment. Importantly, Ncor1 expression is not exclusive to luminal epithelium of mouse prostate, the site of Pbsn-cre expression. Similar to what we saw in LNCaP and LAPC4 cells, NCOR1 deletion in mouse prostate did not reduce AR mRNA levels. However, some of the AR target genes that are highly expressed in prostate epithelium (1, 47) lost their response to bicalutamide in mutant mice. In agreement with the previous report that Siah2 regulates gene expression by removing NCOR1 complexes from a subset of genes (1), Siah2-regulated AR target genes, ApoE, Nbs3.1, and Spink1, lost their ability to respond to bicalutamide treatment in Ncor1 knockout mouse prostate, while Tmprss2 expression did not change.

NCOR1 has emerged as an important regulatory protein in prostate and other cancers. Analysis of changes in signaling pathways with prostate cancer progression by Taylor and colleagues showed that NCOR1 is lost in 8% of primary and 16% metastatic prostate tumors due to deletions, loss of expression, and/or mutations (2, 33, 48). A comparison of high-grade untreated localized prostate cancers and lethal metastatic CRPC showed a high level of NCOR1 somatic mutations and copy number alterations in CRPC, while none were detected in untreated tumors (33). Additional evidence for NCOR1’s contribution to the development of resistance to antihormonal treatments was found in breast cancer. In Ehr−positive breast tumors, low NCOR1 expression is an independent predictor of tamoxifen resistance (49). Loss of NCOR1 at the protein level also correlated with acquired tamoxifen resistance in mouse models of breast
cancer (50) and with breast cancer recurrence in human patients (51). Tamborero and colleagues identified NCO1 as a high-confidence cancer-driver gene using three unbiased screening techniques: MuSIC-SMG, OncodriveFM, and Active Driver (48). We have compared NCO1 protein expression in normal prostate and prostate cancer in more than 400 paired tissues (Fig. 6). NCO1 was detected mostly in the prostate secretory epithelium (Supplementary Fig. S8). Consistent with previous reports, we observed NCO1 staining in both the cytoplasm and the nuclei of the cells (10, 11), and levels significantly declined in both compartments in malignant cells (Fig. 6A). Significantly, the NCO1-regulated transcriptional signature declines in advanced prostate cancer in multiple cohorts (Fig. 6D–F). Similar to thyroid cancer cells (11), we observe a highly significant negative correlation between cytoplasmic levels of NCO1 and levels of pAkt staining in prostate cancer tissues (Fig. 6B). These data suggest that a decline in NCO1 protein occurs with prostate cancer progression and may contribute to patients’ response to androgen treatments.

Our data illustrate the importance of NCO1 in prostate response to bicalutamide in two cell-based and in Ncor1-deficient mice in vivo. Together with the observation that NCO1 levels decline during prostate cancer progression in men, we demonstrate that NCO1 is involved in the development of resistance to bicalutamide treatment and provide an additional mechanism to explain the better response to MDV3100.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: A.I. Agoulnik, N.L. Weigel, I.U. Agoulnik
Development of methodology: I.U. Agoulnik
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.M. Lopez, A.I. Agoulnik, M. Zhang, E. Suarez, G.A. Gandarillas, R. Li, M.M. Ittmann, N.L. Weigel, I.U. Agoulnik
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.M. Lopez, A.I. Agoulnik, M. Zhang, L.E. Peterson, A. Frolow, K. Rajapakse, C. Coarfa, I.U. Agoulnik
Writing, review, and/or revision of the manuscript: S.M. Lopez, A.I. Agoulnik, A. Frolow, K. Rajapakse, C. Coarfa, M.M. Ittmann, N.L. Weigel, I.U. Agoulnik
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.M. Lopez, E. Suarez, G.A. Gandarillas, I.U. Agoulnik
Study supervision: I.U. Agoulnik

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