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Transcription of *Nrdp1* by the androgen receptor is regulated by nuclear filamin A in prostate cancer

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Abstract

Prostate cancer (PCa) progression is regulated by the androgen receptor (AR); however, patients undergoing androgen-deprivation therapy (ADT) for disseminated PCa eventually develop castration-resistant PCa (CRPC). Results of previous studies indicated that *AR*, a transcription factor, occupies distinct genomic loci in CRPC compared with hormone-naïve PCa; however, the cause of this distinction was unknown. The E3 ubiquitin ligase *Nrdp1* is a model AR target modulated by androgens in hormone-naïve PCa but not in CRPC. Using *Nrdp1*, we investigated how AR switches transcription programs during CRPC progression. The proximal *Nrdp1* promoter contains an androgen response element (ARE); we demonstrated AR binding to this ARE in androgen-sensitive PCa. Analysis of hormone-naive human prostatectomy specimens revealed correlation between *Nrdp1* and AR expression, supporting AR regulation of NRDP1 levels in androgen-sensitive tissue. However, despite sustained AR levels, AR binding to the *Nrdp1* promoter and *Nrdp1* expression were suppressed in CRPC. Elucidation of the suppression mechanism demonstrated correlation of NRDP1 levels with nuclear localization of the scaffolding protein filamin A (FLNA) which, as we previously showed, is itself repressed following ADT in many CRPC tumors. Restoration of nuclear FLNA in CRPC stimulated AR binding to *Nrdp1* ARE, increased its transcription, and augmented NRDP1 protein expression and responsiveness to ADT, indicating that nuclear FLNA controls AR-mediated androgen-sensitive *Nrdp1* transcription. Expression of other AR-regulated genes lost in CRPC was also re-established by nuclear FLNA. Thus, our results indicate that nuclear FLNA promotes androgen-dependent AR-regulated transcription in PCa, while loss of nuclear FLNA in CRPC alters the AR-regulated transcription program.

Key Words
- castration-resistant prostate cancer
- AR/androgen receptor
- FLRF/RNF41/Nrdp1
- ABP280/filamin A
- HER3/ErbB3

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Introduction

Prostate cancer (PCa) development and progression are regulated by the androgen receptor (AR), a steroid nuclear receptor, in both early and advanced stages of the disease (Yuan *et al*. 2014). While localized PCa is mostly treated by surgery or radiation therapy, AR inhibition is a cornerstone of treatment for disseminated PCa. Although initially effective, patients undergoing androgen deprivation therapy (ADT) eventually fail this treatment due to the development of castration-resistant PCa (CRPC) (Nelson 2012, Mitsiades 2013). Most CRPC patients develop relapsed tumors with high levels of AR-regulated activity, as evidenced by elevated serum levels of prostate-specific antigen (PSA), an AR-dependent gene (Karantanos *et al*. 2013, Yuan *et al*. 2014). With the advent of strong AR antagonists, such as enzalutamide, and androgen synthesis inhibitors, such as abiraterone acetate, it was observed that 65–70% CRPC patients initially responded to these drugs (de Bono *et al*. 2011, Scher *et al*. 2012), confirming continued AR activity in CRPC.

One of the perplexing aspects of PCa progression observed using patient-derived tissues is that AR target genes identified in low-grade localized PCa are often downregulated in high-grade, high risk PCa and in metastasis, despite continued AR expression (Tomlins *et al*. 2007). In addition, results of previous studies indicated that the AR occupies a distinct set of genomic loci in CRPC compared with those occupied in androgen-dependent cells (Wang *et al*. 2009*a*, Decker *et al*. 2012, Hu *et al*. 2012). AR-binding sites in untreated PCa were lost upon ADT initiation, and although a proportion of these were regained with the emergence of CRPC and AR resurgence, others were not (Sharma *et al*. 2013). AR mutations and alternately spliced AR variants that lack the ligand-binding domain (LBD; Guo *et al*. 2009, Hu *et al*. 2012) offer partial explanation for the change in expression of targets, but this discrepancy is observed even in tumors that do not harbor altered AR forms. A consequence of the altered AR transcriptome is that pathways not activated by AR in hormone-naïve tumors are upregulated in CRPC, promoting tumor progression (Wang *et al*. 2009*a*, Decker *et al*. 2012, Hu *et al*. 2012). The overall goal of these studies was to understand how the AR regulates a different transcription program in CRPC and whether this altered program can be reversed.

We have shown previously that in androgen-dependent cells, the AR suppresses the levels of the receptor tyrosine kinase ERBB3 by stimulating the E3 ubiquitin ligase NRDP1 (Chen *et al*. 2010*a*), which causes ERBB3 degradation (Wu *et al*. 2004, Yen *et al*. 2006, Cao *et al*. 2007). However, in CRPC cells, NRDP1 levels were reported to be downregulated despite continued AR expression (Chen *et al*. 2010*a*). In this study, we identify *Nrdp1* as an AR target gene in hormone-naive PCa, but not in some CRPC tumors. Using *Nrdp1* as a model, we investigated why the AR did not induce the transcription of certain genes in CRPC cells though they were transcribed in hormone-naïve cells.

Transcriptional activity of the AR is tightly regulated via interaction with co-regulators (van de Wijngaart *et al*. 2012, Parker *et al*. 2013). The presence or absence of co-regulators determines transcriptional efficiency of the AR, independent of AR splicing or mutations. In this study, we show that a scaffolding protein, filamin A (FLNA), affects AR-regulated transcription of *Nrdp1*. FLNA is a 280 kDa protein consisting of an actin-binding domain followed by 24 repeats of 96-amino acid units (Loy *et al*. 2003). Upon proteolysis, FLNA cleaves to a 170 kDa N-terminal and an 110 kDa C-terminal fragment which further cleaves to a 90 kDa fragment (Loy *et al*. 2003). The 90 kDa C-terminal fragment binds to AR and translocates to the nucleus (Ozanne *et al*. 2000), whereas the N-terminal fragment remains cytoplasmic (Loy *et al*. 2003). Results of our previous studies indicated that nuclear FLNA is observed in more than 75% of localized tumors but fewer than 45% of metastatic CRPC lesions (Bedolla *et al*. 2009). We demonstrated that in the presence of nuclear FLNA, CRPC cells were sensitized to anti-androgens (Wang *et al*. 2007), but ADT inhibited FLNA proteolysis, thereby preventing FLNA translocation to the nucleus, which persisted in CRPC (Mooso *et al*. 2012). Thus, loss of FLNA nuclear localization is one characteristic of CRPC development, and in cells where resistance to anti-androgen therapy was FLNA-regulated, restoration of nuclear FLNA reinstated androgen-sensitive cell growth (Wang *et al*. 2007, Mooso *et al*. 2012).

In this study, we demonstrate that *Nrdp1* is a direct AR transcriptional target, but only in the presence of nuclear FLNA, which is present in normal prostate and in hormone-naïve PCa but is reduced in most CRPC. Furthermore, we observe that this influence of nuclear FLNA is also effective in the transcription of various other AR-regulated genes whose expression is reduced in CRPC, but is restored when nuclear FLNA levels are increased. In addition, our results indicate that nuclear FLNA-induced AR transcriptional activity is ligand-dependent; thus, expression of FLNA-upregulated genes can be suppressed by the use of anti-androgens, thereby restoring androgen-sensitivity to CRPC cells. In contrast, in the absence of

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nuclear FLNA, the expression of AR-transcribed genes, including *PSA*, is not suppressed by anti-androgens. These results indicate that loss of nuclear FLNA is one reason why in some CRPC cells, AR induces an altered transcriptional program, and that this program can be restored when FLNA is induced to re-enter the nucleus.

Materials and methods

Patient characteristics

All data was collected with approval from the University of California, Davis (UCD) or VA Northern California Health Care System (VANCHCS) Institutional Review Board. The sections from formalin fixed paraffin-embedded prostate tumors of 157 patients who underwent prostatectomy at UCD (79) or VANCHCS (78) were analyzed for these studies. The patient characteristics are listed in Table 1. Tumor and non-tumor areas were identified by a pathologist, and 60 μm core samples were extracted. The specimens were arranged in triplicate in a tissue microarray (TMA) using a Beecher Instruments Manual Tissue Arrayer (Sun Prairie, WI, USA). Hematoxylin–eosin staining was used as a reference for interpreting the additional sections of the TMA stained with antibodies to NRDP1 and AR.

Cell culture and materials

LNCaP, CWR22Rv1 (ATCC, Manassas, VA, USA), C4-2 (UroCor, Oklahoma City, OK, USA), C4-2B (MDA Cancer Center, Houston, TX, USA), CWR-R1 (Dr Elizabeth Wilson, University of North Carolina), LNCaP-AI (Wang *et al.* 2007), and pRNS1-1 (Dr Johng Rhim, University of the Health Sciences, Bethesda, MD, USA) cells were cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS) and 1% antibiotic–antimycotic solutions. Stable transfectants of pRNS1-1 cells expressing WT-AR could only be cultured in the media containing 10% charcoal-stripped serum (CSS) as they were growth-inhibited by the levels of hormones present in FBS. Stable transfectants of pRNS1-1 expressing AR(T877A) and C4-2 cells expressing FLNA(16–24) were cultured in RPMI+10% FBS. All cell lines used in this study were investigated for the presence of contaminants and their cellular origins were verified before use. The cells were transiently transfected using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's specifications. Casodex (bicalutamide) was kindly provided by AstraZeneca. Antibodies to the following proteins were employed: NRDP1 (US Biologicals, San Antonio, TX, USA); ERBB3, lamin A, and α-tubulin (Cell Signaling Technology, Beverly, MA, USA); AR and β-actin (Santa Cruz Biotechnology); FLNA (Abcam, Cambridge, MA, USA); and GAPDH (Millipore, Billerica, MA, USA). The primers are described in Supplementary Tables 1 and 2, see section on supplementary data given at the end of this article.

Table 1 Patient characteristics. Of the 157 patients included in this study, matching tumor and nontumor tissue was available for 78, while for the remainder, only tumor tissue was available.

No. of patients	157		
Race			
Caucasian	122		
African American	22		
Undeclared	13		
Mean BMI	28.3±4.69	MAX: 45.5	MIN: 19.7
Mean Pre-op PSA	9.946±8.114	MAX: 57.8	MIN: 1.0
Gleason			
Gleason 5–6	72		
Gleason 7	67		
Gleason 8–9	18		
Stage			
Stage T1	43		
Stage T2	88		
Stage T3	18		
Positive margins	34/114		
PSA failure	53/143		

Plasmids

pCMV-FLNA, FLNA(16–24), and FLNA(1–15) plasmids were kindly provided by Dr E W Yong, National University of Singapore, Singapore, and human PSA-luciferase (hPSA-luc) construct containing two androgen response elements (AREs) in the proximal *PSA* promoter was kindly provided by Dr XuBao Shi, UCD. Human NRDP1-luciferase constructs pGL4.11 ARE3 and mutated ARE3 were constructed as follows: a 500 bp fragment immediately upstream of the *NRDP1* transcriptional start site was amplified from LNCaP genomic DNA using primers CA TCA GAT GCGC GGT ACC GGT TAC GAA GCT CTG GGA TGC T and CA TCA GAT GCGC GCT AGC GAA GAC TCC TAC CAC TCG TCG C and then directionally cloned into the KpnI- and NheI-cut pGL4.11 reporter construct (Promega). Mutagenesis was performed using the Stratagene QuikChangeII Kit from Agilent Technologies (Santa Clara, CA, USA) according to the manufacturer's instructions. Mutagenic primers were designed using the Agilent Technologies QuikChange Primer Design program and were used to amplify nascent plasmid containing the desired mutation(s). All mutant plasmids described were fully sequenced for confirmation.

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RNA inhibition

LNCaP cells were plated in 60 mm dishes and transfected with 50 pmol of a pool of three duplexes sold as FLNA siRNA (siRNA1, Santa Cruz Biotechnology) with the following sequences: strand #1: 5′-CCAUCACUGA-CAACAAAGA-3′, strand #2: 5′-CUGCAGAGUUUAU-CAUUGA-3′, and strand #3: 5′-GCUACCUCAUCUCCAU-CAA-3′. A pool of four scrambled nonspecific siRNA duplexes (Santa Cruz Biotechnology) was used as control.

Mouse studies

All experiments were conducted as approved by the UC Davis Institutional Animal Care and Use Committee (IACUC). 4–5-week-old *Nu/Nu* athymic male mice were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN, USA) and sustained release testosterone pellets (12.5 mg, 90-day release; Innovative Research of America, Sarasota, FL, USA) were implanted subcutaneously. Suspensions of CWR22 or CWR22Rv1 cells were made in 50% Matrigel-solubilized basement membrane (BD Biosciences, Bedford, MA, USA) and xenografts were established by s.c. injections of 2.5×10^6 cells/site. When palpable tumors were observed, the testosterone pellets were removed and animals were followed for approximately 4 weeks, after which the mice were killed and the tumors were collected. Part of each tumor was processed for paraffin embedding for immunohistochemistry (IHC), while the rest was lysed and homogenized for western blotting in cell lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 1% NP-40, and protease inhibitors: 0.1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml each of phenathroline, leupeptin, aprotinin, and pepstatin A) and phosphatase inhibitors: 20 mM β-glycerol phosphate, 1 mM Na-orthovanadate, and 10 mM NaF. Proteins were quantitated using a BCA assay (Pierce, Rockford, IL, USA) and fractionated using 29:1 acrylamide-*bis* SDS–PAGE.

Chromatin immunoprecipitation

The cells were treated using the Magna ChIP Chromatin Immunoprecipitation Kit (Millipore) according to the manufactures protocol. The lysates were sonicated using the Bioruptor UCD-200 (Diagenode, Denville, NJ, USA) and immunoprecipitated using 3 μg of ChIPAP+ AR (Millipore). After immunoprecipitation, DNA was size selected by DNA electrophorisis between 100- and 300-bp and purified using a Gel Extraction Kit (Qiagen, Inc.). All experiments were conducted using a negative control: either ARarfneg2, representing a region of the *p14ARF* gene that the AR does not bind to, or *ZNF333*, a non-AR transcribed zinc-finger region, as per studies indicating the use of an unresponsive region as negative control (Kidder *et al.* 2011).

QPCR

Total RNA was prepared using the RNeasy Mini Kit (Qiagen, Inc.) and a method based on the manufacturer's protocol. cDNA was prepared using the Verso cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA), according to manufacturer's instructions. The expression levels were determined using the Luminaris Color HiGreen qPCR Master Mix (Thermo Scientific) and StepOnePlus Real-Time PCR System (Applied Biosystems).

Other methods

Western blotting, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assays, immunofluorescence, IHC, and flow cytometry were performed as described previously (Mooso *et al.* 2012). AR transcriptional activity was estimated in cells transfected with 2 μg of pGL3-hPSA-luc, pGL4.11-ARE3, or pGL4.11-mutated ARE3 and 1 μg β-galactosidase with or without co-transfection of 2 μg FLNA vectors as described previously (Chen *et al.* 2010*a*). Subcellular fractionation into cytoplasmic and nuclear fractions was conducted using the NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol.

Statistical analyses

For immunohistochemical analysis of patient samples, median staining levels were compared between cancer and non-cancer cells from the same subject using Wilcoxon signed-rank tests. Median staining levels were classified as high or low and analyzed using χ^2 test for association, logistic regression or log-linear models, and compared between levels of categorical demographic characteristics using Wilcoxon rank-sum tests for the case of demographic characteristics with two levels, or using Kruskal–Wallis tests for the case of demographic characteristics with more than two levels. The correlations between staining levels and continuous demographic characteristics were estimated using Spearmans' ρ. Analyses were conducted using R, version 3.1.2 (University of California, Los Angeles, USA) or SAS version 9.3 (SAS Institute, Cary, NC). Tumor data for mice were analyzed by normalization of all measurements to pre-operation (sham or castration) measurements for each individual mouse, then

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mean and S.E.M. calculated for the aggregate group. For staining analysis, associations were based on Pearson's product moment correlation coefficient. Similar statistical considerations had been reported previously in more detail (Kreisberg *et al*. 2004, Bedolla *et al*. 2007).

Results

Nrdp1 is a transcriptional target of the AR in hormone-sensitive PCa

We previously demonstrated that expression of the E3 ubiquitin ligase NRDP1 was androgen-regulated in hormone-naïve PCa cells (Chen *et al*. 2010*a*). Prolonged culture of androgen-sensitive LNCaP cells in CSS (which was significantly stripped of various hormones including androgens) decreased the expression of NRDP1 (both 36 and 28 kDa isoforms) compared with FBS (Fig. 1A). As CSS reduces a number of hormones and growth factors, to determine the specific effect of androgens, the cells were treated with the androgen dihydrotestosterone (DHT), a strong AR ligand, which significantly replenished NRDP1 levels (Fig. 1A). These results demonstrated hormone sensitivity of NRDP1 expression.

We identified a 209 bp ARE upstream of the transcriptional start site (ARE3), which is a 15 bp bipartite palindromic sequence very similar to ARE1 in the *PSA* proximal promoter (Cleutjens *et al*. 1996, 1997; Fig. 1B). In LNCaP cells, the

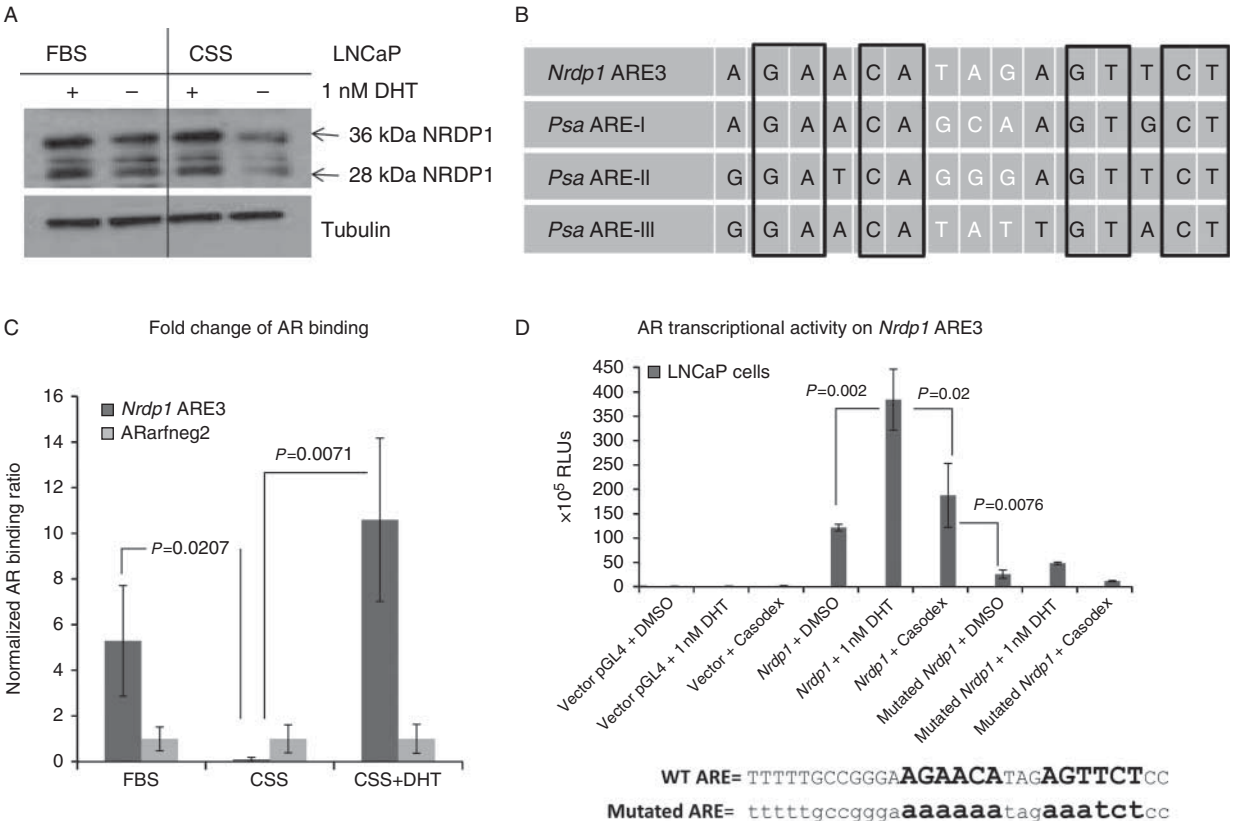

Figure 1

Nrdp1 is a transcriptional target of the AR in androgen-dependent LNCaP cells. (A) LNCaP cells were cultured in complete media containing FBS or CSS with and without DHT (1 nM) for 72 h. The cell lysates were blotted for NRDP1 and two isoforms were detected of approximately 36 and 28 kDa. Exposure to CSS caused a decrease in both 36 and 28 kDa, NRDP1 but this effect was restored by DHT, indicating that NRDP1 is androgen-regulated. (B) We identified an ARE in the proximal promoter of *Nrdp1*. Comparison of *Psa* AREs and *Nrdp1* ARE show that the *Psa* AREs and *Nrdp1* ARE3 both contain a 15 bp-palindromic ARE. (C) ChIP assay of AR binding in LNCaP cells to *Nrdp1* ARE3. LNCaP cells were cultured in media containing CSS for 48 h and then switched to complete media containing FBS or CSS with or without DHT. Chromatin samples were immunoprecipitated with an anti-AR antibody and analyzed by qPCR with primers flanking the *Nrdp1* ARE3 region against a negative control (ARarfneg2). In LNCaP cells grown in normal FBS media, the AR binds to the *Nrdp1* ARE3 sequence, this binding no longer occurs in CSS media, but is restored in CSS media with the addition of DHT. In contrast, the negative control is unaffected by these manipulations. (D) LNCaP cells were transfected with plasmids expressing luciferase under the control of the WT *Nrdp1* ARE3 promoter, or the mutant *Nrdp1* ARE3 promoter (as shown), or with the parental vector (pGL4), and assayed for luciferase activity in the presence of DMSO vehicle, or 1 nM DHT, with or without 10 μM bicalutamide (Casodex). The luciferase activity of *Nrdp1* ARE3 in LNCaP cells was responsive to androgens but was abolished by the mutations.

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results of chromatin immunoprecipitation (ChIP) analysis indicated AR binding to *Nrdp1* ARE3, but not to a negative control. Furthermore, there was a decrease in AR binding to the *Nrdp1* ARE3 in CSS compared with FBS and a restoration of AR binding in CSS with DHT (Fig. 1C). A luciferase reporter containing *Nrdp1* ARE3 was transfected into LNCaP cells, and further treated with vehicle, DHT, or the anti-androgen bicalutamide. Significantly, AR transcriptional activity of the *Nrdp1* promoter increased 3.158-fold following the addition of DHT ($P=0.007$), but was suppressed by addition of bicalutamide ($P=0.02$). In contrast, there was little to no luciferase activity when LNCaP cells were transfected with a construct containing a mutated ARE3 (fold change=1.85, $P=0.186$), indicating that the site was required for AR-dependent transcription (Fig. 1D).

As LNCaP cells carry a mutated AR(T877A), the androgen sensitivity of *Nrdp1* transcription was tested in other cell lines as well. pRNS1-1 is a cell line derived from a normal prostate (Shi *et al.* 2007), which express low (normal) levels of WT-AR. Examination of stable pRNS1-1 transfectants expressing an empty vector, WT-AR, or the AR(T877A) mutant (Fig. 2A) indicated

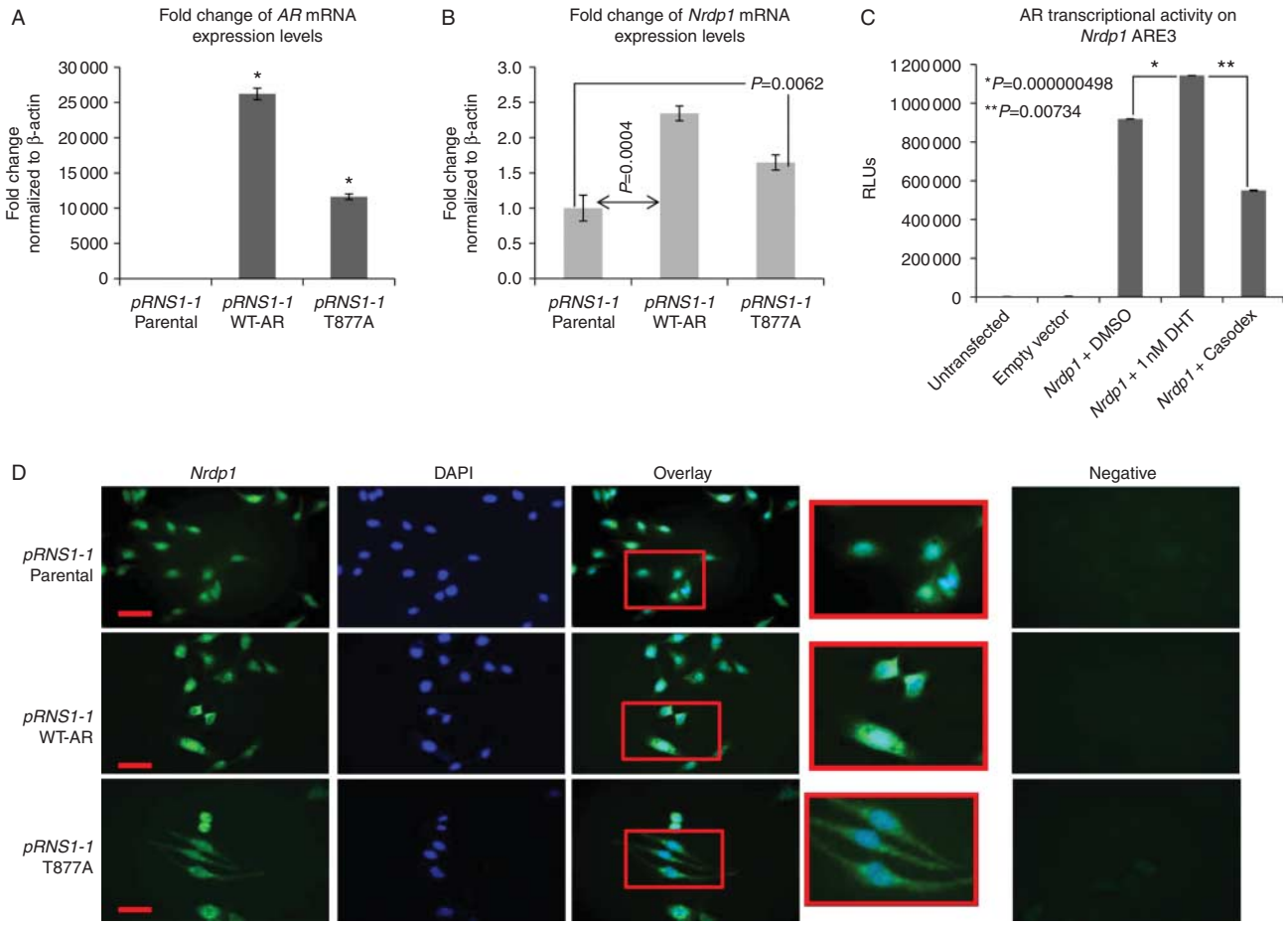

Figure 2

Nrdp1 is transcribed by both WT and mutated AR in a normal prostate-derived cell line. (A) Parental pRNS1-1 cells derived from a normal prostate were stably transfected with an empty vector, WT-AR, or mutant AR(T877A). QPCR to determine AR expression in parental pRNS1-1 cells, or those stably expressing AR expression increases up to 120-fold in PRNS1-1 WT-AR cells (*$P\leq0.0001$) and 53-fold in PRNS1-1 AR(T877A) (*$P\leq0.0001$) compared with PRNS1-1 parental cells. AR transcript levels were normalized to the corresponding values for β-actin. (B) QPCR for *Nrdp1* expression in parental pRNS1-1 cells, or those stably expressing WT-AR or AR(T877A). *Nrdp1* expression increases up to 2.3-fold in pRNS1-1 WT-AR cells ($P=0.0004$) and 1.6-fold in pRNS1-1 AR(T877A) ($P=0.0062$) compared with parental pRNS1-1 cells. *Nrdp1* transcript levels were normalized to the corresponding values for β-actin. (C) pRNS1-1 AR(T877A) cells were transfected with plasmids expressing luciferase under the control of the *Nrdp1* ARE3 promoter or with the parental vector (pGL4), and assayed for luciferase activity in the presence of DMSO (vehicle), 1 nM DHT, or 10 μM bicalutamide (Casodex), and indicates responsiveness to androgens. (D) Immunofluorescence of parental pRNS1-1 cells, or those stably expressing WT-AR or AR(T877A). The cells were stained for *Nrdp1* (green) or DAPI (blue) to indicate the location of the nucleus. (Top) Note that while parental pRNS1-1 express very little *Nrdp1*, (middle) the expression of WT-AR, or (bottom) AR(T877A) increased NRDP1 in the cytoplasm. Negative control(s) were stained with secondary antibody alone. Scale bar=20 μm.

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that the expression of WT or mutant AR significantly increased endogenous NRDP1 levels (Fig. 2B), thereby demonstrating that this effect is not due to the expression of the mutated AR alone. Results of a luciferase assay in AR-expressing pRNS1-1 cells indicated that AR transactivation of the *NRDP1* promoter was androgen-sensitive, similar to that observed in LNCaP (Fig. 2C). (Similar experiments could not be conducted in pRNS1-1 cells overexpressing WT-AR, because they have to be cultured in CSS and their activation with DHT induced cell death). Baseline levels of NRDP1 protein were observed in all cells, indicating the influence of other transcription factors on *NRDP1* expression; however, its levels increased when the cells were transfected with WT or mutant AR (Fig. 2D), confirming AR-dependence. Taken together, these results demonstrate that the *NRDP1* is a novel AR target gene regulated in a hormone-sensitive manner in androgen-dependent cells.

NRDP1 expression is elevated in localized human PCa tissue compared with non-tumor prostate and correlates with active (nuclear) AR levels

We next investigated the levels of NRDP1 in primary prostate tissues obtained from 157 individual patients. Of these, matched tumor and surrounding non-tumor tissues were available from 78 patients. Using a scoring system (0–3) based on IHC, where 0 represents no staining and 3 represents 100% staining, we observed that NRDP1 was strongly expressed in the epithelial cells of the prostate, and could be observed in both the nucleus and the cytoplasm (Fig. 3A; specificity of the NRDP1 antibody was

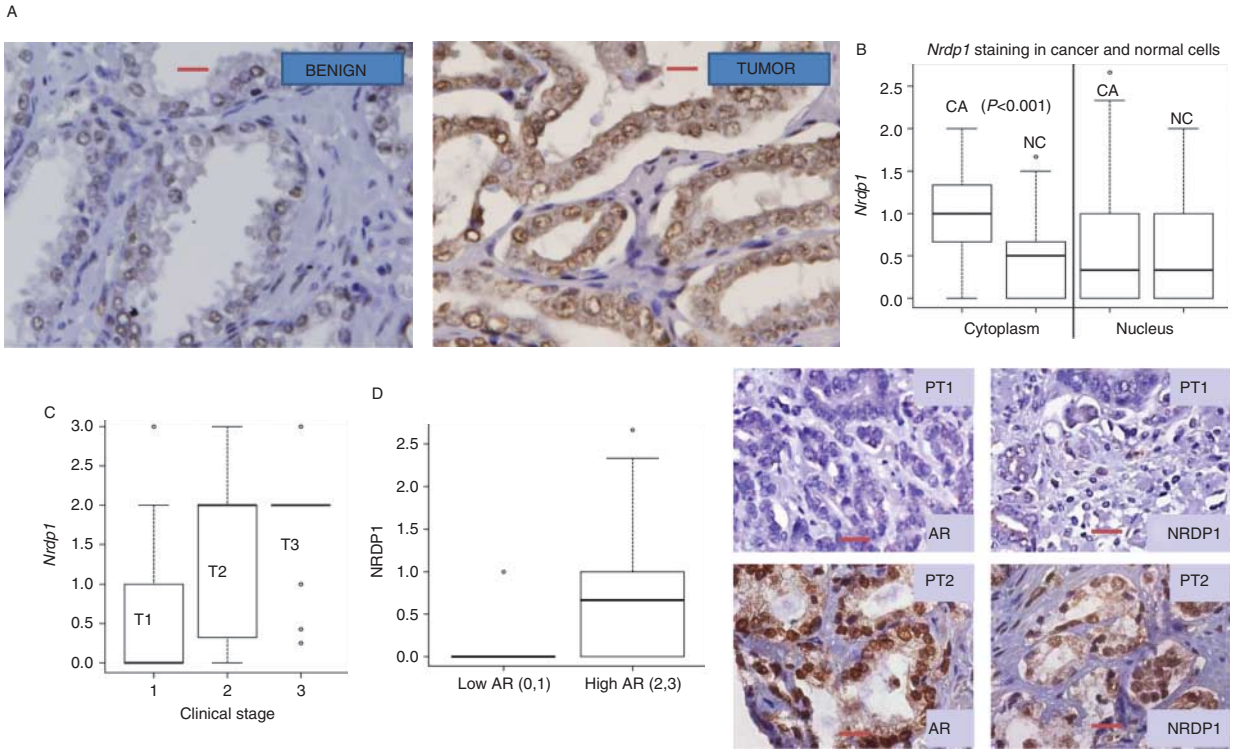

Figure 3

Nrdp1 is highly expressed in hormone-naïve localized tumors from patients with PCa and correlates with intratumoral AR. (A) Formalin-fixed paraffin-embedded human localized prostate cancer specimens obtained by prostatectomy were arranged in a tissue microarray and stained with anti-*Nrdp1* antibody. *Nrdp1* expression (brown staining) was observed in the nucleus, cytoplasm, or both and was scored on a scale of 0–3 in both benign and cancerous prostate tissues. Typical staining from a benign section (score 1, left) and a section showing Gleason grade 3 tumor (score 3, right), scale bars = 20 μm, are shown. (B) Boxplot depicting the distribution of *Nrdp1* in the nucleus or cytoplasm of tumor compared with non-tumor tissue ($n = 78$) indicating that the expression of nuclear *Nrdp1* remains the same in both cancerous and non-tumor tissues, whereas cytoplasmic expression of *Nrdp1* increases in tumor compared with non-tumor tissue. (C) Nuclear *Nrdp1* levels differed significantly with respect to clinical stage ($P < 0.001$), with *post hoc* testing showing significantly higher expression in stage 2 (T2) and stage 3 (T3) patients than in stage 1 (T1) patients ($P = 0.001$). (D, left) Boxplots showing the correlation between NRDP1 and AR in tumor tissue ($P < 0.001$). (D, right) IHC of AR and NRDP1 in two patients with high and low AR versus NRDP1. Note that the patient who showed little expression of AR also little expression of NRDP1, whereas strong AR staining correlated with strong NRDP1 expression. Scale bars = 50 μm.

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Table 2 Statistical analysis of protein staining in prostate tumors and non-tumor prostate tissues

	Cytoplasm			Nucleus		
Protein	Median in cancer cells ($n=78$)	Median in non-cancer cells ($n=78$)	*P* value (Wilcoxon's signed rank test)	Median in cancer cells ($n=78$)	Median in non-cancer cells ($n=78$)	*P* value (Wilcoxon's signed rank test)
Nrdp1	1.0	0.5	<0.001	0.3	0.3	0.134
AR	1.0	0.8	<0.001	2.0	2.0	0.071

Nuclear and cytoplasmic levels of both NRDP1 and AR were analyzed individually. Cytoplasmic but not nuclear AR and *NRDP1* expression increase in human prostate tumor tissues compared with non-tumor prostate tissues. *P* values <0.05 were considered to be significant.

verified with control and *NRDP1* shRNA (Supplementary Fig. 1, see section on supplementary data given at the end of this article)). Cytoplasmic NRDP1 was significantly increased in tumor versus non-tumor specimens ($P<0.001$), although low levels of nuclear NRDP1 were observed in all samples (Fig. 3B and Table 2). Within the tumor tissues, comparison with clinical stage in all 157 patients showed that NRDP1 expression increased with the stage of the tumor (T1<T2 and T3; $P<0.001$; Fig. 3C and Table 3).

Comparison of AR and NRDP1 protein levels revealed a significant correlation between NRDP1 levels and nuclear (active) AR (cytoplasmic NRDP1 versus nuclear AR: pairwise correlation coefficient: 0.42; $P<0.001$ and nuclear NRDP1 versus nuclear AR: pairwise correlation coefficient: 0.26; $P=0.035$; Fig. 3D). Examination of Oncomine datasets indicated a similar correlation between *NRDP1* and *AR* mRNA levels in patients on comparing prostate tumor versus non-tumor prostate tissue, as determined by various investigators (Supplementary Fig. 2, see section on supplementary data given at the end of this article), supporting our observations. Taken together, these results indicate strong association between AR and NRDP1 expression in localized human PCa, and support AR-dependence of NRDP1 expression.

NRDP1 levels are reduced in CRPC compared with hormone-sensitive PCa

We also investigated whether the correlation between NRDP1 and AR is observed in CRPC. Well-characterized CRPC sublines of LNCaP cells (Wu *et al*. 1994, Denmeade *et al*. 2003, Vinall *et al*. 2006) were analyzed for comparison of levels of NRDP1. Although the AR in the CRPC sublines is known to be active (Ghosh *et al*. 2005, Vinall *et al*. 2006), NRDP1 expression was decreased in all three, indicating dissociation between AR and NRDP1 in these CRPC lines (Fig. 4A). Comparison of the AR-binding site of the *NRDP1* promoter in LNCaP and C4-2 revealed that the site was identical in the two cell lines (Supplementary Fig. 3, see section on supplementary data given at the end of this article); however, AR binding to the *NRDP1* ARE was severely decreased in C4-2 cells compared with LNCaP cells ($P<0.0001$), despite the level of AR protein in the C4-2 cells being similar to that in LNCaP, as we have shown elsewhere (Ghosh *et al*. 2005, Wang *et al*. 2008). Thus, the decrease in AR binding is not caused by a mutation in the AR-binding site, and is not a result of a significantly different AR protein level. It may be noted that in CRPC cells, the *NRDP1* promoter is regulated by other transcription factors that take over once the cell achieves a CRPC phenotype. As a result of the loss of AR binding to the *NRDP1* ARE, the levels of NRDP1 mRNA and protein were significantly lower, but because it is now transcribed by other transcription factors, NRDP1 is not completely eliminated.

We next investigated whether loss of *NRDP1* expression in CRPC is also observed in other models. A tumor line, CWR22, and its CRPC derivative CWR22Rv1 were implanted into nude mice and the tumors excised when the volume exceeded 150 cm^3. The CWR22Rv1 tumors grew at a significantly more rapid rate compared with CWR22 when normalized to day 1 ($P=0.003$; Fig. 4C). In addition, by IHC, expression of NRDP1 was significantly lower in castration-resistant CWR22Rv1 tumors (median=0, $n=6$) compared with androgen-sensitive CWR22 tumors (median=1, $n=6$) ($P=0.0157$; Fig. 4D) despite expression of full-length AR in both CWR22 and CWR22Rv1 (although CWR22Rv1 tumors in addition expressed AR splice variants; Li *et al*. 2013; Fig. 4E). We also determined whether NRDP1 in the

Table 3 NRDP1 levels with respect to clinical stage

	Stage T1 median (range)	**Stage T2 median** (range)	**Stage T3 median** (range)	**Kruskal–Wallis test *P* value**
Number of patients	43	88	18	
NRDP1	0 (0–3)	2 (0–3)	2 (0.3–3)	<0.001

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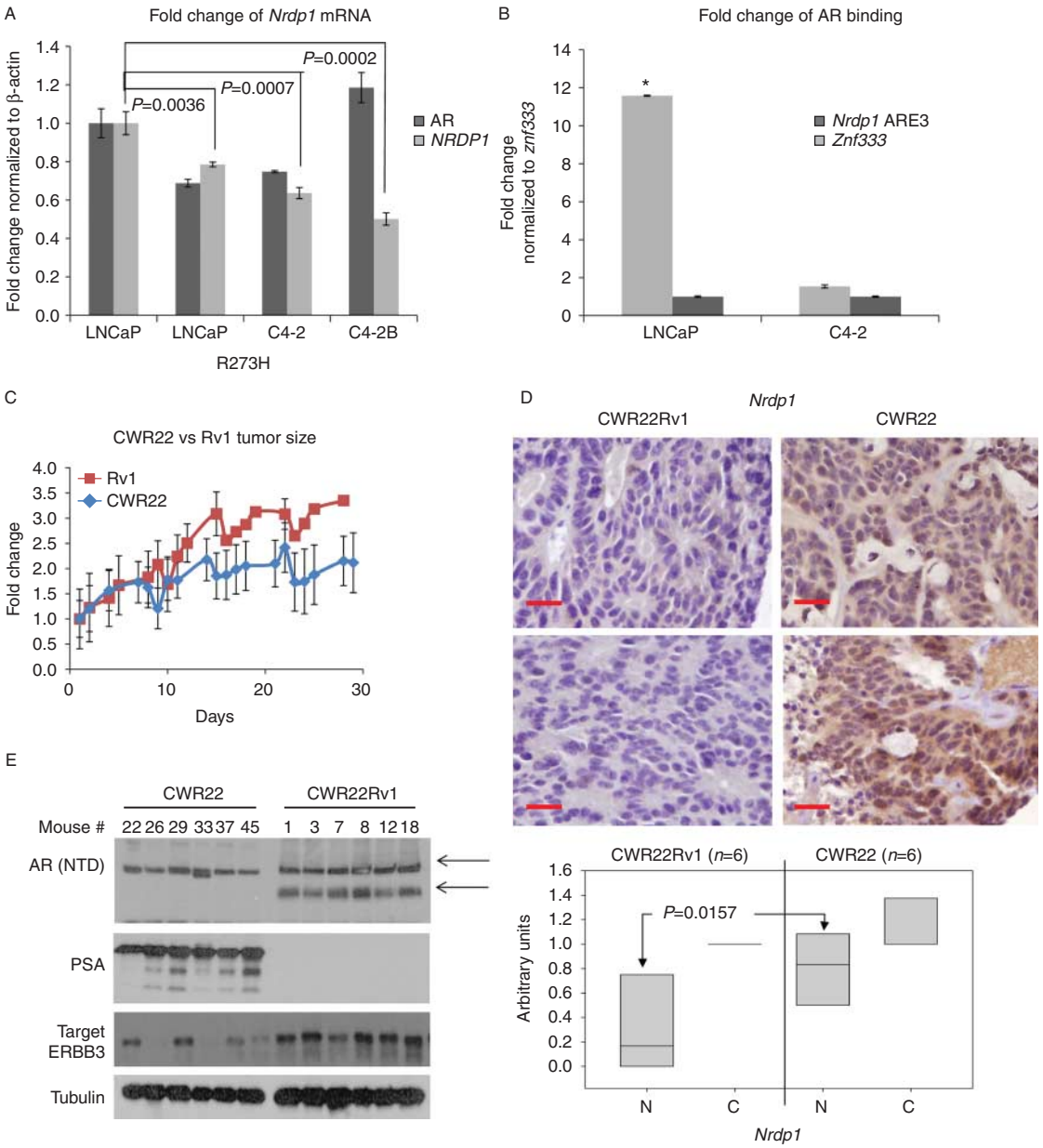

Figure 4

Loss of *NRDP1* expression and AR regulation of *NRDP1* transcription in CRPC compared with hormone-naïve tumors. (A) Products of qPCR comparing *NRDP1* expression in LNCaP cells compared with those in LNCaP R273H ($P=0.0036$), C4-2 ($P=0.0007$), and C4-2B ($P=0.0002$). Note the decrease in NRDP1 levels in the latter three cell lines, which are all CRPC. *Nrdp1* transcript levels were normalized to the corresponding values for β-actin. (B) Comparison of AR binding to the *Nrdp1* ARE3 in LNCaP versus C4-2 cells. Note the sharp decrease in AR binding in C4-2 compared with LNCaP (*$P<0.0001$). Chromatin samples were immunoprecipitated with an anti-AR antibody and analyzed by qPCR with primers flanking the *Nrdp1* ARE3 region with *Znf333* as a negative control. (C, top) Nude mice received subcutaneous implants of either CWR22 cells (right) or CWR22Rv1 (left) tumor and the tumors were allowed to grow for up to 29 days; the mice were killed and the tumors excised when tumors exceeded 150 cm^3 or at the end of that period. Tumor size was measured as described and plotted over time. The Rv1 tumors were more aggressive compared with CWR22 ($P=0.003$). (D) Formalin fixed paraffin-embedded tumor specimens were stained with anti-NRDP1 antibody. Note that the CWR22 cells stained strongly for *Nrdp1* (brown), while the CWR22Rv1 cells did not (scale bars = 30 μm). (Bottom) Boxplot of *Nrdp1* in CWR22 ($n=6$) vs CWR22Rv1 ($n=6$) in the nucleus (N) and cytoplasm (C). Primary CWR22 tumors expressed higher levels of nuclear NRDP1 compared with recurrent CWR22Rv1 tumors ($P=0.0157$). (E) Whole-cell lysates of xenografted tumors were run on a western blotted and stained with AR, PSA, and ERBB3, while tubulin levels were used as a loading control. Results indicate that despite the sharp change in expression of *Nrdp1* between the two tumor types, there was no significant difference in AR levels (except that CWR22Rv1 tumors also expressed the alternately spliced forms). However, AR transcriptional activity in CWR22Rv1 was significantly suppressed, as indicated by a decrease in PSA levels. In support of the decrease in NRDP1 in CWR22Rv1 compared with CWR22, the former expressed higher levels of the NRDP1 degradation target ERBB3.

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tumors (detected by IHC) was active by examining the levels of its ubiquitination target ERBB3. As expected, ERBB3 levels were higher in CWR22Rv1 compared with CWR22 (Fig. 4E), thereby demonstrating negative correlation with NRDP1. In all, these results indicate that despite the AR-dependence of NRDP1 in hormone-naïve PCa, this correlation is lost in advanced disease.

Expression of NRDP1 and AR binding to the *NRDP1* promoter correlates with expression of the 90 kDa C-terminal FLNA fragment

We now investigated the molecular mechanism leading to reduction of the expression of NRDP1 in advanced PCa. In support of the decreased AR binding to *NRDP1* ARE3 shown in Fig. 4B, C4-2 cells expressed lower levels of NRDP1 protein compared with LNCaP (Fig. 5A). As C4-2 cells express the same AR mutation as LNCaP, this difference cannot be attributed to an AR mutation. However, we previously demonstrated that FLNA expression was mostly nuclear in LNCaP cells, whereas in C4-2, it was mostly cytoplasmic (Supplementary Fig. 4, see section on supplementary data given at the end of this article; Wang *et al*. 2007, Bedolla *et al*. 2009). Comparison of the two cell lines revealed that the levels of 90 kDa FLNA correlated with NRDP1 levels (Fig. 5A). These results indicate a possible role for FLNA in determining the transcriptional activity of AR on *NRDP1*.

To determine whether 90 kDa FLNA indeed plays a role in AR transcription of *Nrdp1*, we compared two CRPC lines, CWR22Rv1 and CWR-R1, both derived from relapsed CWR22 tumors. Both lines expressed AR variants that lacked the LBD, and were essentially androgen-independent in phenotype (Chen *et al*. 2010*b*). However, CWR-R1 cells express higher levels of FLNA compared with CWR22Rv1 (Fig. 5B). Both lines express similar levels of total AR (detected using primers against the DNA-binding domain of the AR, $P>0.05$), while CWR-R1 cells expressed higher levels of *FlnA* mRNA ($P=0.0002$) compared with CWR22Rv1 (Fig. 5C). Results of a ChIP assay indicated that in CWR-R1, but not in CWR22Rv1, the AR strongly bound to the *Nrdp1* promoter, versus a negative control (Fig. 5D). These results indicated correlation between *Nrdp1* transcription with AR and 90 kDa *FlnA*. In support of a role for FLNA in androgen sensitivity, CWR-R1 which express high levels of FlnA, but not CWR22Rv1 cells which express low levels of FLNA, responded partially to the treatment with the anti-androgen bicalutamide (Casodex; Fig. 5E). Therefore, despite the castration-resistance in both lines, the response of the cells correlated with the expression of 90 kDa FLNA.

Expression of 90 kDa FLNA isoform restored the expression of *Nrdp1* in CRPC cells

As CWR22Rv1 cells expressed higher levels of the low-molecular-weight AR variants compared with CWR-R1, to distinguish between the effects of the splice variants and 90 kDa FLNA in the regulation of *Nrdp1* transcription by AR, we used cells that did not express AR splice variants. Transfection of C4-2 cells with full-length FLNA (280 kDa), N-terminal FLNA (FLNA(1–15), 170 kDa) or C-terminal FLNA (FLNA(16–24), 90 kDa) revealed that only FLNA(16–24) localized to the nucleus (Fig. 6A). These results were verified by isolation of C4-2 cytoplasmic and nuclear fractions, which also revealed that FLNA(16–24), but not FLNA(1–15), was expressed in the nuclear fragment (Fig. 6B).

Having established that FLNA(16–24) transfection induces nuclear expression of this protein, we next investigated whether nuclear FLNA altered the expression of *Nrdp1*. Significantly, only FLNA(16–24), but not FLNA(1–15), restored NRDP1 protein levels in C4-2 cells (Fig. 6B), indicating a role for nuclear FLNA in this process. To demonstrate a role for FLNA in *Nrdp1* expression independent of AR splice variants, we used CWR-R1 cells that expressed the splice variant. When CWR-R1 cells were transfected with full-length FLNA, FLNA(1–15) and FLNA(16–24), expression of the 90 kDa FLNA band was seen only in the latter, and we observed the highest levels of NRDP1 as determined by protein expression in cells expressing FLNA(16–24) (Fig. 6C). *FlnA*(16–24) affected the rate of transcription as determined by the change in mRNA expression (Fig. 6D) and by the extent of AR binding to the *Nrdp1* promoter in the presence of FLNA(16–24) (Supplementary Fig. 5A, see section on supplementary data given at the end of this article). Our results indicate that nuclear expression of 90 kDa FLNA regulates NRDP1 levels by modulating its transcription. In addition, Fig. 6E shows that NRDP1 and therefore ERBB3 levels are slightly androgen-dependent in CWR-R1 cells (left), but not in CWR22Rv1 cells (right). These results support the observed AR binding to the *Nrdp1* promoter in CWR-R1 but not CWR22Rv1 cells shown in the previous figure.

We next investigated whether other AR targets are also affected by the presence of FLNA. The best known AR target is PSA, which is known to be decreased upon bicalutamide treatment in androgen-dependent LNCaP cells (Ghosh *et al*. 2005). Downregulation of FLNA

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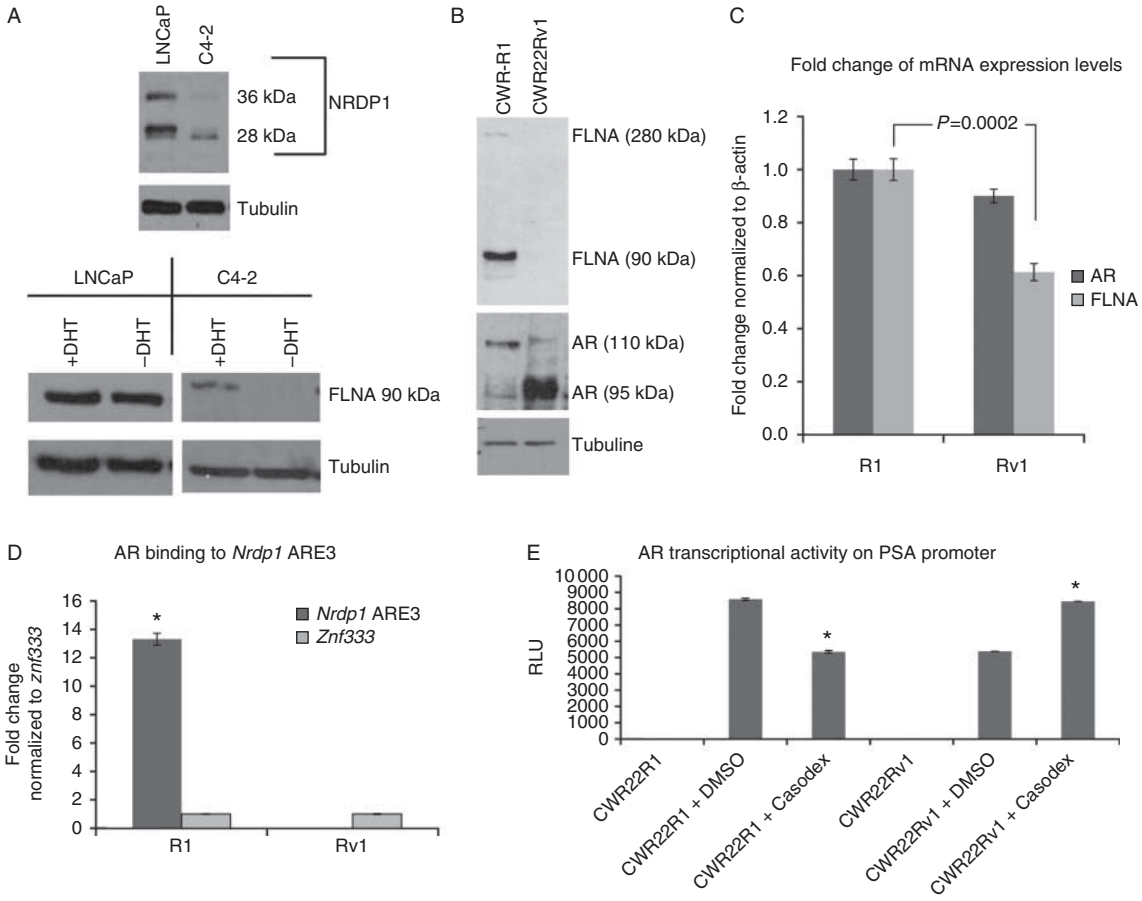

Figure 5

Correlation between NRDP1 levels and expression of a 90 kDa FLNA isoform: (A, upper) comparison of NRDP1 protein levels in LNCaP and C4-2 cell lines cultured in FBS. The cell lysates were immunoblotted with anti-NRDP1 and anti-tubulin antibodies. Note that C4-2 cells expressed lower levels of this protein compared with LNCaP. (A, lower) Correspondingly, the levels of 90 kDa FLNA was also assessed in the presence or absence of 1 nM DHT. LNCaP cells cultured in FBS showed no difference in FLNA levels with DHT; however, in C4-2 cells these levels were much lower, and change with DHT was immediately obvious. (B) Protein expression of FLNA in CWR-R1 and CWR22Rv1 cells cultured in FBS. Cell lysates were immunoblotted with anti-FLNA, anti-AR, and anti-tubulin antibodies. Whole-cell lysates of CWR-R1 and CWR22Rv1 demonstrate the decreased expression of the 90 kDa fragment of FLNA in CWR22Rv1 cells. (C) QPCR for AR and FLNA expression in CWR-R1 and CWR22Rv1. Higher expression of FLNA was observed in CWR-R1 cells ($P=0.0002$), although AR expression in the two cell lines was comparable ($P>0.05$). AR and FLNA transcript levels were normalized to the corresponding values for β-actin. (D) ChIP assay of AR binding to ARE3 in CWR-R1 and CWR22Rv1 cells. AR binds to NRDP1 ARE3 in CWR22R1 cells but not in CWR22Rv1 cells. Chromatin samples were immunoprecipitated with an anti-AR antibody and analyzed by qPCR with primers flanking the *Nrdp1* ARE3 region ($*P<0.0001$), and *Znf333* ($P>0.05$) as a negative control. (E) Androgen sensitivity in CWR-R1 vs CWR22Rv1 cells. AR transcriptional activity was tested in untransfected cells, or cells transfected cells with luciferase driven by the *Psa* ARE. The decrease in luciferase activity in CWR-R1 cells but not in CWR22Rv1 cells in the presence of 10 μM bicalutamide indicates that CWR-R1 cells are more androgen sensitive compared with CWR22Rv1 cells, though both are considered castration resistant (bicalutamide decreased AR transcriptional activity on the *PSA* promoter in CWR-R1 cells by ~38%, $*P<0.0001$).

expression by siRNA revealed that bicalutamide failed to suppress AR activity on the *PSA* promoter in the absence of FLNA (Fig. 6F). The above results did not reveal which fragment of FLNA was needed to show a PSA response. Therefore, LNCaP cells were transfected with either FLNA(1–15) or FLNA(16–24). Similar to control cells (transfected with an empty vector), those transfected with FLNA(16–24) responded to bicalutamide, whereas the cells transfected with FLNA(1–15) did not (Supplementary Fig. 5B). Therefore, it is the C-terminal fragment that is needed for PSA response.

Nuclear FLNA regulates AR-mediated transcription of *Nrdp1* in CRPC cells

As *Nrdp1* is transcriptionally regulated by the AR, and also by FLNA(16–24), we investigated whether AR-regulated *Nrdp1* transcription is modulated by nuclear FLNA.

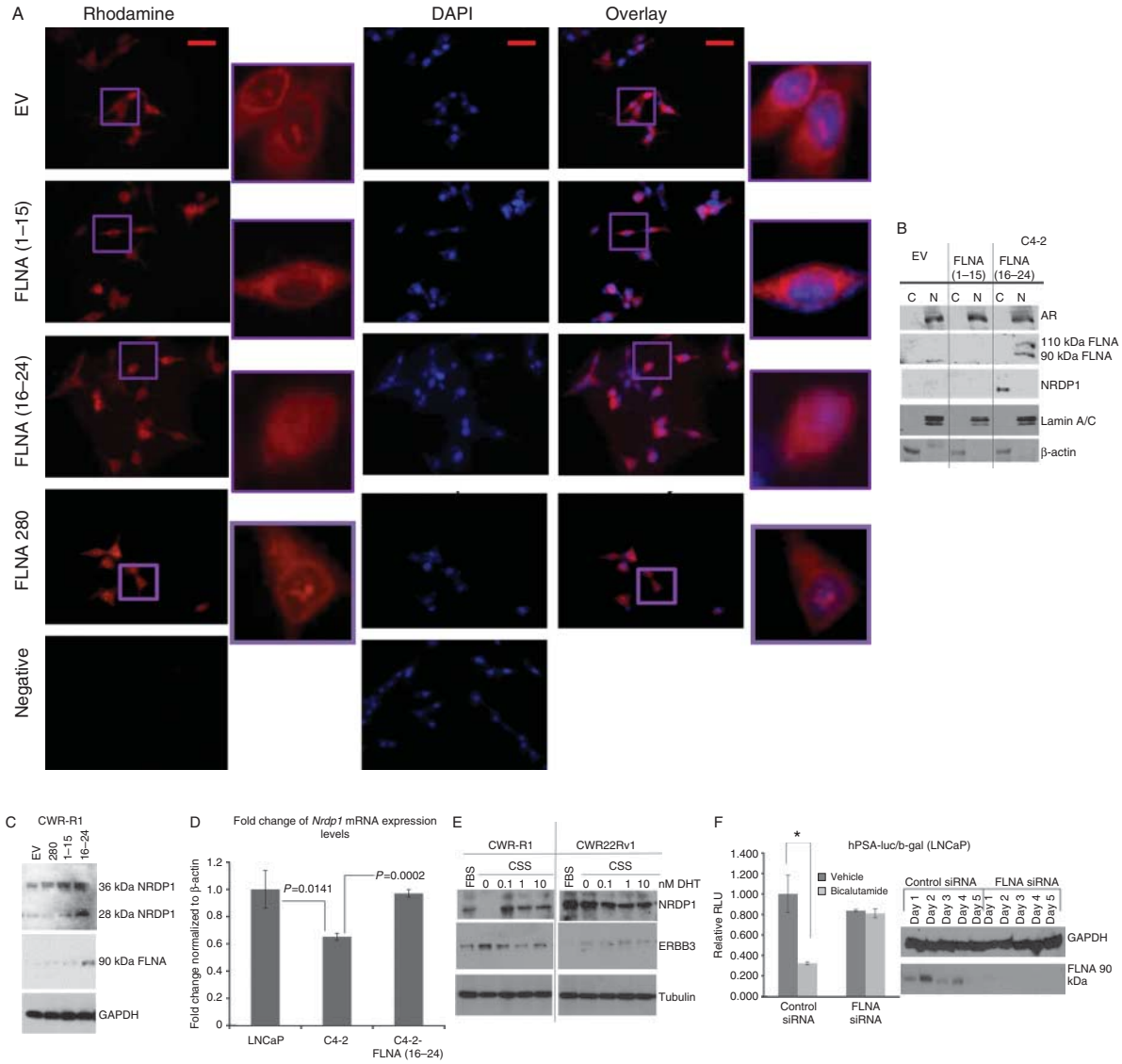

Figure 6

The 90 kDa FLNA isoform localized to the nucleus and promoted apoptosis and growth arrest in a ligand-dependent manner. (A) Immunofluorescence of C4-2B cells transfected with empty vector (EV), FLNA(1–15), or FLNA (16–24). The cells were stained for C-terminal FLNA or with DAPI to identify the location of the nucleus. (Top row) Control cells (transfected with empty vector) only express cytoplasmic FLNA, as demonstrated by unstained nuclear regions in FLNA stained cells, (second row) and transfection of FLNA(1–15) did not affect the localization, (third row) while those transfected with FLNA(16–24) express both cytoplasmic and nuclear FLNA. (Fourth row) Transfection of full-length FLNA did not restore nuclear localization completely. (Fifth row) Negative controls were treated identically, except that the anti-FLNA antibody was omitted (scale bars 30μm). (B) Subcellular fractionation of C4-2 cells transfected with empty vector, FLNA(1–15), or FLNA(16–24). Fractionated cell lysates were immunoblotted with anti-NRDP1, anti-AR, anti-FLNA (C-terminal), anti-β-actin (to demonstrate specificity of cytoplasmic fraction), and anti-lamin A/C (to demonstrate specificity of nuclear fraction) and indicates that transfection of FLNA(16–24) caused nuclear expression of FLNA and restored NRDP1 protein in C4-2 cells, although AR levels were not altered. (C) Protein expression of NRDP1 in CWR-R1 cells is regulated by the 90 kDa FLNA. Whole-cell lysates of CWR-R1 cells that were transfected with empty vector, full-length FLNA, FLNA(1–15), or FLNA(16–24) were immunoblotted with anti-FLNA, anti-NRDP1, and anti-tubulin antibodies. NRDP1 protein levels increased with the increased levels of the 90 kDa FLNA fragment. (D) FLNA restores NRDP1 expression in CRPC cells. QPCR for *Nrdp1* expression in LNCaP, C4-2, and stably transfected C4-2-FLNA(16–24) showed that *Nrdp1* expression was reduced in C4-2 compared with LNCaP cells ($P=0.0141$), but expression of FLNA(16–24) in C4-2 cells restored *Nrdp1* expression to a level similar to that of LNCaP cells ($P=0.0002$ compared with C4-2). *Nrdp1* transcript levels were normalized to the corresponding values for β-actin. (E) Comparison of *Nrdp1* response to changes in AR in CWR-R1 and CWR22Rv1 cells cultured in FBS, CSS, or CSS treated with increasing doses of DHT as indicated. Lysates were immunoblotted with anti-NRDP1, anti-ERBB3, and anti-tubulin antibodies. While the levels of Nrdp1 in CWR22Rv1 cells were unaltered despite culture in CSS, in CWR-R1 these levels altered slightly. (F, left) Reporter gene activity of AR on a luciferase-tagged PSA promoter section demonstrates that in control LNCaP cells, 10 μM bicalutamide is able to suppress AR activity whereas in cells where FLNA is downregulated by siRNA, bicalutamide failed to affect AR activity, $*P<0.0001$. (F, right) Western blotting demonstrating the efficacy of FLNA siRNA used.

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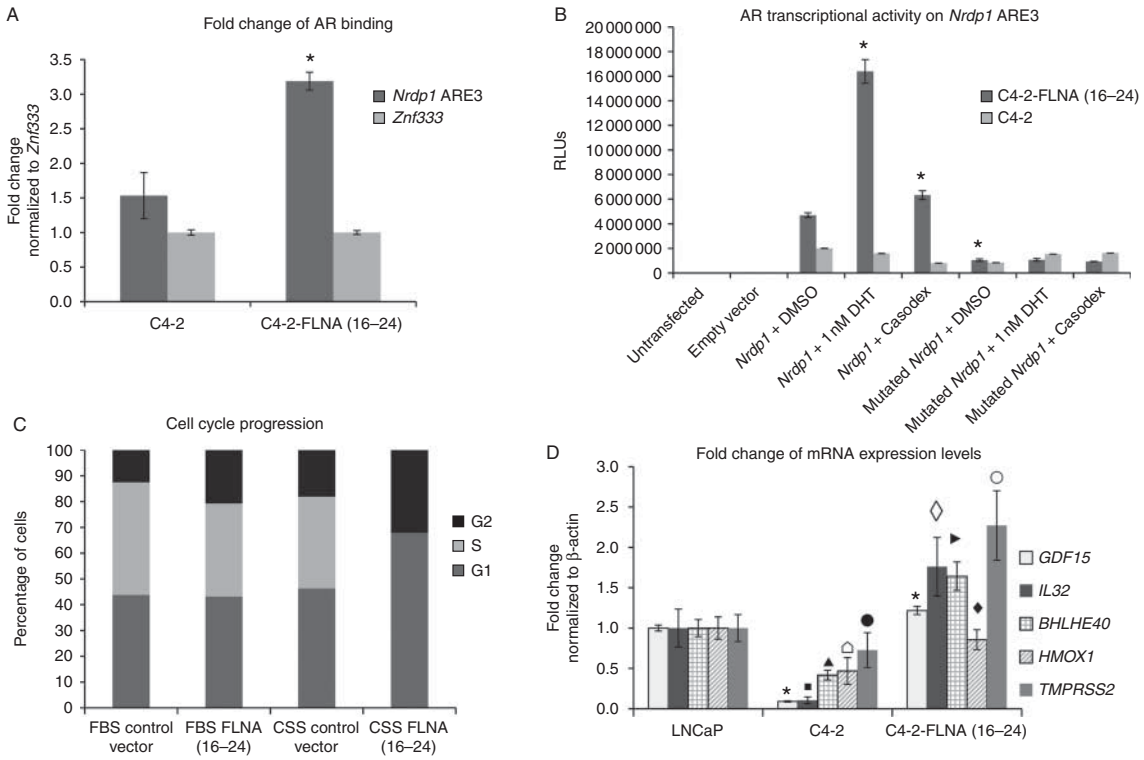

Figure 7

Expression of the 90 kDa FLNA isoform restored AR regulation of *Nrdp1* transcription. (A) AR binds to *Nrdp1* ARE3 in the presence of FLNA(16–24) (90 kDa). Chromatin samples were immunoprecipitated with an anti-AR antibody and analyzed by qPCR with primers flanking the *Nrdp1* ARE3 region with *Znf333* as a negative control. Note that AR binding increased twofold upon FLNA(16–24) transfection (*$P<0.0001$). (B) AR transcriptional activity of *Nrdp1* ARE3 is androgen regulated in the presence of FLNA(16–24). C4-2 cells transfected with empty vector or FLNA(16–24) were cultured in FBS medium and transfected with control vector, normal *Nrdp1* ARE3, or mutant *Nrdp1* ARE3. AR transcriptional activity was measured by luciferase assay. Cells were also treated with DMSO, 1 nM DHT, or 10 μM bicalutamide (Casodex). Luciferase was increased in the presence of FLNA(16–24), and regulated in an androgen-dependent manner with normal *Nrdp1* ARE3, *$P<0.0001$. (C) Flow cytometric analysis in PI-stained, ethanol-fixed C4-2 cells to determine the effect of transfection with FLNA(16–24) on cell cycle. Cells were grown in FBS or CSS and transfected with either empty vector or FLNA(16–24). Transfection with FLNA(16–24) has the same effect as growing the cells in CSS, however the combination of the two almost completely depleted the cell in S phase. (D) FLNA acts as a regulator of mRNA expression levels in multiple genes. Results of qPCR for *GDF15*, *IL32*, *TMPRSS2*, *BHLHE40*, and *HMOX1* expression in LNCaP, C4-2, and C4-2 FLNA(16–24). The expression of four of five genes was significantly decreased in C4-2 compared with LNCaP cells (*GDF15*, $P<0.0001$ (*); *IL32*, $P=0.0029$ (filled square); *BHLHE40*, $P=0.0012$ (filled triangle); and *HMOX1*, $P=0.0129$ (open pentagon)) while that for *TMPRSS2* was not significant ($P>0.05$; filled circle). The expression of FLNA(16–24) in C4-2 cells restored expression of these genes to a level similar or higher than LNCaP cells (GDF15, $P<0.0001$ (*); *IL32*, $P=0.0014$ (open diamond); *BHLHE40*, $P=0.0003$ (filled right triangle); *HMOX1*, $P=0.0052$ (filled diamond); and *TMPRSS2*, $P=0.0312$ (open circle)). All transcript levels were normalized to the corresponding values for β-actin.

Transfection of FLNA(16–24) in C4-2 cells increased AR binding to *Nrdp1* ARE3 (Fig. 7A). Importantly, this re-establishment of AR binding restored androgen sensitivity of AR-induced *Nrdp1* transcription. C4-2 cells sham transfected or transfected with FLNA(16–24) and either WT or mutated *Nrdp1* ARE3-luciferase constructs were treated with vehicle, DHT, or bicalutamide. Untransfected C4-2 cells showed very little AR transcriptional activity on *Nrdp1* ARE3, and no response to either the androgen or the anti-androgen ($P>0.05$), whereas C4-2 cells transfected with FLNA(16–24) responded to them. The mutant ARE3 construct showed little AR-dependent transactivation, but in C4-2 FLNA(16–24) cells transfected with WT ARE3, transcription was increased in the presence of DHT and inhibited by bicalutamide, indicating a restoration of androgen sensitivity (Fig. 7B). In contrast, AR transcriptional activity stimulated by FLNA(1–15) (cytoplasmic) was ligand-independent as indicated by the lack of response when cells were cultured in CSS (unlike nuclear FLNA(16–24)) (Supplementary Fig. 5B), and is probably caused by increased actin cross-linking, which promotes AR transcriptional activity (de Vere White *et al.* 1997, McGrath *et al.* 2013). Thus, nuclear FLNA induced androgen-sensitive AR transcriptional activity

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while in the absence of nuclear FLNA, AR activity was ligand-independent.

Next, we investigated whether nuclear FLNA-regulated AR activity had any functional role in tumor response. Investigation of the effect of FLNA(16–24) on cell proliferation by flow cytometric analysis revealed that in CRPC C4-2 cells, FLNA(16–24) by itself had no significant effect, neither did the removal of hormones (which includes androgens) by charcoal stripping; however, in the absence of hormones, FLNA(16–24) induced severe growth arrest, causing cells to arrest in both G1 and G2 phases, with very few cells in S phase (Fig. 7C). FLNA(16–24) also induced a threefold increase in apoptosis (Supplementary Fig. 6, see section on supplementary data given at the end of this article).

Finally, we determined whether other genes were also similarly regulated by FLNA. Five genes known to contain an ARE in the proximal promoter (interleukin 32 (*IL32*), heme oxygenase 1 (*HMOX1*), growth differentiation factor 15 (*GDF15*), basic loop–helix–loop E40 (*BHLHE40*), and *TMPRSS2*, see Supplementary Table 3, see section on supplementary data given at the end of this article) were examined to determine whether FLNA(16–24) affected their transcription (Fig. 7D). The expression of GDF15, a member of the transforming growth factor beta superfamily, and IL32 is known to induce apoptosis (Podar *et al.* 2007, Park *et al.* 2012, Wang *et al.* 2012, Yun *et al.* 2013), while TMPRSS2 is a known AR target suppressed in CRPC. HMOX1 counteracts oxidative and inflammatory damage and is implicated in the adhesive and morphological properties of tumor cells (Gueron *et al.* 2014), while BHLHE40 is a transcription factor involved in the regulation of cell differentiation, response to hypoxia, and carcinogenesis (Wu *et al.* 2015). Results of qPCR indicated that each of these genes were underexpressed in C4-2 compared with LNCaP, while expression of nuclear FLNA restores their expression (Fig. 7D). These results indicate that FLNA regulates a subset of genes associated with AR transcriptional activity in PCa.

Discussion

The AR regulates a very different transcription program in androgen-dependent PCa versus CRPC, even in tumors that do not harbor AR mutations or alternately spliced forms (Wang *et al.* 2009*a*, Decker *et al.* 2012, Hu *et al.* 2012). To examine the cause of AR reprograming, we used *NRDP1* as a model gene whose transcription is regulated by AR in hormone-naïve PCa, but not in CRPC. NRDP1 was first identified as an E3 ubiquitin ligase that caused ERBB3 degradation in breast cancer cells (Wu *et al.* 2004, Yen *et al.* 2006, Cao *et al.* 2007). Since then, this RING-finger-containing protein has been found to regulate a number of other targets, including pro-inflammatory cytokines (Wang *et al.* 2009*b*), type 1 cytokine receptor (Wauman *et al.* 2011), inhibitor of apoptosis proteins (Qiu *et al.* 2004), parkin (Zhong *et al.* 2005), and CCAAT/enhancer-binding protein beta (Ye *et al.* 2012). Results of previous studies of breast cancer indicated that NRDP1 is lost during normal-to-tumor transition (Wu *et al.* 2004, Yen *et al.* 2006, Cao *et al.* 2007); however, our results indicated that it increased in PCa compared with non-tumor prostate, indicating alternate pathways regulating its levels in various organs. Despite the initial increase in NRDP1 with AR in hormone-naïve tumors, we observed reduced NRDP1 expression in CRPC, although AR expression persisted in the latter. The current study resulted in three important findings. i) In androgen-sensitive PCa, NRDP1 is a direct transcription target of AR, and is increased in localized PCa, where AR levels increase compared with nontumor prostate. ii) In CRPC, despite the further increase in AR activity, NRDP1 levels decrease because the AR no longer regulates its transcription and iii) this difference in AR-induced transcription is regulated by the availability of nuclear FLNA.

Full-length FLNA is a 280 kDa actin-binding protein that acts as a scaffold to enable the interaction of actin with other proteins to regulate diverse functions such as cell rigidity, adhesion, and migration (van der Flier & Sonnenberg 2001, Stossel *et al.* 2001). Full-length FLNA is important for proper embryonic development (Robertson *et al.* 2003), but probably promotes metastasis if over-expressed in the cytoplasm of cancer cells (Castoria *et al.* 2011, McGrath *et al.* 2013). Actin-binding proteins are known to regulate AR transcriptional activity (de Vere White *et al.* 1997), and FLNA has been suggested to be a putative AR co-regulator (Parker *et al.* 2013). Androgen stimulation of quiescent NIH3T3 cells caused cytoplasmic FLNA binding to AR and co-localization of the FLNA–AR complex at intermediate actin filaments, leading to extranuclear AR-mediated RAC1 activation and subsequent cell motility (Castoria *et al.* 2011). However, in normal epithelial cells of the adult prostate FLNA cleaves to a 90 kDa fragment which localizes to the nucleus and regulates AR transcriptional activity (Ozanne *et al.* 2000, Loy *et al.* 2003). Importantly, in the presence of nuclear FLNA, AR regulated its transcription only upon ligand-binding (Supplementary Fig. 5B). FLNA is a scaffolding protein, and binds to a very large number of proteins of diverse functions. One way that FLNA can promote AR

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binding to target genes is by regulating the interaction of the AR with other co-regulators. Both actin-binding proteins and DNA-repair proteins act as AR co-factors (van de Wijngaart *et al*. 2012, Parker *et al*. 2013), and FLNA is known to interact with both these classes of proteins (van der Flier & Sonnenberg 2001, Stossel *et al*. 2001). The significance of this observation is that in the presence of nuclear FLNA, treatment with anti-androgens such as bicalutamide or enzalutamide will prevent tumor growth or progression, whereas in the absence of nuclear FLNA, the anti-androgens are ineffective.

Basal levels of *Nrdp1* are seen in all cells, as apart from AR, *Nrdp1* promoter has binding sites for several other transcription factors. Thus in cells where *Nrdp1* is transcribed by AR, its expression is androgen-dependent, while in other cells, its expression is regulated by other transcription factors in an androgen-independent manner. A finding of this study is that AR binding and increased transcription in an androgen-dependent manner was seen only in the presence of nuclear FLNA. It may be remarked that both cytoplasmic and nuclear FLNA promoted AR transcriptional activity; however, AR transcriptional activity induced by cytoplasmic FLNA was ligand-independent, whereas AR activity caused by nuclear FLNA was androgen-dependent. One mechanism by which nuclear FLNA can affect AR transcriptional activity is by its scaffolding action in regulating the interaction of the AR with other co-regulators. While we (Wang *et al*. 2007) and others (Loy *et al*. 2003) have demonstrated direct interaction between AR and FLNA, the mechanism of AR interaction with FLNA in the nucleus by which androgen-sensitivity is maintained is yet to be identified. AR binds to C-terminal FLNA (Ozanne *et al*. 2000), and although nuclear FLNA induced AR transcriptional activity, it prevented inappropriate activation of AR by nonspecific ligands or by ligand-independent activation, thereby demonstrating anti-tumorigenic properties (Loy *et al*. 2003, Wang *et al*. 2007, Bedolla *et al*. 2009, Mooso *et al*. 2012, Sun *et al*. 2014).

A relevant question is – how does FLNA(16–24) cause growth arrest and apoptosis in the absence of hormones? In this paper, we have not addressed this issue – but it is well known that in CRPC cells, AR regulates cell cycle progression as well as cell survival, and anti-androgens are known to promote apoptosis and induce growth arrest. Now, full-length FLNA is required for cell cycle progression and cell survival as well; therefore, we believe that one way FLNA(16–24) can induce apoptosis and growth arrest is by preventing cell survival and cell cycle progression in cells where anti-androgens induce apoptosis and growth arrest, which would have been possible with full-length FLNA. On that note, FLNA is also known to be a major factor in DNA damage repair by interaction with BRCA1 (Velkova *et al*. 2010) and BRCA2 (Yue *et al*. 2009). As anti-androgens also prevent DNA double-strand-break repair (Polkinghorn *et al*. 2013), it is expected that FLNA(16–24) will also promote ionizing-radiation-induced apoptosis.

In a previous study, we had shown that the expression of nuclear FLNA is also regulated by the AR (Mooso *et al*. 2012). FLNA proteolysis is prevented by its phosphorylation at S2152 (van der Flier & Sonnenberg 2001). Our results indicated that in LNCaP cells, FLNA is not phosphorylated and undergoes proteolysis to form the 90 kDa fragment which then translocates to the nucleus. Prevention of FLNA phosphorylation appears to require AR activity. On the other hand, in C4-2 cells, which were developed by implantation of LNCaP cells into castrated mice, FLNA is phosphorylated, which probably occurred during its progression to castration-resistant growth. Therefore, the phosphorylated FLNA in C4-2 cells does not undergo proteolysis and remains cytoplasm-bound (Mooso *et al*. 2012). It may be noted that FLNA has also been shown to localize to the nucleolus and to associate with the RNA polymerase I transcription machinery to suppress rRNA gene transcription (Deng *et al*. 2012). However, we found nucleolar FLNA to be present in C4-2 as well as in LNCaP cells (Supplementary Fig. 4), and when transfected with either the empty vector or a FLNA plasmid (Fig. 6A), *Nrdp1* expression seems to be independent of the FLNA fraction localizing to the nucleolus, although it is dependent on the fraction localizing to the nucleoplasm.

FLNA's effect on broad gene expression is obvious from its effects on *TMPRSS2*, *HMOX1*, *BHLHE40*, *GDF15*, and *IL32*, each of which has been identified to have an ARE in the proximal promoter. Results of previous studies indicated that *TMPRSS2* mRNA expression, but not *TMPRSS2–ERG* gene fusion, is decreased in CRPC, because the gene fusion probably causes an increase in ERG expression instead of *TMPRSS2* (Cai *et al*. 2009). FLNA also upregulates two other genes, *GDF15* and *IL32* that are also associated with increased apoptosis (Park *et al*. 2012, Wang *et al*. 2012, Yun *et al*. 2013). Therefore, it is likely that nuclear FLNA induces apoptosis by increasing GDF15 and IL32 levels. It is important to note that the absence of BHLHE40, a transcription factor, has been identified as being involved in the regulation of cell differentiation and prevention of carcinogenesis (Wu *et al*. 2015), which may be one way by which it has an effect on tumor cell progression.

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In conclusion, in this study, we demonstrated that FLNA(16–24) regulates *NRDP1* transcription by the AR by acting as a co-activator of its transcriptional action. The regulation of *Nrdp1* by AR in PCa cells that express 90 kDa FLNA, its loss in those that do not, and the adjustment upon re-introduction of FLNA into the nucleus are an example of retooling of the transcriptional program regulated by the AR in PCa cells. We demonstrated that it is possible to restore the original androgen-sensitive program by re-introducing a key co-regulator that is frequently lost in CRPC (Bedolla *et al.* 2009). The AR is known to repress cell growth and induce differentiation in various tissues (Batch *et al.* 1992, Govoroun *et al.* 2001, Holdcraft & Braun 2004), but to promote tumorigenesis in prostate cells (Berger *et al.* 2004). Our results indicate that the availability of co-regulators may dictate which genes are transcribed by the AR.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-15-0021.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported. The work reported here does not represent the views or opinions of the Department of Veteran Affairs or the United States Government.

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Author contribution statement
P M Ghosh contributed to conception and design of this study; P M Ghosh, M Mudryj, and K L Carraway, III developed the methodology; R M Savoy, L Chen, S Siddiqui, M K Jathal, T M Steele, S Bose, B A Mooso, L S D'Abronzo, and W H Fry contributed to data acquisition; F U Melgoza, B Durbin-Johnson, C Drake, and P M Ghosh analyzed and interpreted data; and R M Savoy and P M Ghosh wrote, reviewed and/or revised the manuscript.

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