

Nrdp1-Mediated Regulation of ErbB3 Expression by the Androgen Receptor in Androgen-Dependent but not Castrate-Resistant Prostate Cancer Cells

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Abstract

Patients with advanced prostate cancer (PCa) are initially susceptible to androgen withdrawal (AW), but ultimately develop resistance to this therapy (castration-resistant PCa, CRPC). Here, we show that AW can promote CRPC development by increasing the levels of the receptor tyrosine kinase ErbB3 in androgen-dependent PCa, resulting in AW-resistant cell cycle progression and increased androgen receptor (AR) transcriptional activity. CRPC cell lines and human PCa tissue overexpressed ErbB3, whereas downregulation of ErbB3 prevented CRPC cell growth. Investigation of the mechanism by which AW augments ErbB3, using normal prostate-derived pRNS-1-1 cells, and androgen-dependent PCa lines LNCaP, PC346C, and CWR22 mouse xenografts, revealed that the AR suppresses ErbB3 protein levels, whereas AW relieves this suppression, showing for the first time the negative regulation of ErbB3 by AR. We show that AR activation promotes ErbB3 degradation in androgen-dependent cells, and that this effect is mediated by AR-dependent transcriptional upregulation of neuregulin receptor degradation protein-1 (Nrdp1), an E3 ubiquitin ligase that targets ErbB3 for degradation but whose role in PCa has not been previously examined. Therefore, AW decreases Nrdp1 expression, promoting ErbB3 protein accumulation, and leading to AR-independent proliferation. However, in CRPC sublines of LNCaP and CWR22, which strongly overexpress the AR, ErbB3 levels remain elevated due to constitutive suppression of Nrdp1, which prevents AR regulation of Nrdp1. Our observations point to a model of CRPC development in which progression of PCa to castration resistance is associated with the inability of AR to transcriptionally regulate Nrdp1, and predict that inhibition of ErbB3 during AW may impair CRPC development. *Cancer Res*; 70(14); 5994–6003. ©2010 AACR.

Introduction

Because prostate cancer (PCa) cells are initially dependent on androgens for growth, the standard therapy for recurrent PCa is the pharmacologic removal of circulating androgens (androgen withdrawal, AW). Although initially effective, this therapy ultimately fails, indicating the development of castration-resistant PCa (CRPC). The treatment options for

patients who fail AW therapy are limited; hence, there is an urgent need for the elucidation of molecular pathways leading to CRPC. Previous studies concluded that AW resulted in cell cycle arrest, whereas CRPC is associated with a release from that arrest (1). In this study, we show that AW results in an increase in the receptor tyrosine kinase ErbB3, which induces an increase in androgen receptor (AR) transcriptional activity and cell cycle progression.

The ErbB receptor tyrosine kinase family regulates proliferation and survival in PCa (2). It consists of the type 1 tyrosine kinases ErbB1/human epidermal growth factor receptor (HER)1/epidermal growth factor receptor (EGFR), ErbB2/HER2/neu, ErbB3/HER3, and ErbB4/HER4 (3). PCa cells express EGFR, HER2, and ErbB3, but not ErbB4 (2, 3), which are activated by ligand binding, dimerization, and phosphorylation. ErbB receptors, except HER2, have specific ligands (4); however, ErbB3 is unique in that its tyrosine kinase domain is functionally defective so it must heterodimerize with other ErbB receptors for signaling activity (4). Despite this, multiple studies suggested that ErbB3 plays a role in promoting PCa. Treatment of androgen-dependent LNCaP cells with the cytokine interleukin-6,

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known to promote PCa progression, stimulates HER2 and ErbB3 (5). Phosphatidylinositol 3-kinase, which regulates cell survival by activating Akt, associates with ErbB3 (6), whereas microarray analysis showed increased ErbB3 expression in PCa compared with normal prostate (7), and immunohistochemical analysis showed that ~90% of PCa tissues displayed significant ErbB3 staining (8–10). Alternate splicing caused multiple soluble, truncated forms of ErbB3 (11, 12), and this receptor tyrosine kinase was also shown to localize to the nucleus (8, 13). These studies point to the importance of ErbB3 in PCa signaling.

Substantial evidence also underscores a link between ErbB3 activation and AR activity. The AR is known to remain active in CRPC, and activation of ErbB3 in a mouse model of PCa was associated with AR phosphorylation (10), whereas activation of HER2/ErbB3 heterodimers modulated AR transcriptional activity (14) and promoted AR transactivation of reporter genes (15). The effects of ErbB3 on the AR are likely mediated by suppression of the ErbB3-binding protein Ebp1 (16, 17), which inhibits both E2F1 and AR activity (17).

In this study, we make the novel observation that ErbB3 levels are increased during AW, and that the effect of AR on ErbB3 is mediated by the RING finger E3 ubiquitin ligase neuregulin receptor degradation protein-1 (Nrdp1), which was discovered as an ErbB3-interacting protein by yeast two-hybrid analyses (18), but was not, until now, investigated in PCa. Nrdp1 associates with ErbB3 and mediates its ubiquitination and rapid degradation in a ligand-independent manner (19), thus regulating steady-state levels in breast cancer cells (20). We show that although the AR regulates Nrdp1 transcription in androgen-dependent cells, this regulation is lost in AR-positive CRPC cells due to the suppression of Nrdp1 by elevated ErbB3. Our data explain how ErbB3 levels are increased in CRPC: during AW, AR levels are decreased, and the suppressive effect of the AR on ErbB3 is relieved, resulting in an increase in ErbB3 levels. However, in AR-positive CRPC cells, ErbB3 levels remain high because elevated ErbB3 suppresses Nrdp1 expression and the AR loses control of Nrdp1 transcription; therefore, it cannot suppress ErbB3 levels even when AR levels rebound.

Materials and Methods

Patients and tissues used

Tissue microarrays from the University of Michigan Prostate Cancer Specialized Program of Research Excellence were constructed from benign prostate ($n = 36$ cores in triplicate, i.e., total 108), high-grade prostatic intraepithelial neoplasia [HGPI; $n = 21$ cores in triplicate (63 total)], prostate tumor tissue [$n = 65$ cores in triplicate (195 total)] obtained by prostatectomy from men with localized PCa, and CRPC metastatic lesions [$n = 68$ cores in triplicate (204 total)] from “warm” autopsies (mean 3 h lapsed from death to commencement of autopsy) of patients succumbing to castrate-resistant disease (21, 22). Tissue details, immunohistochemical techniques, and statistical analyses were described earlier (23) and in Supplementary Materials.

Cell culture and pharmacologic treatments

LNCaP, PC-3, DU-145, CWR22Rv1, and RWPE-1 cells were purchased from the American Type Culture Collection, whereas C4-2 cells were from UroCor. pRNS-1-1 cells were from Dr. Johng Rhim, University of the Health Sciences, Bethesda, MD (24), whereas PC-346C cells were provided by Dr. W.M. van Weerden, Josephine Nefkens Institute, Erasmus MC, Rotterdam, the Netherlands. All cells were cultivated in RPMI 1640 with 5% serum unless otherwise noted. CRPC sublines of LNCaP cells (LNCaP-AI cells) were developed by prolonged culture of LNCaP cells in phenol red-free RPMI 1640 with 5% charcoal-stripped fetal bovine serum (CSS; ref. 25). The cell lines were not tested or authenticated specifically for this study; however, they have been authenticated elsewhere (24–27). RPMI 1640 and fetal bovine serum (FBS) was from Invitrogen, whereas CSS was from Gemini Bioproducts. 4,5 α -Dihydrotestosterone (DHT), cycloheximide, and concanamycin were obtained from Sigma-Aldrich. Casodex (bicalutamide) was from AstraZeneca. Rabbit polyclonal EGFR, HER2, ErbB3, β -actin, AR, and α -tubulin antibodies were from Santa Cruz Biotechnology. Rabbit polyclonal anti-phospho-Akt (Ser 473), anti-phospho-EGFR (Y1068), anti-phospho-HER2 (Y1248), and phospho-ErbB3 (Y1289) were from Cell Signaling Technology. Affinity-purified rabbit antibodies to Nrdp1 were previously described (18).

Transfections, plasmids, and small interfering RNA

Plasmids expressing wtAR or AR(T877A) resulted from cloning into pCEP4, the full-length wild-type AR cDNA or AR cDNA whose protein product contains a Thr \rightarrow Ala mutation at the 877 residual position isolated from LNCaP cells (24). pCDNA-HER2 and pCDNA3-ErbB3 plasmids were from Dr. John Koland, University of Iowa, Iowa City, IA (28). Wild-type AR (pAR0) plasmid was from Dr. Albert Brinkman, Erasmus University, the Netherlands (29). Plasmids encoding human Nrdp1 COOH terminally tagged with a FLAG epitope and a pSuper-Nrdp1 RNAi were described earlier (18, 20, 30). Another small interfering RNA (siRNA) pool against human Nrdp1 was from Santa Cruz Biotechnology (against 5'-CAA-GCA-GUA-UCC-CUG-UUC-ATT-3', 5'-CUG-UGA-CUG-UAG-UUA-GUU-ATT-3', and 5'-CUU-CCU-CUC-UUC-CUG-UGA-ATT-3'). siRNA against human AR (Invitrogen): hAR1, 5'-GAC-UCC-UUU-GCA-GCC-UUG-CUC-UCU-A-3' and 5'-UAG-AGA-GCA-AGG-CUG-CAA-AGG-AGU-C-3'; hAR2, 5'-GCC-UUG-CUC-UCU-AGC-CUC-AAU-GAA-C-3' and 5'-GUU-CAU-UGA-GGC-UAG-AGA-GCA-AGG-C-3'. siRNA against ErbB3 (Santa Cruz Biotechnology): 5'-CCA-AUA-CCA-GAC-ACU-GUA-CTT-3'. Control siRNA: pool of four scrambled nonspecific siRNA (siCONTROL, Santa Cruz Biotechnology). Reverse transcription-PCR (RT-PCR) was carried out using the following primers: c-erbB3-F, 5'-GGA-GTA-CAA-ATT-GCC-AAG-GG-TA-3'; c-erbB3-R, 5'-CAG-GTC-TGG-CAA-GTA-TGG-AT-3'; EGFR-F, 5'-GAG-AGG-AGA-ACT-GCC-AGA-A-3'; EGFR-R, 5'-GTA-GCA-TTT-ATG-GAG-AGT-G-3'; β -actin-F, 5'-ACT-CTT-CCA-GCC-TTC-GTT-C-3'; β -actin-R, 5'-ATC-TCC-TTC-TGC-ATC-CTG-TC-3'; Nrdp1-F, 5'-GCA-GTG-GAG-TCT-TGG-AGG-AG-3'; Nrdp1-R, 5'-GCC-TTT-AGC-AGC-TGG-ATG-TC-3'.

Mouse studies

Mice (4–5-week-old, *nu/nu* athymic male) were obtained from Harlan Sprague-Dawley, Inc. and implanted s.c. with sustained release testosterone pellets (12.5 mg, 90-d release; Innovative Research of America). Suspensions of CWR22 cells were made in 50% Matrigel solubilized basement membrane (BD Biosciences), and xenografts were established by s.c. injections of 2.5×10^6 cells/site into both flanks. When palpable tumors were observed, animals were treated with (a) vehicle or (b) bicalutamide, delivered by oral gavage at a dose of 50 mg/Kg, 100 μ L per dose, five times per week, dissolved in ethanol, and delivered as a suspension in peanut oil. After 2 weeks, the mice were euthanized, tumors were collected and divided into sections for paraffin embedding, and snap frozen in liquid nitrogen.

For additional methods, see previous publications (31, 32) and Supplementary Text.

Results

Increased expression of ErbB3 in castration-sensitive PCa and CRPC

We stained tissue microarrays representing benign prostates, HGPIN, and localized prostate tumors obtained by prostatectomy, as well as metastatic lesions from warm autopsies of men who died with hormone refractory PCa, with an anti-ErbB3 antibody that recognizes cytoplasmic ErbB3. Only the tumor tissue stained strongly for ErbB3, which was seen exclusively in the epithelial cells (Fig. 1, top). The extent of ErbB3 staining increased from benign prostate (mean staining score, 1.65 ± 0.80) to PIN (1.9 ± 0.61) to localized PCa (2.37 ± 0.63) to metastatic CRPC specimen (2.51 ± 0.74 ; benign versus localized tumors: $P = 0.0001$; Benign versus CRPC: $P = 0.0001$; PIN versus loc. tumors: $P = 0.0039$; PIN versus CRPC: $P = 0.0005$; Supplementary Table S1; Fig. 1,

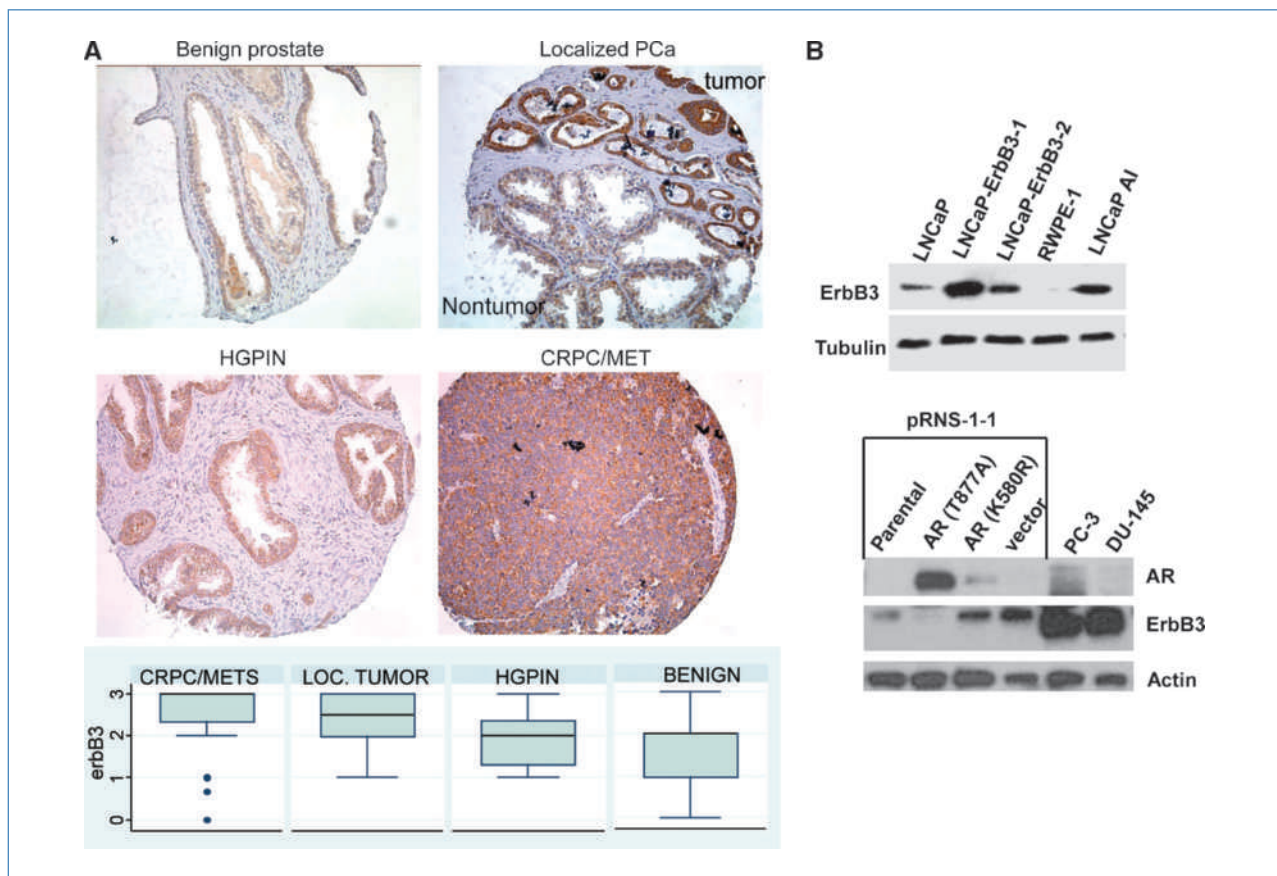


Figure 1. ErbB3 levels increase with PCa progression. A, tissue microarrays representing (a) benign prostate ($n = 36$), (b) HGPIN ($n = 21$), (c) localized tumors ($n = 65$) obtained by prostatectomy, and (d) prostatic tissues ($n = 68$; CRPC/MET) from warm autopsies of men who died of CRPC were immunostained with anti-ErbB3 antibody (brown staining) and counterstained with hematoxylin (blue staining). Top, sections stained with anti-ErbB3. Note the strong ErbB3 stain in the tumor containing regions, whereas the benign tissue alongside stained only weakly. The antibody used did not stain the nuclei in these tissues ($\times 20$ magnification). Bottom, box plots representing range of ErbB3 expression in benign prostate, HGPIN, localized tumors, and metastatic and localized tissues from warm autopsies of men who died of CRPC. B, increased ErbB3 expression in PCa cells compared with lines derived from normal prostate. Top, normal prostate derived RWPE-1 cells, androgen-dependent LNCaP, and its CRPC subline LNCaP-AI cells compared with stable LNCaP sublines overexpressing ErbB3 (LNCaP-ErbB3-1 and LNCaP-ErbB3-2). Bottom, ErbB3 expression in pRNS-1-1 cells derived from a normal prostate, which upon culture lost the expression of the AR, and AR-null PC-3 and DU-145 cells. pRNS-1-1 cells were transfected with vector only, or mutant AR(T877A) or AR(K580R).

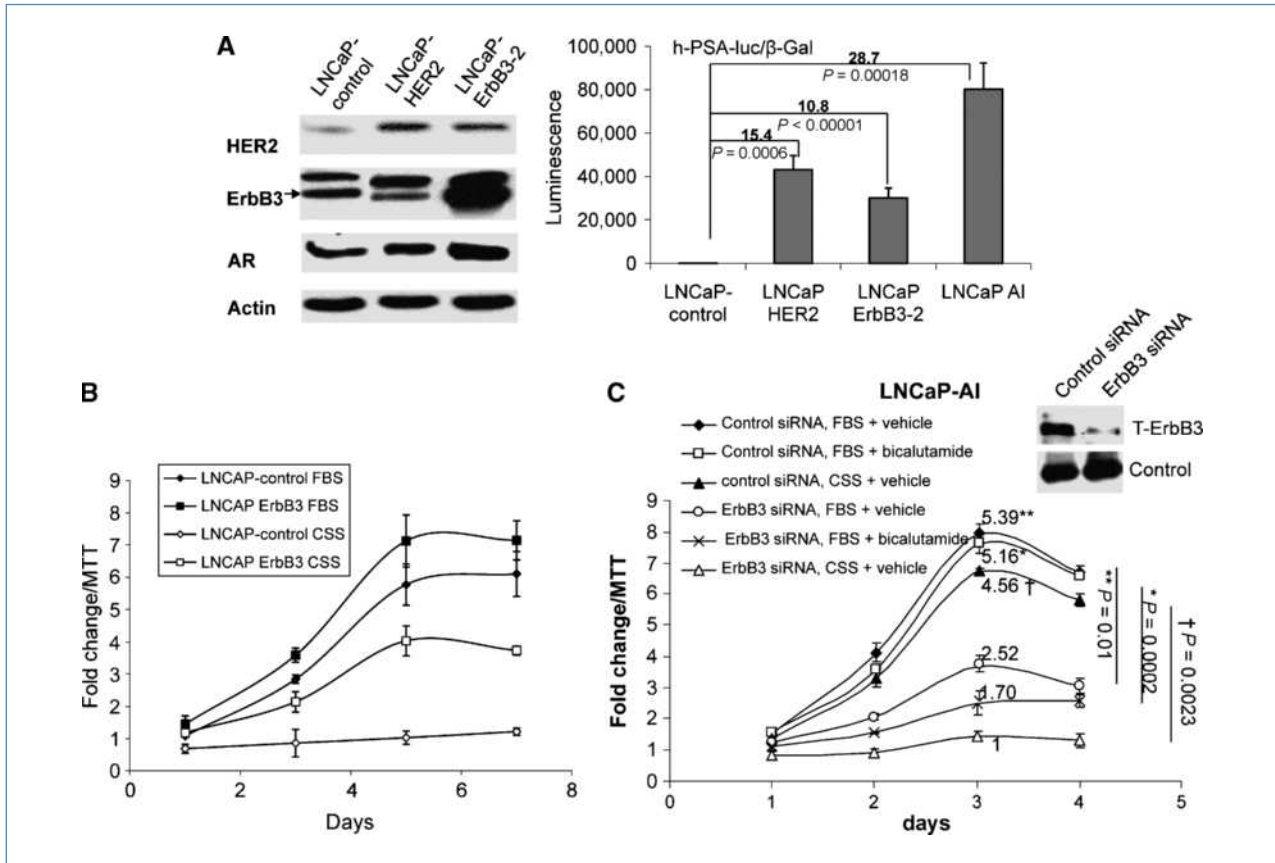


Figure 2. Overexpression of ErbB3 leads to increased AR transcriptional activity and cell proliferation. A, left, increased AR levels in stable LNCaP cell lines overexpressing HER2 or ErbB3 (LNCaP-HER2 and LNCaP-ErbB3) overexpressing plasmids (pcDNA3-HER2 or pcDNA3-ErbB3) in LNCaP cells. Overexpression of HER2 and ErbB3 was confirmed by Western blotting. Right, increased AR transcriptional activity in LNCaP-HER2 and LNCaP-ErbB3 compared with LNCaP. Cells were cultured in CSS-containing medium, and AR transcriptional activity was measured on a prostate-specific antigen promoter by luciferase assay. LNCaP-AI cells were used as positive control. B, ErbB3 overexpression stimulates proliferation in LNCaP cells. MTT assay showed increased growth rates of LNCaP-ErbB3-2 cells versus LNCaP in medium containing FBS versus CSS. Points, mean of three independent readings; bars, SD. C, downregulation of ErbB3 by siRNA suppressed cell growth as estimated through MTT assay in cells cultured in FBS or CSS, in the presence or absence of 10 $\mu\text{mol/L}$ bicalutamide. Points, mean of three independent readings; bars, SD. Inset, immunoblot showing ErbB3 downregulation by siRNA.

bottom). Similarly, comparison of ErbB3 levels in various prostate-derived cell lines revealed that “normal-like” RWPE-1 cells expressed very little ErbB3, whereas its expression was significantly higher in androgen-dependent LNCaP PCa cells and further increased in its castration-resistant subline LNCaP-AI (see Supplementary Fig. S1A and B; Fig. 1A, top). Similarly, CRPC cell lines PC-3 and DU-145 cells expressed very high levels of ErbB3 compared with pRNS-1-1 cells derived from a normal prostate (Fig. 1A, bottom), supporting the assertion that ErbB3 increased with PCA progression.

Overexpression of ErbB3 increased AR transcriptional activity and cell proliferation

To determine the functional significance of ErbB3 increase, LNCaP cells were stably transfected with plasmids encoding ErbB3 (pcDNA3-ErbB3) or HER2 (pcDNA3-HER2; Fig. 2A). The overexpressors used in subsequent experiments

were chosen from a screen of multiple clones to select the ones that expressed HER2 and ErbB3 at levels comparable with that in LNCaP-AI cells (two ErbB3 clones shown in Fig. 1B). Overexpression of ErbB3 in LNCaP cells increased AR expression and AR transcriptional activity as determined by reporter gene assay on a prostate-specific antigen promoter construct (Fig. 1B) in an EGFR- and HER2-dependent manner (Supplementary Fig. S1C).

AW in LNCaP cells was induced by culture in CSS-containing medium, causing growth arrest, which was relieved by ErbB3 overexpression, (Supplementary Fig. S2B; Fig. 2A) similar to HER2 (Supplementary Fig. S2A), whereas inhibition of ErbB3 using a human ErbB3 siRNA pool in LNCaP-AI cells significantly decreased their growth rates (Fig. 2C). Overexpression of ErbB3 in normal-like RWPE-1 cells also resulted in increased proliferation (data not shown). These results indicate that ErbB3 regulates cell proliferation in PCa cells.

AW induces ErbB3 increase in androgen-dependent PCa by preventing ErbB3 degradation

We next investigated the cause for ErbB3 increase in PCa cells with a history of AW exposure. Prolonged culture in CSS-containing medium decreased AR expression, but increased ErbB3 levels (Fig. 3A, left). To investigate whether this effect was caused by AR downregulation, AR levels were suppressed in LNCaP cells by two different RNAi, both stimulated ErbB3 levels, although to different extent (Fig. 3A, right). These results indicated that the AR suppresses ErbB3 levels in LNCaP cells, likely to prevent AR-independent cell signaling, whereas AW relieves this suppression.

Similarly, transfection with increasing amounts of wild-type AR (pAR0) in pRNS-1-1, a cell line derived from benign prostate tissue that had lost the expression of its endogenous

AR, decreased ErbB3 levels, but not EGFR (Fig. 3B). Stable transfection with AR(T877A) (a mutant AR found in LNCaP cells, which remains ligand dependent), but not the vector alone, also downregulated ErbB3 levels in pRNS-1-1 cells (Fig. 3C, left). DHT, a strong AR ligand, suppressed ErbB3 in pRNS-1-1/AR(T877A), whereas prolonged culture in CSS-containing medium upregulated ErbB3 (Fig. 3C, right). These results show that the AR is a negative regulator of ErbB3 expression in normal prostate also.

Our data indicated that the AR regulated ErbB3 levels by a posttranscriptional, rather than a transcriptional mechanism (Supplementary Fig. S3). To determine whether AR affected ErbB3 degradation, pRNS-1-1 cells transfected with vector alone or with wtAR (Fig. 3D) were treated with the protein synthesis inhibitor cycloheximide, and the rate of ErbB3

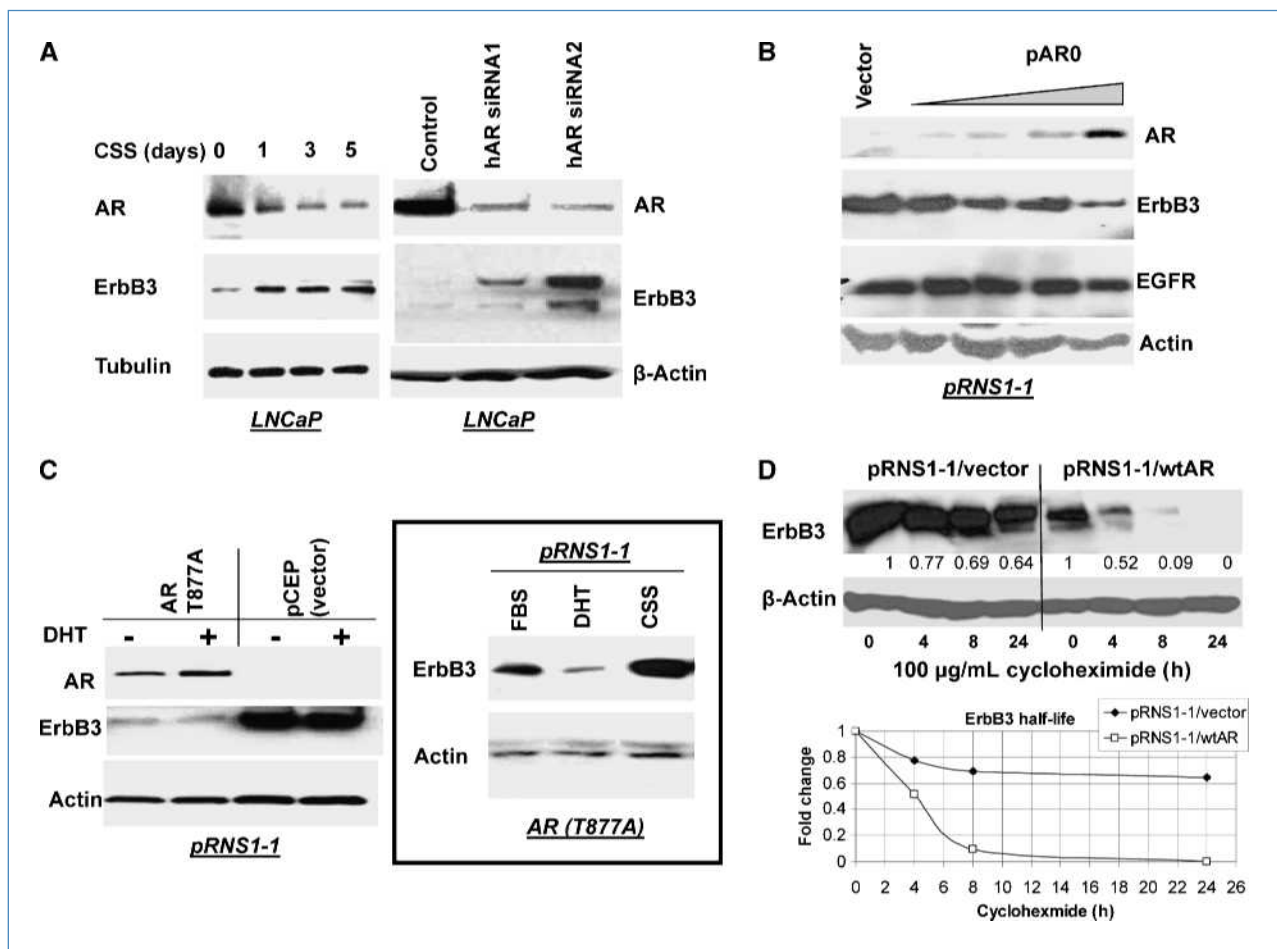


Figure 3. AR negatively regulates ErbB3 expression in androgen-dependent PCa cells. A, left, increased ErbB3 expression with decreasing AR levels in LNCaP cells cultured in CSS-containing medium over a period of 5 d. Right, stimulation of ErbB3 expression in LNCaP cells following AR downregulation with two different AR siRNA duplexes (hAR1 and hAR2). B, increasing AR levels in parental AR-null pRNS1-1 cells transfected with vector alone or with increasing amounts of pAR0 revealed decreasing ErbB3 expression, whereas EGFR levels were not altered. C, expression of AR(T877A) but not vector alone in pRNS1-1 cells caused increased expression of ErbB3. Inset, treatment of pRNS1-1 cells stably expressing AR(T877A) with 10 nmol/L DHT suppressed ErbB3 levels, whereas 5-d culture in CSS-containing medium stimulated ErbB3. D, decreased ErbB3 half-life upon expression of wild-type AR. pRNS1-1 cells stably transfected with vector only or wtAR were treated with 100 μ g/mL cycloheximide for the indicated times. Top, lysates were blotted with anti-ErbB3 or β -actin, and the bands were quantitated. Bottom, ErbB3 half-life was calculated by fitting the data to a single exponential. Results indicate that in pRNS1-1 cells expressing vector alone, ErbB3 levels were stabilized, whereas in those expressing wtAR, ErbB3 half-life was \sim 4 h.

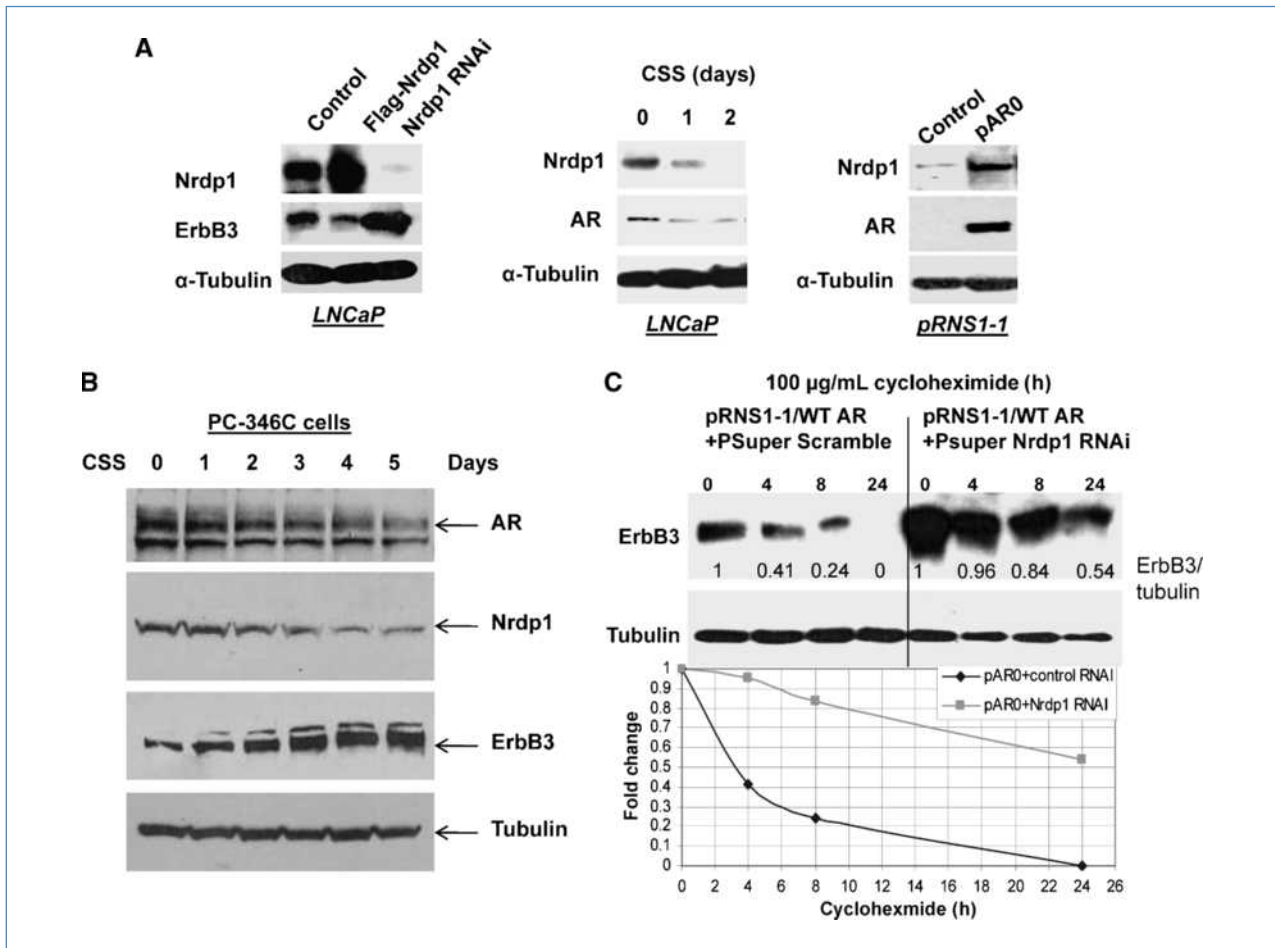


Figure 4. Negative regulation of ErbB3 by AR is mediated by Nrdp1. **A**, left, expression of the ubiquitin E3 ligase Nrdp1 corresponds to a decrease in ErbB3 expression. LNCaP cells were transiently transfected with plasmids encoding Flag vector, Flag-Nrdp1, or pSuper-Nrdp1 short hairpin RNA for 48 h, after which the lysate was collected and blotted with anti-Nrdp1, anti-ErbB3, or α -Tubulin as control. Middle, Nrdp1 levels correspond to AR expression. Culture of LNCaP cells over 2 d in CSS shows a decrease in Nrdp1 with lower AR. Right, expression of AR in pRNS-1-1 cells stimulates Nrdp1. Parental pRNS1-1 cells were transiently transfected with vector alone or pAR0. Cell lysate was collected after 48 h and blotted with anti-Nrdp1 and anti-AR. **B**, the AR positively regulates Nrdp1 and negatively regulates ErbB3 levels in androgen-dependent PC-346C cells. Note the expression of a lower molecular weight AR product in these cells. **C**, decreased half-life of ErbB3 induced by AR expression is mediated by Nrdp1. pRNS1-1 cells stably transfected with wtAR were further transfected with pSuper-scramble or pSuper-Nrdp1 RNAi plasmids for 24 h. ErbB3 half-life in pRNS-1-1/wtAR cells expressing control RNAi remained <4 h, whereas in the absence of Nrdp1 expression, the half-life of ErbB3 increased to >24 h.

degradation in the absence of *de novo* synthesis was determined over time. In cells transfected with vector alone, inhibition of protein synthesis with cycloheximide caused a 36% decline in ErbB3 after 24 hours; however, transfection with wtAR greatly reduced ErbB3 half-life (<4 h; Fig. 3D). These results show that the increase in ErbB3 expression in the absence of AR is due to a decrease in protein degradation rates.

Nrdp1 mediates AR-induced ErbB3 degradation in androgen-dependent cells

Previous studies identified the RING finger E3 ubiquitin ligase Nrdp1 as a promoter of ErbB3 degradation in breast cancer (18, 20). Nrdp1 overexpression in LNCaP cells decreased ErbB3 levels (Fig. 4A, left) and decreased cell proliferation (Supplementary Fig. S4A), whereas Nrdp1 downregulation (19, 20) increased ErbB3 (Fig. 4A, left), indi-

cating an inverse relationship between ErbB3 and Nrdp1 in PCA as well. Hence, we hypothesized that the effect of AR on ErbB3 may be mediated by Nrdp1.

Culture in CSS medium decreased Nrdp1 expression, whereas transfection of wtAR into AR-null pRNS-1-1 cells increased Nrdp1 expression (Fig. 4A). We also tested this effect in androgen-dependent PC-346C cells derived from a non-treated human prostate tumor extracted by transurethral resection of the prostate (26, 27). Similar to LNCaP, culture of PC-346C cells in CSS medium decreased AR and Nrdp1, whereas ErbB3 increased (Fig. 4B). These results confirmed that the AR positively regulated Nrdp1 expression in androgen-dependent cells. Hence, we investigated whether the effect of AR on ErbB3 half-life is mediated by Nrdp1. ErbB3 half-life in pRNS-1-1 cells stably expressing wtAR was ~3.5 hours, whereas downregulation of Nrdp1 increased ErbB3

half-life to >24 hours (Fig. 4C). Taken together, these results show that AR-regulated decrease in ErbB3 half-life is mediated by AR-induced Nrdp1 transcription. Thus, during AW, AR levels sharply decline, decreasing Nrdp1 levels, which in turn increased ErbB3 levels.

Regulation of Nrdp1 and ErbB3 expression by the AR is lost in AR and ErbB3-overexpressing CRPC cells

If AR always negatively regulated ErbB3 levels, then as AR increased during CRPC development, ErbB3 levels should decrease. However, we see that both LNCaP-AI and C4-2, another androgen-independent subline of LNCaP cells (33), expressed higher AR, as well as ErbB3, compared with LNCaP cells (Fig. 5A). Therefore, we compared the effect of AR stimulation on ErbB3 in LNCaP and LNCaP-AI cells. Increasing doses of DHT in LNCaP stimulated AR, as well as Nrdp1

protein and mRNA levels, but suppressed ErbB3 (Fig. 5B, top left). However, in LNCaP-AI cells, DHT did not affect Nrdp1 or ErbB3 levels (Fig. 5B, top right), although AR activity increased (Fig. 5B, bottom). Our results showed that the AR regulates the levels of Nrdp1, and, as a consequence that of ErbB3 expression, in LNCaP cells, but not in LNCaP-AI.

Both LNCaP-AI and C4-2 expressed higher ErbB3 and lower levels of Nrdp1 compared with LNCaP cells. In addition, overexpression of ErbB3 in LNCaP cells also decreased Nrdp1 (Fig. 5C, top). We conclude that ErbB3 overexpression suppressed Nrdp1 by an AR-independent mechanism, which prevented AR-mediated Nrdp1 transcription, underscored by the observation that AR-independent Nrdp1 decrease by short hairpin RNA prevented stimulation by DHT (Fig. 5C, bottom). Thus, despite increased AR, inability

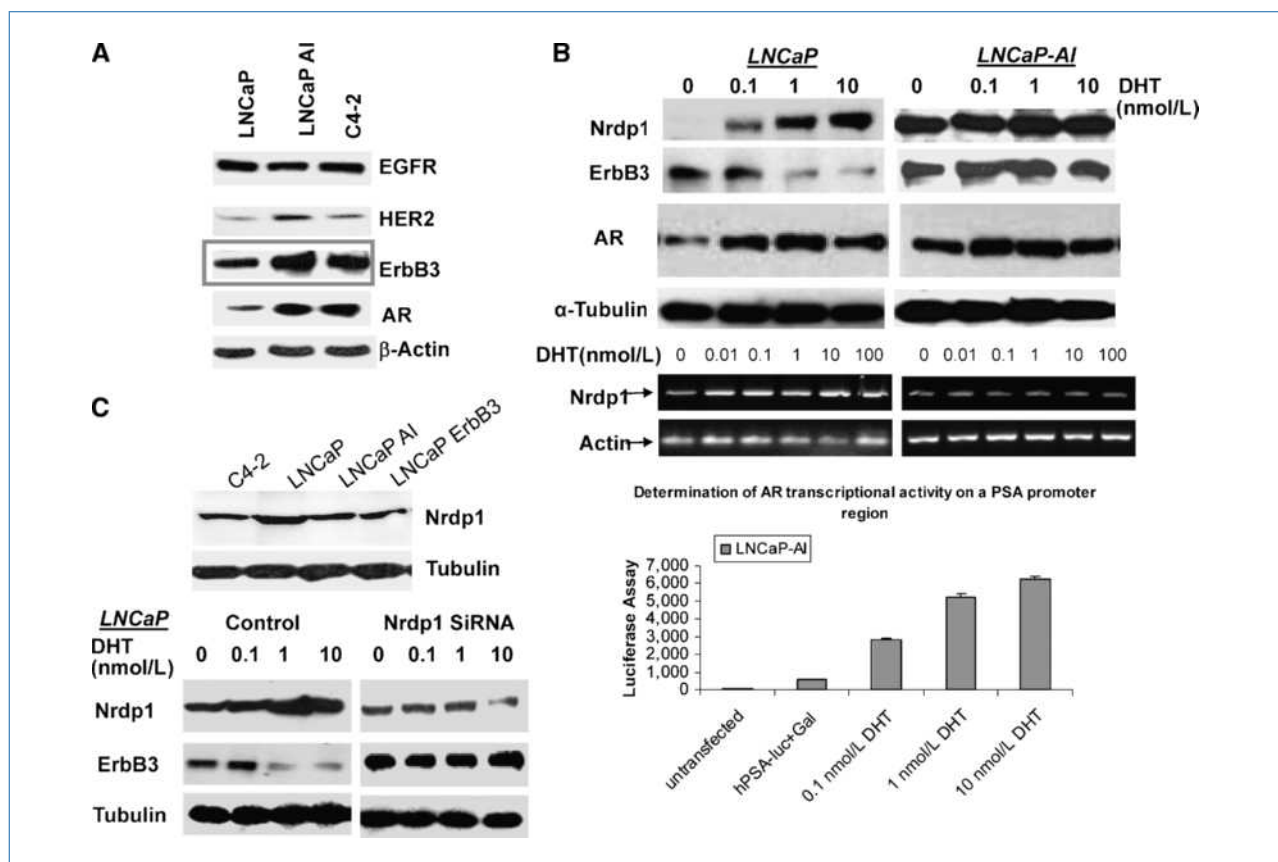


Figure 5. AR regulation of ErbB3 and Nrdp1 is not seen in CRPC cells with high AR transcriptional activity. A, expression of EGFR, HER2, ErbB3, and AR were compared in androgen-dependent LNCaP cells, and its CRPC sublines LNCaP-AI and C4-2 cells by Western blotting, and confirms overexpression of ErbB3 and AR in the CRPC lines. B, stimulation of AR expression with increasing DHT increases Nrdp1 and decreases ErbB3 in LNCaP but not LNCaP-AI. Top, LNCaP and LNCaP AI cells were cultured in CSS-containing medium overnight, then the indicated concentrations of DHT were added for another 48 h. Cell lysates were immunoblotted with anti-ErbB3, anti-Nrdp1, anti-AR, and anti-tubulin antibodies. Middle, AR increases Nrdp1 transcription in LNCaP but not LNCaP AI cells as determined from mRNA levels by RT-PCR after treatment with increasing doses of DHT. Bottom, this is despite increased AR transcriptional activity on a human prostate-specific antigen (PSA) promoter as determined by luciferase assay upon DHT stimulation in LNCaP-AI cells. C, AR fails to regulate Nrdp1 and ErbB3 levels in CRPC cells due to decreased Nrdp1 levels caused by ErbB3 overexpression. Top, suppression of Nrdp1 expression in LNCaP AI, C4-2, and LNCaP-ErbB3 cells compared with LNCaP. Cells were cultured in FBS medium up to 75% confluence and collected for Western blotting. Bottom, DHT-induced suppression of ErbB3 levels in LNCaP cells is mediated by high levels of Nrdp1. This effect was not seen when LNCaP cells were transfected with Nrdp1 siRNA (in CSS) for 24 h, followed by DHT treatment for another 48 h. Cell lysate was collected and blotted with anti-ErbB3 and anti-Nrdp1.

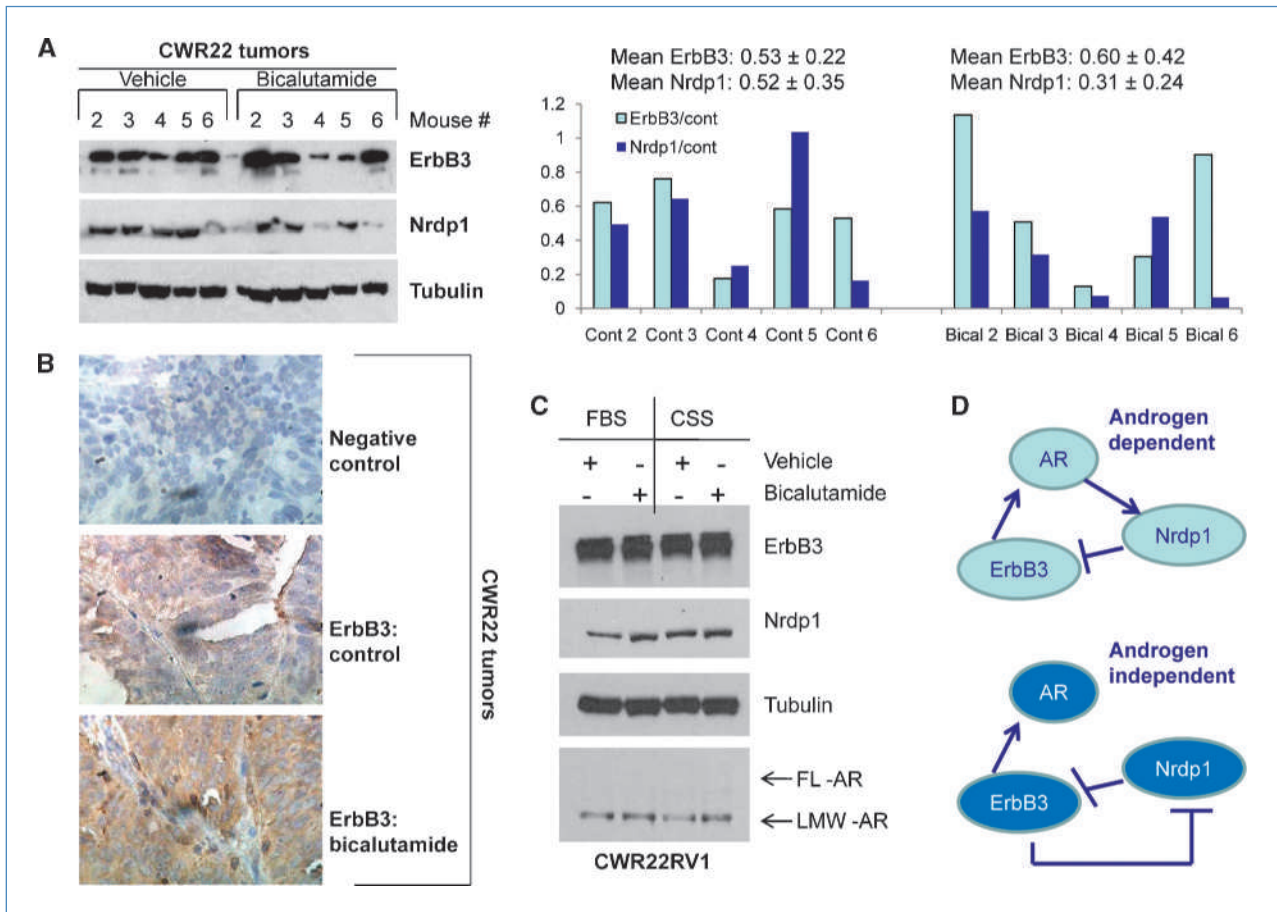


Figure 6. Effect of AR activity on ErbB3 and Nrdp1 expression seen in castration-sensitive CWR22 tumors but not in CRPC cell lines derived from recurrent tumors from castrated mice. **A** and **B**, CWR22 xenograft tumors were established by s.c. injections of cell suspensions (2.5×10^6 cells in Matrigel; 1:1, v/v) bilaterally into the flanks of 4- to 5-wk-old *nu/nu* athymic male mice ($n = 10$) previously implanted with sustained release testosterone pellets. When palpable tumors were observed, animals were treated with (a) vehicle (peanut oil) or (b) the AR antagonist bicalutamide ($n = 5$ /group). After 2 wk on this treatment, the mice were euthanized, and tumors were harvested and divided into sections that were paraffin embedded and snap frozen in liquid nitrogen. Paraffin-embedded tumors were analyzed by immunohistochemistry for ErbB3, whereas frozen tumors were excised and lysed, and protein levels were determined by Western blotting. **A**, Western blotting revealed that ErbB3 levels increased whereas Nrdp1 levels decreased with bicalutamide treatment. **B**, immunohistochemistry showing that ErbB3 levels increased in tumors extracted from the bicalutamide-fed mice. Brown staining, ErbB3; blue staining, counterstaining with hematoxylin. Negative control showed no ErbB3 staining; the xenograft from the vehicle-fed mouse showed lower ErbB3 expression (scored +1) compared with the bicalutamide-treated one (scored +3). **C**, in contrast, CWR22Rv1, a CRPC cell line derived from a CWR22-relapsed tumor grown in a castrated mouse, failed to respond to bicalutamide, and in these cells, Nrdp1 and ErbB3 were not androgen regulated. **D**, scheme describing androgen regulation of Nrdp1 transcription in androgen-dependent but not androgen-independent cells.

of AR to regulate Nrdp1 prolonged ErbB3 half-life in LNCaP-AI (Supplementary Fig. S4B).

Androgen regulation of ErbB3 and Nrdp1 in androgen-dependent CWR22 tumors but not in CRPC CWR22-Rv1 cells

We also tested the effect of AR on Nrdp1 and ErbB3 in androgen-dependent CWR22 tumors in nude mice (34) and in CRPC CWR22Rv1 cells, derived from a relapsed CWR22 tumor (35). CWR22 xenograft tumors were cultivated in the flanks of athymic *nu/nu* mice treated with vehicle or 50 mg/kg bicalutamide (an AR antagonist). Bicalutamide did not affect AR expression but severely decreased AR transcriptional activity in androgen-sensitive cells (32, 36).

After 2 weeks, the mice were euthanized and the tumors were extracted, part was paraffin-embedded, and the rest were frozen. ErbB3 levels determined by Western blot (Fig. 6A) reflected only the epithelial cells, as immunohistochemical studies revealed ErbB3 staining only in epithelial cells (Fig. 6B) and showed higher ErbB3 in bicalutamide-treated animals both by immunoblotting and immunohistochemistry, whereas Nrdp1 levels were significantly suppressed. These results indicate that ErbB3 and Nrdp1 levels were regulated by AR in CWR22 tumors as well. However, in CRPC CWR22Rv1 cells, which express very low levels of Nrdp1 (Supplementary Fig. S3C), neither bicalutamide nor culture in CSS medium affected either Nrdp1 or ErbB3 (Fig. 6C). These results show that, similar

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to LNCaP and PC-346C cells, AR regulates Nrdp1 and ErbB3 in androgen-dependent CWR22 PCa cells but not in its CRPC subline CWR22Rv1.

Discussion

Men who undergo AW therapy are known to become refractory to this therapy, although investigators continue to debate the causes leading to the development of castration resistance. The present study identifies ErbB3 as a likely mediator of increased proliferation during CRPC development, which releases cells from cell cycle arrest imposed by AW therapy, because increased ErbB3 resulted in enhanced proliferation both in the presence and the absence of androgens. We show that ErbB3 is increased during AW, and that this is caused by a novel mechanism involving the negative regulation of ErbB3 by the AR. In androgen-dependent cells, AR suppresses ErbB3, likely because although the AR promotes androgen-dependent cell cycle progression, it also resists the activation of androgen-independent pathways that promote cell growth. AW therapy relieves this suppression, thereby elevating ErbB3 levels, which stimulate proliferation and likely promote resistance to this therapy. Our data identifies the inability of the AR to suppress ErbB3 expression as one cause of CRPC.

The case for ErbB3 as a mediator of cell cycle release is strengthened by previous studies emphasizing the importance of HER2 in the development of castration resistance (37–45) and by the identification of ErbB3, rather than EGFR, as a major binding partner for HER2 in PCa (5, 14, 15). Reports described transcriptional as well as posttranslational increase of HER2 during AW (46); hence, the major binding partner of HER2 in CRPC would also be expected to increase. Our study shows that the AR regulates ErbB3 levels in PCa by promoting its degradation, whereas during AW, ErbB3 levels are increased due to decreased degradation.

We show that the AR promotes ErbB3 degradation by regulating Nrdp1 transcription. Importantly, we show that the AR transcriptionally regulates Nrdp1 expression in androgen-dependent but not in CRPC cells. Nrdp1 was originally identified as a RING finger domain-containing protein that interacts with ErbB3 (18, 47), binding to the cytoplasmic tail of ErbB3, and promoting its degradation by mediating ligand-independent ubiquitination. Nrdp1 was expressed in multiple androgen-regulated tissues including prostate, testicles (47), and skeletal muscles (18). Our data showed an inverse relationship between ErbB3 and Nrdp1 in androgen-dependent PCa cells. Nrdp1 promotes ErbB3 degradation (19, 47), and we show that it mediates AR regulation of ErbB3 half-life. These results indicate for the first time that Nrdp1 is a transcriptional target of the AR.

References

1. Agus DB, Cordon-Cardo C, Fox W, et al. Prostate cancer cell cycle regulators: response to androgen withdrawal and development of androgen independence. *J Natl Cancer Inst* 1999;91:1869–76.
2. Robinson D, He F, Pretlow T, Kung HJ. A tyrosine kinase profile of prostate carcinoma. *Proc Natl Acad Sci U S A* 1996;93:5958–62.
3. Grasso AW, Wen D, Miller CM, Rhim JS, Pretlow TG, Kung HJ. ErbB kinases and NDF signaling in human prostate cancer cells. *Oncogene* 1997;15:2705–16.
4. Olayioye MA, Neve RM, Lane HA, Hynes NE. The ErbB signaling network: receptor heterodimerization in development and cancer. *EMBO J* 2000;19:3159–67.

We also show that AR regulation of Nrdp1 and ErbB3 is lost in CRPC cells, thereby maintaining elevated ErbB3 in CRPC cells despite high AR activity. Our data show that ErbB3 overexpression suppressed Nrdp1 levels and likely prevented AR regulation of Nrdp1 transcription. This indicates androgen-dependent regulation of Nrdp1 in cells with high Nrdp1 and low ErbB3, and androgen independence of Nrdp1 transcription in cells expressing high ErbB3 and low Nrdp1. Therefore, our data explain why intermittent AW therapy, in which the effects of AW on ErbB3 may be reversed during frequent “off-cycle” phases, while Nrdp1 transcription is still AR regulated, may prolong androgen dependence (48), and indicates Nrdp1 regulation by AR as a hallmark of androgen-sensitive cell growth.

In summary, this study shows that induction of ErbB3 occurs as a result of AR inactivation during AW. However, even after AR levels rebound, ErbB3 remains elevated, eventually promoting castration-resistant proliferation of PCa cells. We show that Nrdp1 mediates the regulation of ErbB3 levels by the AR in androgen-dependent cells and is itself transcriptionally regulated by the AR, whereas this regulation is lost in AR-positive CRPC cells, likely due to high ErbB3 levels caused by AW therapy that may have suppressed Nrdp1 levels in an androgen-independent manner (Fig. 6D). These studies point to the importance of ErbB3 in PCa progression and identify it as a possible target of therapy for prevention of CRPC development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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5. Qiu Y, Ravi L, Kung HJ. Requirement of ErbB2 for signalling by interleukin-6 in prostate carcinoma cells. *Nature* 1998;393:83–5.
6. Lin J, Adam RM, Santiestevan E, Freeman MR. The phosphatidylinositol 3'-kinase pathway is a dominant growth factor-activated cell survival pathway in LNCaP human prostate carcinoma cells. *Cancer Res* 1999;59:2891–7.
7. Chaib H, Cockrell EK, Rubin MA, Macoska JA. Profiling and verification of gene expression patterns in normal and malignant human prostate tissues by cDNA microarray analysis. *Neoplasia* 2001;3:43–52.
8. Koumakpayi IH, Diallo JS, Le Page C, et al. Expression and nuclear localization of ErbB3 in prostate cancer. *Clin Cancer Res* 2006;12:2730–7.
9. Lyne JC, Melhem MF, Finley GG, et al. Tissue expression of neu differentiation factor/hergulin and its receptor complex in prostate cancer and its biologic effects on prostate cancer cells *in vitro*. *Cancer J Sci Am* 1997;3:21–30.
10. Li Z, Szabolcs M, Terwilliger JD, Efstratiadis A. Prostatic intraepithelial neoplasia and adenocarcinoma in mice expressing a probasin-Neu oncogenic transgene. *Carcinogenesis* 2006;27:1054–67.
11. Srinivasan R, Leverton KE, Sheldon H, Hurst HC, Sarraf C, Gullick WJ. Intracellular expression of the truncated extracellular domain of c-erbB-3/HER3. *Cell Signal* 2001;13:321–30.
12. Lee H, Akita RW, Sliwkowski MX, Maihle NJ. A naturally occurring secreted human ErbB3 receptor isoform inhibits heregulin-stimulated activation of ErbB2, ErbB3, and ErbB4. *Cancer Res* 2001;61:4467–73.
13. Koumakpayi IH, Diallo JS, Le Page C, et al. Low nuclear ErbB3 predicts biochemical recurrence in patients with prostate cancer. *BJU Int* 2007;100:303–9.
14. Mellingerhoff IK, Vivanco I, Kwon A, Tran C, Wongvipat J, Sawyers CL. HER2/neu kinase-dependent modulation of androgen receptor function through effects on DNA binding and stability. *Cancer Cell* 2004;6:517–27.
15. Gregory CW, Whang YE, McCall W, et al. Heregulin-induced activation of HER2 and HER3 increases androgen receptor transactivation and CWR-R1 human recurrent prostate cancer cell growth. *Clin Cancer Res* 2005;11:1704–12.
16. Zhang Y, Hamburger AW. Specificity and heregulin regulation of Ebp1 (ErbB3 binding protein 1) mediated repression of androgen receptor signalling. *Br J Cancer* 2005;92:140–6.
17. Zhang Y, Wang XW, Jelovac D, et al. The ErbB3-binding protein Ebp1 suppresses androgen receptor-mediated gene transcription and tumorigenesis of prostate cancer cells. *Proc Natl Acad Sci U S A* 2005;102:9890–5.
18. Diamonti AJ, Guy PM, Ivanof C, Wong K, Sweeney C, Carraway KL, III. An RBCC protein implicated in maintenance of steady-state heregulin receptor levels. *Proc Natl Acad Sci U S A* 2002;99:2866–71.
19. Cao Z, Wu X, Yen L, Sweeney C, Carraway KL, III. Neuregulin-induced ErbB3 downregulation is mediated by a protein stability cascade involving the E3 ubiquitin ligase Nrdp1. *Mol Cell Biol* 2007;27:2180–8.
20. Yen L, Cao Z, Wu X, et al. Loss of Nrdp1 enhances ErbB2/ErbB3-dependent breast tumor cell growth. *Cancer Res* 2006;66:11279–86.
21. Rubin MA, Putzi M, Mucci N, et al. Rapid ("warm") autopsy study for procurement of metastatic prostate cancer. *Clin Cancer Res* 2000;6:1038–45.
22. Shah RB, Mehra R, Chinnaiyan AM, et al. Androgen-independent prostate cancer is a heterogeneous group of diseases: lessons from a rapid autopsy program. *Cancer Res* 2004;64:9209–16.
23. Bedolla RG, Wang Y, Asuncion A, et al. Nuclear versus cytoplasmic localization of filamin A in prostate cancer: immunohistochemical correlation with metastases. *Clin Cancer Res* 2009;15:788–96.
24. Shi XB, Xue L, Tepper CG, et al. The oncogenic potential of a prostate cancer-derived androgen receptor mutant. *Prostate* 2007;67:591–602.
25. Mikhailova M, Wang Y, Bedolla R, Lu XH, Kreisberg JI, Ghosh PM. AKT regulates androgen receptor-dependent growth and PSA expression in prostate cancer. *Adv Exp Med Biol* 2008;617:397–405.
26. van Weerden WM, de Ridder CM, Verdaasdonk CL, et al. Development of seven new human prostate tumor xenograft models and their histopathological characterization. *Am J Pathol* 1996;149:1055–62.
27. Limpens J, Schroder FH, de Ridder CM, et al. Combined lycopene and vitamin E treatment suppresses the growth of PC-346C human prostate cancer cells in nude mice. *J Nutr* 2006;136:1287–93.
28. Vijapurkar U, Cheng K, Koland JG. Mutation of a Shc binding site tyrosine residue in ErbB3/HER3 blocks heregulin-dependent activation of mitogen-activated protein kinase. *J Biol Chem* 1998;273:20996–1002.
29. Brinkmann AO, Blok LJ, de Ruiter PE, et al. Mechanisms of androgen receptor activation and function. *J Steroid Biochem Mol Biol* 1999;69:307–13.
30. Wu X, Yen L, Irwin L, Sweeney C, Carraway KL, III. Stabilization of the E3 ubiquitin ligase Nrdp1 by the deubiquitinating enzyme USP8. *Mol Cell Biol* 2004;24:7748–57.
31. Ghosh PM, Malik SN, Bedolla RG, et al. Signal transduction pathways in androgen-dependent and -independent prostate cancer cell proliferation. *Endocr Relat Cancer* 2005;12:119–34.
32. Wang Y, Mikhailova M, Bose S, Pan CX, deVere White RW, Ghosh PM. Regulation of androgen receptor transcriptional activity by rapamycin in prostate cancer cell proliferation and survival. *Oncogene* 2008;27:7106–17.
33. Hsieh JT, Wu HC, Gleave ME, von Eschenbach AC, Chung LW. Autocrine regulation of prostate-specific antigen gene expression in a human prostatic cancer (LNCaP) subline. *Cancer Res* 1993;53:2852–7.
34. Nagabhushan M, Miller CM, Pretlow TP, et al. CWR22: the first human prostate cancer xenograft with strongly androgen-dependent and relapsed strains both *in vivo* and in soft agar. *Cancer Res* 1996;56:3042–6.
35. Tepper CG, Boucher DL, Ryan PE, et al. Characterization of a novel androgen receptor mutation in a relapsed CWR22 prostate cancer xenograft and cell line. *Cancer Res* 2002;62:6606–14.
36. Wang Y, Kreisberg JI, Bedolla RG, Mikhailova M, deVere White RW, Ghosh PM. A 90 kDa fragment of filamin A promotes Casodex-induced growth inhibition in Casodex-resistant androgen receptor positive C4-2 prostate cancer cells. *Oncogene* 2007;26:6061–70.
37. Dankort D, Jeyabalan N, Jones N, Dumont DJ, Muller WJ. Multiple ErbB-2/Neu phosphorylation sites mediate transformation through distinct effector proteins. *J Biol Chem* 2001;276:38921–8.
38. Klapper LN, Waterman H, Sela M, Yarden Y. Tumor-inhibitory antibodies to HER-2/ErbB-2 may act by recruiting c-Cbl and enhancing ubiquitination of HER-2. *Cancer Res* 2000;60:3384–8.
39. Myers RB, Brown D, Oelschlagel DK, et al. Elevated serum levels of p105(erbB-2) in patients with advanced-stage prostatic adenocarcinoma. *Int J Cancer* 1996;69:398–402.
40. Osman I, Scher HI, Drobnjak M, et al. HER-2/neu (p185neu) protein expression in the natural or treated history of prostate cancer. *Clin Cancer Res* 2001;7:2643–7.
41. Oxley JD, Winkler MH, Gillatt DA, Peat DS. Her-2/neu oncogene amplification in clinically localised prostate cancer. *J Clin Pathol* 2002;55:118–20.
42. Signoretti S, Montironi R, Manola J, et al. Her-2-neu expression and progression toward androgen independence in human prostate cancer. *J Natl Cancer Inst* 2000;92:1918–25.
43. Wen Y, Hu MC, Makino K, et al. HER-2/neu promotes androgen-independent survival and growth of prostate cancer cells through the Akt pathway. *Cancer Res* 2000;60:6841–5.
44. Yeh S, Lin HK, Kang HY, Thin TH, Lin MF, Chang C. From HER2/Neu signal cascade to androgen receptor and its coactivators: a novel pathway by induction of androgen target genes through MAP kinase in prostate cancer cells. *Proc Natl Acad Sci U S A* 1999;96:5458–63.
45. Zhou BP, Hu MC, Miller SA, et al. HER-2/neu blocks tumor necrosis factor-induced apoptosis via the Akt/NF- κ B pathway. *J Biol Chem* 2000;275:8027–31.
46. Berger R, Lin DI, Nieto M, et al. Androgen-dependent regulation of Her-2/neu in prostate cancer cells. *Cancer Res* 2006;66:5723–8.
47. Qiu XB, Goldberg AL. Nrdp1/FLRF is a ubiquitin ligase promoting ubiquitination and degradation of the epidermal growth factor receptor family member, ErbB3. *Proc Natl Acad Sci U S A* 2002;99:14843–8.
48. Abrahamsson PA. Potential benefits of intermittent androgen suppression therapy in the treatment of prostate cancer: a systematic review of the literature. *Eur Urol* 57:49–59.