

Androgen Deprivation Increases p300 Expression in Prostate Cancer Cells

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Abstract

Standard therapy for nonorgan confined prostate cancer aims to block the production or action of androgens. Although initially successful, antiandrogen therapy eventually fails and androgen depletion independent (ADI) disease emerges. Remarkably, ADI prostate cancers still rely on a functional androgen receptor (AR). Aberrant expression of coregulatory proteins required for the formation of productive AR transcriptional complexes is critical for ADI AR activation. Previously, we have shown that the transcriptional coactivator p300 is required for ADI activation of the AR and is up-regulated in prostate cancer, in which its expression is associated with cell proliferation and predicts aggressive tumor features. The mechanism responsible for the deregulated expression of p300, however, remains elusive. Here, we show that p300 expression in prostate cancer cells is subject to androgen regulation. In several prostate cancer model systems, addition of synthetic and natural androgens led to decreased expression of p300 in a time-dependent and dose-dependent manner. Experiments using AR antagonists or small interfering RNA targeting the AR revealed that down-regulation of p300 depends entirely on the presence of a functional AR. It is noteworthy that androgens down-regulated p300 protein expression while leaving messenger levels unaltered. Conversely, both short-term and long-term androgen deprivation resulted in marked up-regulation of p300 expression. The androgen deprivation-induced increase in p300 expression was not affected by the addition of cytokines or growth factors or by cotreatment with antiandrogens. Moreover, increased p300 expression upon androgen starvation is crucial for prostate cancer cell proliferation, as loss of p300 expression severely reduces expression of cyclins governing G₁-S and G₂-M cell cycle transition and decreases 5-bromo-2'-deoxyuridine incorporation. [Cancer Res 2007;67(7):3422–30]

Introduction

Prostate adenocarcinoma cells depend on androgens for their survival. For several decades, this characteristic has been exploited for therapeutic intervention in locally advanced and metastatic prostate cancers, which can no longer be treated efficiently by surgery or radiation therapy. Androgen ablation

therapy either targets the production of androgens by means of surgical or medical castration, interferes with the activity of androgens by administration of androgen receptor (AR) antagonists that inhibit proper functioning of the AR, or involves a combination of both of these approaches (1). Although androgen ablation therapy is initially successful in the majority of cases leading to a decrease in tumor size and a favorable clinical response, eventually most tumors will find a way to circumvent this form of treatment and start to grow again. At this stage of the disease called androgen depletion independent (ADI) prostate cancer, treatment options are limited and have palliative rather than curative intent (2).

Remarkably, despite the castrate levels of circulating androgens in these patients, a growing body of evidence points toward the AR as a critical determinant for ADI prostate cancer cell proliferation and an attractive target for therapeutic intervention in ADI prostate cancer. The unexpected "reactivation" of the AR in ADI prostate cancer cells has been attributed to mechanisms of AR hypersensitivity (AR amplification and/or mutations), promiscuous activation of the AR (by adrenal androgens, nonandrogenic steroids, and even antiandrogens), and outlaw AR pathways (AR activated by growth factors and cytokines, thereby bypassing the need for androgens). More recently, the importance of the involvement of AR coactivator proteins in ADI AR activation is increasingly being recognized (3–7). Under normal physiologic conditions, coactivators are necessary for the formation of a productive transcriptional AR complex by facilitating DNA occupancy, chromatin remodeling, recruitment of general transcription factors associated with the RNA polymerase II holo-complex, as well as ensuring appropriate folding of the AR, AR protein stability, and/or proper AR subcellular distribution (8). In the progression of prostate cancer, a subset of these coactivators has been shown to be overexpressed; and this overexpression has been shown to substantially contribute to the ADI mechanisms of AR activation described above. Therefore, overexpression of AR coactivators has been suggested as a valuable target for therapeutic intervention (7). What might be driving the increase in expression of these critical cofactors in prostate cancer disease progression, however, remains elusive.

Here, we investigate the regulation of the expression of p300, an AR coactivator that we have previously shown to be required for ADI AR activity and to be overexpressed in prostate cancer cells, in which its expression is associated with prostate cancer cell proliferation and predicts aggressive tumor features, during prostate cancer progression (9, 10). We show that p300 expression is up-regulated by androgen deprivation of prostate cancer cells. These findings suggest that by inducing changes in the expression of AR coactivators, androgen deprivation treatment itself ensures aberrant AR activity in and aggressive behavior of ADI prostate cancer cells.

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Materials and Methods

Cell culture. LNCaP, DU145, and PC-3 cells were purchased from American Type Culture Collection (Manassas, VA). LNCaP and PC-3 cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 9% fetal bovine serum (FBS; Biosource, Rockville, MD), 100 units/mL streptomycin, 100 units/mL streptomycin, and 0.25 $\mu\text{g}/\text{mL}$ amphotericin B (Fungizone; Invitrogen). DU145 cells were maintained in DMEM medium (Invitrogen) supplemented with 9% FBS, 100 units/mL streptomycin, 100 units/mL streptomycin, and 0.25 $\mu\text{g}/\text{mL}$ amphotericin B. The ADI LNCaP cell line LNCaP-Rf was generated and maintained as described previously (11). LAPC-4 cells were a kind gift from Dr. Sawyers and were cultured as described (12). In experiments designed to assess the effects of androgen treatment, LNCaP, PC-3, and DU-145 cells were seeded in medium containing 9% charcoal-stripped serum (CSS), 100 units/mL streptomycin, 100 units/mL streptomycin, and 0.25 $\mu\text{g}/\text{mL}$ amphotericin B. For small interfering RNA (siRNA) transfection studies, antibiotics were left out of the culture medium.

Reagents. Methyltrienolone (R1881) was purchased from DuPont (Boston, MA). Casodex was a kind gift from Zeneca Pharmaceuticals (Wilmington, DE). Mibolerone, lactacystin, MG132, ubiquitin aldehyde, and IKK-NBD peptide was obtained from Biomol (Plymouth Meeting, PA). Mifeprestone (RU486), insulin-like growth factor I (IGF-I), epidermal growth factor (EGF), and staurosporine was purchased from Sigma (St. Louis, MO). Hydroxyflutamide was obtained from Schering (Bloomfield, NJ). Interleukin 6 (IL-6) was purchased from R&D Systems (Minneapolis, MN). Antibodies were obtained from different manufacturers: p300, extracellular signal-regulated kinase 2 (ERK2), β -tubulin, cyclin E, and AR from Santa Cruz Biotechnology (Santa Cruz, CA), phosphorylated signal transducers and activators of transcription 3 (pSTAT3) from Upstate (Temecula, CA), β -actin, ubiquitin (P4D1), poly(ADP-ribose)polymerase (PARP), and bcl-2 from Cell Signaling (Beverly, MA), cyclin A (Ab-5) and cyclin D1 (DSC-6) from NeoMarkers (Fremont, CA), cyclin B from BD Transduction Laboratories (San Jose, CA), and fatty acid synthase (FAS) from Novus Biologicals (Littleton, CO).

Preparation of whole-cell lysates. Cells were washed twice with ice-cold PBS on ice. Whole-cell lysis buffer [10 mmol/L HEPES (pH 7.0), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% NP40, Complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany)] was pipetted onto the culture dish. Cells were incubated with this buffer for 1 min on ice before cells were harvested by scraping. The collected lysate was centrifuged at 13,000 rpm in a microcentrifuge for 10 min at 4°C. The supernatant was aliquoted and stored at -80°C .

Western blotting. Equal amounts of protein were loaded onto NuPage 3–8% Tris-acetate or 10% Bis-Tris gels (Invitrogen), and electrophoresis was done according to the manufacturer's instructions. Proteins were blotted onto nitrocellulose membranes (Bio-Rad, Hercules, CA). Blots were reprobed with antibodies against β -actin or β -tubulin to evaluate potential differences in protein loading.

siRNA transfection. LNCaP cells were seeded at a density of 1.6×10^6 in 100-mm culture dishes in phenol red-free RPMI 1640 medium supplemented with 9% FBS. The next day, cells were transfected with 500 pmol siGenome SMART pool directed against p300 or AR (Dharmacon, Lafayette, CO), 500 pmol of a nontargeting control SMART pool or mock transfected using Lipofectamine 2000 reagent (Invitrogen) following manufacturer's instructions. In experiments assessing the effects of androgens 36 to 48 h after transfection, medium was removed and cells were washed once with medium containing 9% CSS. Fresh medium supplemented with 9% CSS was added to the cells, and cells were treated with androgens or ethanol vehicle for the indicated periods of time.

RNA isolation. LNCaP cells were seeded at a density of 5×10^5 in 60-mm dishes in phenol red-free RPMI 1640 medium supplemented with 9% CSS. Two days later, medium was changed and cells were treated with 1 nmol/L R1881 or ethanol vehicle. At indicated times after treatment, cells were washed with PBS and harvested in Trizol solution (Invitrogen). RNA was isolated according to the manufacturer's instructions.

Real-time reverse transcription-PCR. cDNA was prepared from 3 μg total RNA using the SuperScriptIII first strand synthesis system (Invitrogen)

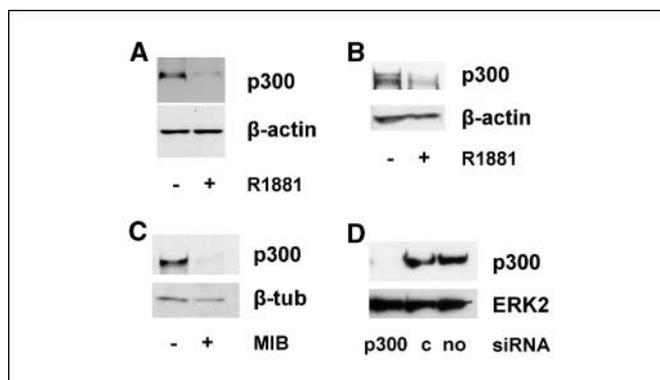


Figure 1. Androgens down-regulate p300 expression in prostate cancer cells. LNCaP cells were seeded in medium supplemented with CSS. Two days later, medium was changed and cells were treated with 5 nmol/L of the synthetic androgens R1881 (A) or mibolerone (MIB; C) or ethanol vehicle for 72 h. Total protein extracts were prepared, and equal amounts of protein were analyzed by Western blotting using an antibody directed against p300. B, LAPC-4 cells were seeded in complete medium. Two days later, cells were washed once with CSS medium, fresh CSS medium was added, and cells were grown in the presence or absence of 5 nmol/L R1881. Seventy-two hours later, cells were harvested and p300 expression was analyzed as described above. D, to confirm specificity of the p300 antibody, LNCaP cells were transfected with siRNAs targeting p300 or control siRNAs (c) or a mock transfection (no siRNA, no) was done. Three days later, total protein extracts were prepared and p300 expression was evaluated. To assess potential intersample loading differences, blots were stripped and reprobed with antibodies recognizing β -tubulin (β -*tub*), β -actin, or ERK2.

following the manufacturer's instructions. Real-time reverse transcription-PCR (RT-PCR) was done using SYBR Green PCR mastermix (Applied Biosystems, Foster City, CA) on an ABI Prism 7700 SDS instrument as described (13).

Cell viability assay. LNCaP and LNCaP-Rf cells were seeded in 96-well tissue culture plates at a density of 4.5 or 6×10^4 per well, respectively, in their regular medium without added antibiotics. The next day, cells were transfected with siGenome SMART pools directed against p300 or a control SMART pool as described. Twelve to 16 h later, medium was changed. At indicated time points, cell viability was assessed by means of a Cell Titer 96 Aquous One solution cell proliferation assay (Promega, Madison, WI) according to the manufacturer's instructions. Values from five wells were measured per treatment group for each time point.

5-Bromo-2'-deoxyuridine incorporation assay. LNCaP-Rf cells were seeded in 96-well tissue culture plates at a density of 6×10^4 per well in medium without added antibiotics. On the next day, cells were transfected with p300 or control siRNAs as described. Twelve to 16 h later, medium was replaced. At indicated time points, cell proliferation was assessed by performing a cell proliferation ELISA (Roche) according to the manufacturer's instructions. Values from five wells were obtained for each treatment group at each time point.

Immunoprecipitation assay. LNCaP cells were seeded in 20-mm dishes at a density of 3×10^6 in RPMI 1640 medium supplemented with 9% CSS. Three days later, medium was changed and cells were treated with 1 nmol/L R1881 or ethanol vehicle. After 45 h of treatment, cells were washed twice on ice with ice-cold PBS and scraped in 1 mL of whole-cell lysis buffer (described above). The lysate was centrifuged at 13,000 rpm in a microcentrifuge for 10 min at 4°C. The supernatant was cleared by gentle agitation with protein G plus agarose (Santa Cruz) at 4°C for 2 h. At that time, the suspension was centrifuged for 10 min at 2,500 rpm at 4°C. The supernatant was transferred to a fresh microcentrifuge tube, 4 μg of antibody (directed against p300 or ubiquitin or nonspecific rabbit IgG) was added, and the mixture was subjected to gentle agitation for 2 h at 4°C. Next, 25 μL of protein G plus agarose slurry was added, and the suspension was shaken for another hour. The suspension was then centrifuged at 2,500 rpm for 10 min at 4°C. The antigen-antibody-agarose pellet was washed with ice-cold PBS and centrifuged for 5 min at 2,500 rpm at 4°C. Washing was repeated two more times. The final pellet was resuspended in 25 μL of

loading buffer 2× [17% glycerol, 8% β-mercaptoethanol, 5% SDS, 0.2 mol/L Tris-HCl (pH 6.7)] and samples were stored at −80°C until Western blot analysis.

Results and Discussion

Androgens down-regulate p300 expression in prostate cancer cells. To explore whether changes in androgen concentrations can affect p300 expression in prostate cancer cells, LNCaP prostate adenocarcinoma cells were cultured in the presence or absence of the synthetic androgen R1881 (methyltrienolone) for 3 days. Whole cell lysates were prepared, and p300 expression levels were evaluated by immunoblotting. As shown in Fig. 1A, androgen exposure led to a marked decrease in p300 expression. Similar effects were observed in a second independent androgen-responsive prostate cancer cell line, LAPC-4 (Fig. 1B). Unlike the LNCaP cell line, which expresses an AR that has a mutation in its ligand-binding domain, LAPC-4 cells express a wild-type AR. The data shown in Fig. 1A and B indicate that androgen-induced down-regulation of p300 expression is not a peculiarity of one particular cell line but represents a more general consequence of androgen action in prostate cancer cells. Moreover, similar suppressive effects on p300 expression were noted when cells were treated with another synthetic androgen mibolerone (Fig. 1C) as well as with the natural androgen dihydrotestosterone (data not shown). As a control for the specificity of the p300 antibody used, LNCaP cells were transfected with siRNA directed against p300 and total protein extracts were analyzed by Western blotting. Confirming the suitability of the antibody used, siRNA-mediated targeting of p300 expression led to a loss of the p300 immunoreactive signal in LNCaP cells. The use of nontargeting control siRNAs or mock transfection of the cells did not affect p300 expression (Fig. 1D).

To better understand this phenomenon, we set out to further characterize the androgen-mediated down-regulation of p300 expression in prostate cancer cells. To this end, a time course was done in which LNCaP cells were treated for up to 72 h in

the presence or the absence of R1881. These studies showed that the androgen-induced decrease in p300 expression is first seen 24 h after treatment and becomes more pronounced after longer periods of treatment with a maximum effect reached at 72 h (Fig. 2A). Dose-response studies using R1881 concentrations ranging from 1 pmol/L to 1 μmol/L showed a R1881 concentration of 1 nmol/L is required for androgens to start repressing p300 expression. Higher R1881 concentrations further suppressed p300 expression, whereas concentrations of 0.1 nmol/L or lower induced a slight increase in p300 levels (Fig. 2B). These findings of elevated p300 expression at androgen concentrations that are associated with LNCaP cell proliferation are consistent with earlier observations in our laboratory linking p300 expression to prostate cancer cell proliferation (10). To assess the involvement of the AR in the observed effects, LNCaP cells were treated with R1881 in the absence or presence of an excess of the antiandrogen Casodex (bicalutamide). Addition of Casodex alone did not notably affect p300 expression in LNCaP cells. In combination with R1881 (1 nmol/L) however, Casodex (10 μmol/L) counteracted the down-regulation of p300 (Fig. 2C). Similar effects were observed when AR expression was targeted. siRNA-mediated loss of AR expression prevented the androgen-mediated suppression of p300 expression (Fig. 2D). As a control for androgen responsiveness of the cells, the expression of FAS, a well-known target gene for androgen action in prostate cancer cells (14), was monitored. As expected, knocking down AR levels prevented androgens from stimulating FAS expression. The use of nontargeting control siRNAs or mock transfection of cells did not affect androgen regulation of either p300 or FAS expression (Fig. 2D). Consistent with the findings described in Fig. 2C and D, androgen treatment of the AR-negative prostate cancer cells lines PC-3 or DU-145 had no effect on p300 expression (data not shown).

Androgens down-regulate p300 protein expression but do not affect p300 mRNA levels. To gain more insight into the molecular mechanism(s) underlying androgen-induced down-regulation of

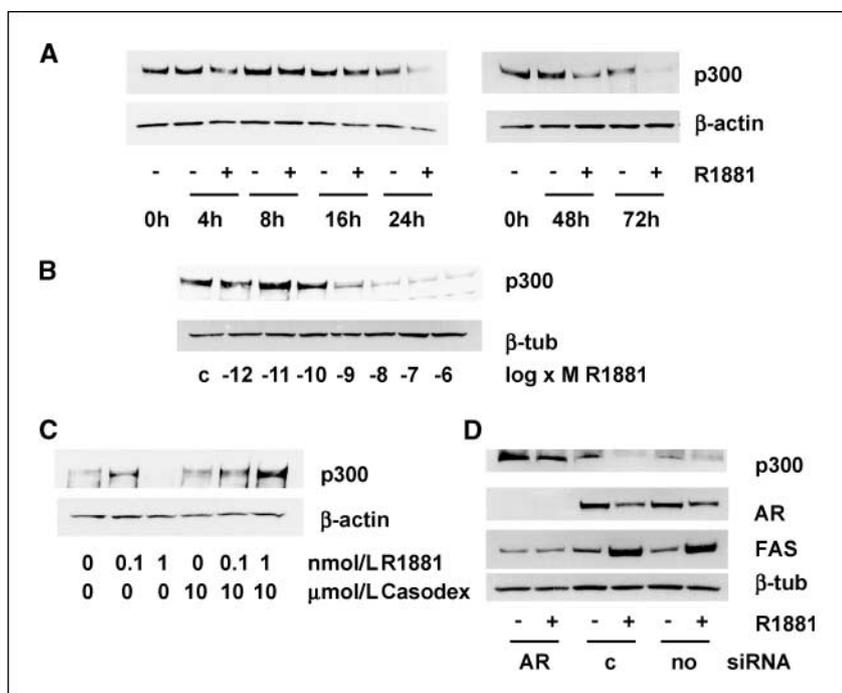
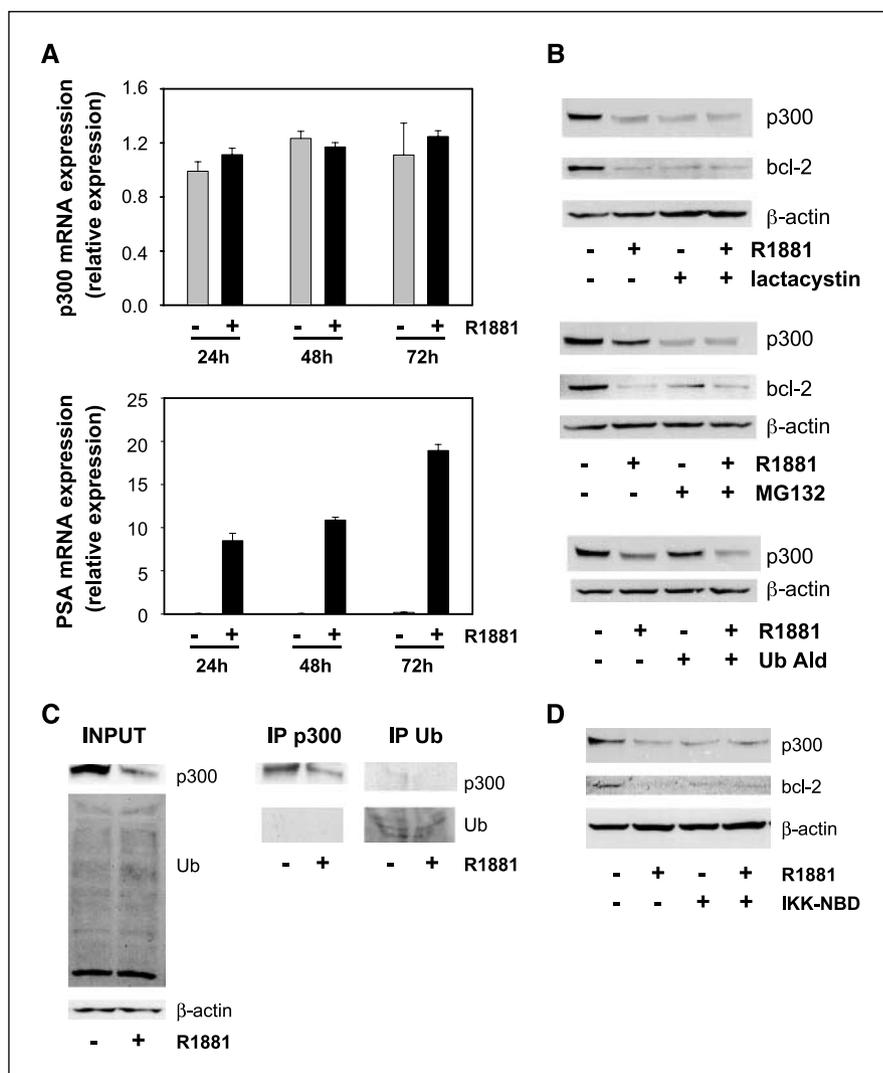


Figure 2. Characterization of the androgen-induced down-regulation of p300 expression. **A**, time course of the effects of androgens on p300 expression. LNCaP cells were seeded in CSS medium. Two days later, medium was changed and cells were treated with 5 nmol/L R1881 for the indicated periods. Total protein extracts were prepared, and equal amounts of protein were analyzed by Western blotting using an antibody directed against p300. **B**, dose dependence of the effects of androgens on p300 expression. LNCaP cells were seeded in CSS medium. Two days later, medium was changed and cells were treated with the indicated concentration of R1881 for 72 h. Cells were harvested, and p300 expression was analyzed as described above. **C** and **D**, involvement of AR in androgen-induced down-regulation of p300 expression. **C**, inhibition of AR activity. Two days after being plated in CSS medium, LNCaP cells received fresh medium and were treated with or without 0.1 or 1 nmol/L R1881 in the presence or absence of 10 μmol/L of the AR antagonist Casodex. After 72 h, whole-cell lysates were prepared and p300 expression was evaluated by immunoblotting as described. **D**, knock down of AR expression. LNCaP cells were seeded in FBS medium without added antibiotics. On the next day, cells were transiently transfected with siRNAs targeting AR or control siRNAs, or mock transfected. Forty-eight hours after transfection, cells were treated with R1881 (5 nmol/L) or ethanol vehicle. Three days later, cells were harvested and p300 expression was assessed as described above. To evaluate potential loading differences, blots were stripped and reprobed with an antibody against β-actin or β-tubulin (A–D).

Figure 3. Mechanism underlying androgen-mediated down-regulation of p300 expression. **A**, no effects of androgens on p300 mRNA expression. LNCaP cells were seeded in CSS medium. Two days later, medium was changed and cells were treated with 1 nmol/L R1881 (black columns) or vehicle (gray columns) for the indicated periods of time. Total RNA was isolated and converted into cDNA. cDNA was amplified using p300 specific primers via real-time RT-PCR. p300 mRNA expression levels were normalized with the values obtained from glyceraldehyde-3-phosphate dehydrogenase expression. p300 mRNA levels are expressed as relative expression values, taking the average value obtained from cells at the 0 h time point as 1. Columns, means of representative experiment done in triplicate; bars, SE. **B**, involvement of the proteasome in the observed effects on p300 protein expression. Two days after being plated in CSS medium, LNCaP cells received fresh medium and were treated with 1 nmol/L R1881 or ethanol vehicle in the presence or absence of 10 μ mol/L lactacystin, 1 μ mol/L MG132, or 5 μ mol/L ubiquitin aldehyde (*Ub Ald*). After 72 h, whole-cell lysates were prepared and p300 expression was evaluated by immunoblotting as described above. **C**, effect of androgens on ubiquitination of p300. LNCaP cells were seeded in medium supplemented with CSS. Three days later, cells were treated with 1 nmol/L R1881 or ethanol vehicle for 45 h. Cell lysates were subjected to immunoprecipitation with antibodies recognizing p300 or ubiquitin, and Western blotting was done as described before. **D**, effect of inhibition of NF- κ B signaling on androgen-induced down-regulation of p300. Three days after being plated in CSS medium, LNCaP cells received fresh medium and were treated with 1 nmol/L R1881 or ethanol vehicle in the presence or absence of 100 μ mol/L IKK-NBD for 72 h. Cells were harvested, and p300 expression was assessed as described above. As an additional control for the efficacy of MG132, lactacystin, and IKK-NBD, blots were stripped and reprobed with an antibody recognizing bcl-2. To evaluate potential loading differences, blots were stripped and reprobed with an antibody against β -actin (B–D).



p300 expression, we evaluated the effect of androgen stimulation on p300 mRNA levels. To this end, real time RT-PCR was done on cDNA samples derived from RNA from LNCaP cells grown in the presence or absence of R1881 for 24, 48, and 72 h. As shown in Fig. 3A, exposure to androgens had no effect on p300 messenger expression. Stimulation of LNCaP cells with R1881 for shorter time periods (4, 8, and 16 h) also did not affect p300 mRNA expression (data not shown). The ability of the primers used to detect p300 mRNA expression has been described before (13) and was verified by experiments in which p300 expression was targeted with specific siRNAs (data not shown). Androgen responsiveness of the cells was confirmed by measuring the induction of PSA mRNA expression by androgens on the same cDNA samples (Fig. 3A, bottom). Taken together, these data indicate that androgens do not exert their effects on p300 expression by negatively regulating transcription of the p300 gene. We therefore examined whether proteasomal degradation of p300 could be responsible for the decrease in p300 protein levels upon androgen stimulation. To this end, LNCaP cells were stimulated with R1881 or vehicle in the absence or presence of the proteasomal inhibitors lactacystin and MG132. The doses of the compounds used were optimized not to jeopardize cell viability. Treatment with either one of these compounds led to a decrease in

basal levels of p300, and addition of androgens did not further affect p300 expression (Fig. 3B), indicating proteasomal involvement in the observed effects. Combination of androgen stimulation with ubiquitin aldehyde, a potent and specific inhibitor of the deubiquitinating enzyme ubiquitin C-terminal hydrolase, on the other hand, had no effect on androgen regulation of p300 expression (Fig. 3B). To assess whether the androgen-induced decrease in p300 expression involved ubiquitination of p300, LNCaP cells were treated with R1881 or ethanol vehicle for 45 h. After this period of androgen stimulation, which allows the decrease in p300 expression to be obvious, while still leaving enough p300 to be readily detectable by Western blot (Fig. 3C and time course in Fig. 2A), LNCaP cells were harvested and cell lysates were subjected to immunoprecipitation with an antibody against p300 or an antibody recognizing ubiquitin as well as monoubiquitinated and polyubiquitinated proteins. The resulting immunoprecipitates were analyzed by Western blotting using the described antibodies. As shown in Fig. 3C, these experiments showed no ubiquitination of p300 upon androgen stimulation, nor did immunoprecipitation with the antiubiquitin(ation) antibody yield a p300 immunoreactive signal. Both antibodies did, however, recognize their target proteins in input samples or in their respective corresponding immunoprecipitates (Fig. 3C). As an

additional control, immunoprecipitation with a nonspecific antibody was done (data not shown). Taken together, these data suggest that androgen-mediated down-regulation of p300 does not involve ubiquitination of p300 but does require proteasomal activity. These observations led us to investigate the potential involvement of nuclear factor- κ B (NF- κ B) signaling in the androgen effects on p300 expression, as inhibition of the proteasome by compounds such as lactacystin and MG132 also inhibits degradation of I κ B and as such inhibits NF- κ B activation. To this end, we combined androgen treatment of LNCaP cells with addition of IKK-NBD, a peptide that blocks interaction of NEMO, an I κ B complex regulatory protein, with the I κ B kinase complex and, thus, selectively inhibits NF- κ B activity. As shown in Fig. 3D, the effects of IKK-NBD on p300 expression were similar to those observed after treatment with the proteasomal inhibitors lactacystin and MG132, suggesting a role for NF- κ B signaling in the androgen regulation of p300. Adding a nontargeting control peptide had no effect on (androgen responsiveness of) p300 expression (data not shown). Taken together, our data favor a proteasome-dependent and NF- κ B-dependent increase in p300 expression upon androgen deprivation (addressed in experiments below). Supporting this possibility, upon treatment with lactacystin, MG132, or IKK-NBD changes similar to those observed for p300 were noted in the expression of bcl-2 (Fig. 3B and D), the expression of which has been described previously to be up-regulated by androgen withdrawal and conversely to be repressed by treatment with androgens and is transcriptionally regulated by NF- κ B (15, 16). The exact molecular machinery by which NF- κ B activity might mediate the effects of changes in the androgenic milieu on p300 expression will be important to explore. Several reports indicate a role for NF- κ B activity in prostate cancer progression, and a requirement for NF- κ B for ligand-independent AR signaling in prostate cancer cells has been described. Higher constitutive NF- κ B DNA binding has been reported in ADI prostate cancer xenografts when compared with their androgen-dependent counterparts. Moreover, the promoter of the PSA gene has been described to contain consensus NF- κ B binding sites that are able to drive transcription of the PSA gene in the absence of androgens. Similar NF- κ B binding sites have been described in several other prostate-enriched genes (including the genes encoding prostatic acid phosphatase, Nkx3.1, MIC-1, and DD3; refs. 17–20).

Androgen deprivation increases p300 expression. Given the suppressive effects of androgens on p300 expression, we wondered whether androgen deprivation would have the opposite effect, i.e., lead to an increase in the expression of p300 in prostate cancer cells. To this end, we investigated the effect of short-term androgen deprivation on p300 expression in LNCaP cells. LNCaP cells were cultured for 3 days in medium supplemented with CSS, depleted of steroids, or in complete medium. As shown in Fig. 4A, passage in androgen-deprived medium led to a marked increase in p300 expression. The effect of long-term androgen deprivation was explored by comparing p300 expression in the LNCaP cell line and its isogenic cell line LNCaP-Rf. The LNCaP-Rf cell line has been established in our laboratory by long-term androgen ablation of LNCaP cells and is considered to be a valuable model for the study of ADI prostate cancer (11). p300 protein levels in ADI LNCaP-Rf cells were considerably higher compared with the parental LNCaP cells (Fig. 4B). These findings are in agreement with the recent observation in the LuCaP35 human prostate cancer xenograft model that progression to ADI prostate cancer after orchiectomy is accompanied with induction of p300 expression (21). In keeping with the results obtained in the parental LNCaP cell line (Figs. 1

and 2), readministration of R1881 to LNCaP-Rf cells down-regulated p300 expression (Fig. 4C). The kinetics of this down-regulation, however, were somewhat slower than those observed in the parental LNCaP line (data not shown).

Next, we sought to determine whether the increase in p300 expression that is induced by androgen deprivation would be affected by the presence of compounds that are relevant to the clinical setting of ADI prostate cancer treatment and progression. Specifically, we were interested in exploring whether antiandrogens (mimicking combined androgen blockade) or growth factors or cytokines would affect p300 expression. To explore the effect of antiandrogens, androgen-deprived LNCaP cells were treated with bicalutamide, hydroxyflutamide, or cyproterone acetate, all commonly used in the clinical setting in combination with androgen ablation strategies (1) or with mifepristone (RU486), a progesterone receptor antagonist that has recently been shown to be an effective AR antagonist (22), which is currently being tested in a clinical trial for prostate cancer. Both cyproterone acetate and hydroxyflutamide treatment led to some decrease in p300 expression, whereas bicalutamide had no effect and mifepristone slightly affected p300 expression in androgen-deprived LNCaP cells (Fig. 5A). Because the AR expressed by LNCaP cells harbors a mutation in its ligand-binding domain that is known to allow stimulation by the latter two antiandrogens, we repeated these experiments using LAPC-4 cells, which express a wild-type AR. In androgen ablated LAPC-4 cells, none of the antiandrogens had an effect on p300 expression. The implications of the mutation status of the AR, as well as its effect on the treatment regimen on p300 expression in the clinical setting will require further study.

Progression of prostate cancer to the ADI state is associated with the overexpression of multiple cytokines and growth factors. Several of these factors, including IL-6, EGF, and IGF-I, have been shown to be able to activate the AR in a ligand-independent

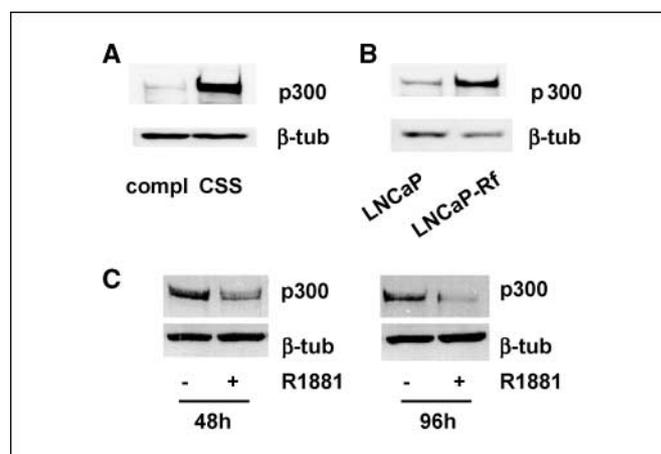
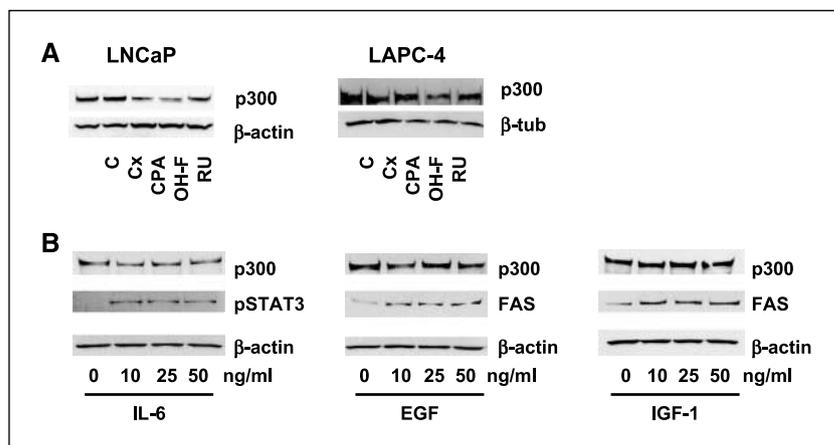


Figure 4. Effect of androgen-deprivation on p300 expression. *A*, effect of short-term androgen deprivation on p300 expression. LNCaP cells were seeded in complete (*compl*) or CSS medium. Three days later, total protein extracts were prepared and equal amounts of protein were analyzed by Western blotting with an antibody directed against p300. *B*, effect of long-term androgen deprivation on p300 expression. LNCaP and LNCaP-Rf cells were seeded in their regular medium. Three days later, cells were harvested and p300 expression was evaluated as described. *C*, effect of readministration of androgen on p300 expression in long-term androgen-deprived prostate cancer cells. LNCaP-Rf cells were seeded in their regular CSS medium. Two days later, medium was changed and cells were treated with R1881 (5 nmol/L) or ethanol vehicle. After 48 and 96 h, cells were harvested and p300 expression was analyzed by Western blot analysis. To evaluate potential loading differences, blots were stripped and reprobbed with an antibody against β -tubulin (*A–C*).

Figure 5. Effects of antiandrogens, growth factors, and cytokines on androgen deprivation–induced p300 expression. **A**, effects of antiandrogens. Cells were seeded in CSS (LNCaP) or complete (LAPC-4) medium. Two days later, medium was replaced (CSS medium) and cells were treated with ethanol vehicle, bicalutamide (Casodex; 10 $\mu\text{mol/L}$), cyproterone acetate (10 $\mu\text{mol/L}$), hydroxyflutamide (10 $\mu\text{mol/L}$), or mifepristone (RU486; 1 $\mu\text{mol/L}$). Three days later, total protein extracts were prepared and equal amounts of protein were analyzed by Western blotting using an antibody directed against p300. **B**, effects of growth factors and cytokines. LNCaP cells were seeded in CSS medium. After 2 d, medium was changed and cells were treated with increasing doses of EGF, IGF-1, or IL-6 for 24 h. Cells were harvested and p300 expression was assessed as described above. To verify viability of the factors used, expression of FAS or pSTAT3 was evaluated. To evaluate potential loading differences, blots were stripped and reprobed with antibodies against β -tubulin or β -actin (**A**, **B**).



manner (23). We therefore investigated the effect of these compounds on the increase in p300 expression after androgen deprivation. As shown in Fig. 5B, despite effects on known target genes, addition of increasing doses of these factors did not affect the level of p300 expression in LNCaP cells. Interestingly, expression of the p300 homologue CBP, another AR coactivator displaying overexpression in prostate cancer disease progression, has previously been reported to be down-regulated upon stimulation of LNCaP cells with IL-6 (24). These findings further emphasize that, despite high levels of homology between p300 and CBP, these proteins should be considered as separate entities and may fulfill non-redundant functions under both physiologic and neoplastic conditions. Moreover, they indicate that although androgens, growth factors, and cytokines are all able to activate the AR, the overlap in number of genes regulated in common by these different ligands may be rather limited, as has recently been shown for androgen and PKA signaling in prostate cancer cells (25). It should be noted that whereas the growth factors and cytokine tested here do not affect expression of p300 in androgen-deprived prostate cancer cells, an effect of these factors on the activity of p300 cannot be ruled out at this time. In fact, only very recently has the neuropeptide bombesin been shown to enhance the histone acetyl transferase activity of the p300 protein, while leaving p300 expression levels unaltered (26). As the p300 histone acetyl transferase moiety is critical for ADI AR activity (9), it will be important to explore whether the stimulatory effect of androgen deprivation on p300 expression can be further enhanced by growth factor–mediated changes in p300 activity.

Increased p300 expression in ADI prostate cancer cells is critical for cell proliferation. Next, we set out to assess the effect of increased p300 expression on prostate cancer cell survival. To this end, we compared the dependency of the isogenic cell lines LNCaP and LNCaP-Rf on p300 expression for cell viability. Cells were transfected with siRNAs targeting p300 or nontargeting control siRNAs. At the indicated time points, cell viability was evaluated by determining metabolic activity by means of a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium (MTS) assay. In LNCaP cells, down-regulation of p300 expression led to minor decreases in cell viability that were first noted 72 h after transfection (Fig. 6A, left). In LNCaP-Rf cells that express higher basal levels of p300 under these culture conditions (see Fig. 4A), loss of p300 expression led to decreases in cell viability with similar kinetics, but in this ADI prostate cancer model the effects were far more pronounced (Fig. 6A, right). As shown in Fig. 6A, these results could not be attributed to marked differences

in the efficiency of the p300 knock-down between these two cell lines. Instead, these data indicate that the increased expression of p300 in ADI prostate cancer cells upon androgen deprivation is advantageous for cell survival.

To further corroborate these findings, we evaluated the effects of loss of p300 on viability of LNCaP cells grown in the absence or presence of wide range of androgen concentrations. To this end, LNCaP cells seeded in medium supplemented with CSS were transfected with siRNAs directed against p300 or control siRNAs. The next day, the medium was changed and cells were stimulated with doses of androgens that have been shown to have no effect on p300 expression (0 and 0.1 nmol/L R1881, see Fig. 2B) or to cause a marked decrease in its expression (1 and 10 nmol/L R1881). At the indicated times after treatment, viability of the cells was evaluated by performing a MTS assay. Consistent with the data on long-term androgen deprivation shown in Figs. 6A and 4B, these experiments revealed overall greater effects of p300 knock-down on viability of LNCaP cells cultured short-term under androgen-deprived conditions. Moreover, when exposed to R1881 concentrations of 1 and 10 nmol/L R1881 for 4 days, the difference in viability of cells treated with siRNAs targeting p300 or control cells was lost, indicating similar effects on cell viability regardless of the manner in which p300 knock-down was achieved (siRNA-dependent or androgen-induced). The observation that it takes 96 h of androgen treatment to observe these effects is consistent with the need for a lag period between the actual occurrence of p300 loss and subsequent effects on cell viability (see Figs. 6A and 2A).

Having established that the increase of p300 expression in prostate cancer cells upon androgen deprivation has severe implications for cell viability, we sought to determine whether this could be due to an effect of p300 on cell death, cell growth, or both. To this end, cell lysates obtained from LNCaP-Rf cells transfected with siRNAs targeting p300 expression or control siRNAs as described above (Fig. 6A) were analyzed for changes in the expression of key regulators of apoptosis and cell proliferation. Cleavage of full-length (116 kDa) PARP, generating an 89-kDa fragment, is a widely recognized marker for cells undergoing apoptosis. As shown in Fig. 6C, loss of p300 expression did not generate a cleaved PARP fragment. This was not due to technical limitations in detecting PARP cleavage, as the same amount of cellular proteins obtained from cells induced to undergo apoptosis upon treatment with staurosporine (Fig. 6C, bottom) yielded immunoreactive bands for an 89-kDa PARP fragment that were readily detectable. Moreover, no proteolytic processing of caspases, central executioners of apoptosis, could be

observed upon transfection with p300 siRNAs (data not shown), confirming that the decrease in cell viability upon down-regulation of p300 expression is not caused by increased apoptosis.

We then investigated whether changes in p300 expression could affect the expression of cyclins, key regulators of the progression

through cell cycle. Using the LNCaP-Rf cell lysates described above, we examined the expression of cyclin D1 and cyclin E (involved in G₁-S transition), as well as the expression of cyclins A and B (gatekeepers for the G₂-M entry; ref. 27). These experiments revealed that concomitantly with the loss of p300 expression, cyclin

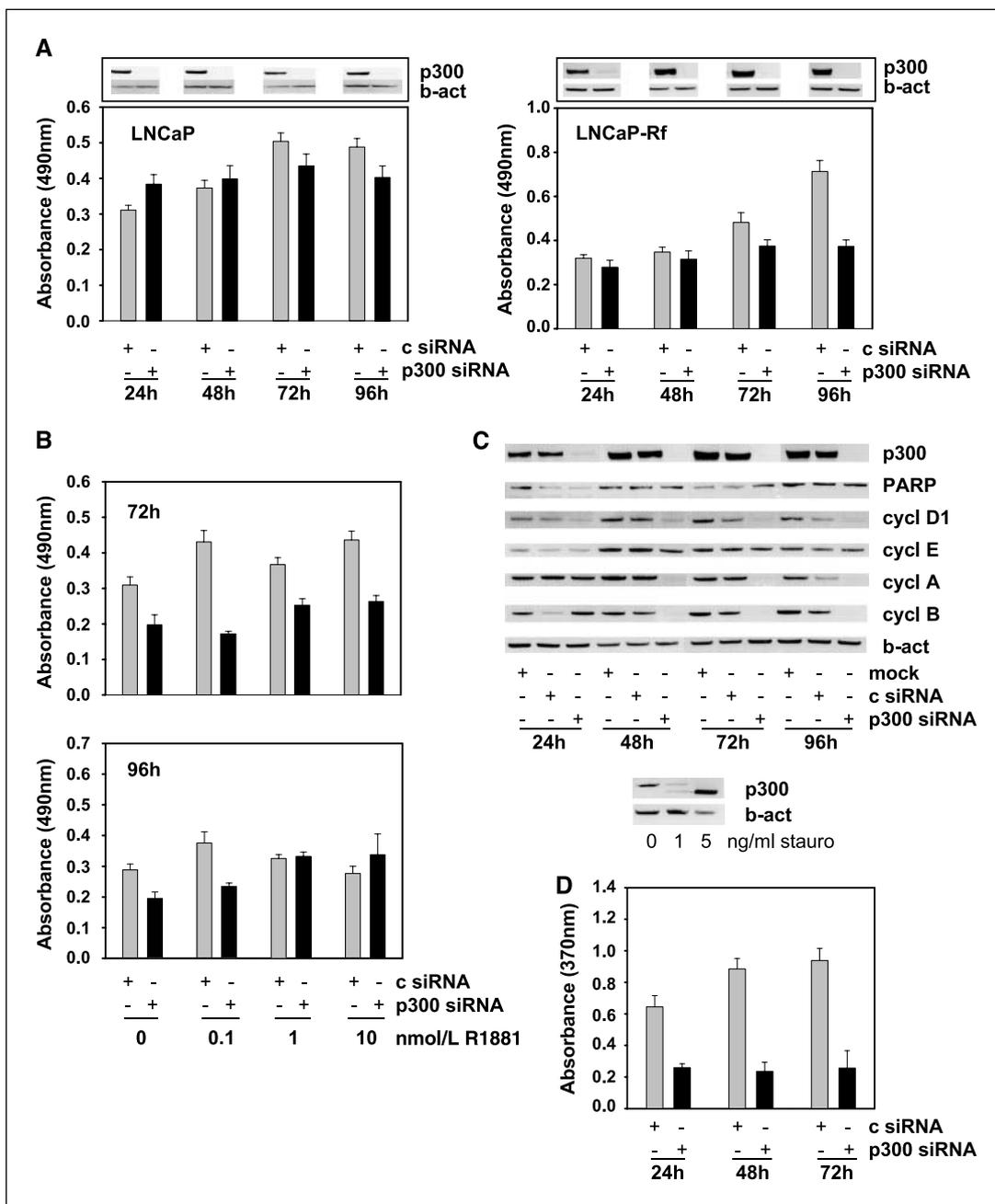


Figure 6. Effect of changes in p300 expression on prostate cancer cell viability. **A**, effect of siRNA-mediated loss of p300 expression on cell viability of LNCaP and LNCaP-Rf cells. Cells were transfected with siRNA against p300 (black columns) or control siRNAs (gray columns) as described. Twelve to 16 h after transfection, medium was changed. Twenty-four, 48, 72, and 96 h later, cell viability was assessed by means of an MTS assay reading absorbance at 490 nm. Columns, means of five individual measurements; bars, SE. **B**, comparison of the effect of androgens and siRNA-mediated loss of p300 expression on cell viability of LNCaP cells. Cells were transfected with siRNA against p300 (black columns) or control siRNAs (gray columns) as described. Twelve to 16 h after transfection, medium was changed and cells were treated with 0, 0.1, 1, or 10 nmol/L R1881 for the indicated periods. Cell viability was assessed by means of an MTS assay, measuring absorbance at 490 nm. Columns, means of five individual measurements; bars, SE. **C**, effect of siRNA-mediated loss of p300 expression on expression of key regulators of apoptosis or cell growth. LNCaP-Rf cells were transfected with siRNA targeting p300 expression or control siRNA. On the next day, medium was replaced. Twenty-four, 48, 72, and 96 h later, cell lysates were prepared and Western blotting was done as described (top). LNCaP-Rf cells were treated with 0, 1, or 5 ng/mL staurosporine for 24 h. Cell lysates were prepared, and Western blotting was done as described above. **D**, effect of siRNA-mediated loss of p300 expression on BrdUrd incorporation. LNCaP-Rf cells were transfected with siRNA against p300 (black columns) or control siRNAs (gray columns) as described. Twelve to 16 h after transfection, medium was changed. Twenty-four, 48, 72, and 96 h later, BrdUrd incorporation was assessed by means of an ELISA assay, reading absorbance at 370 nm. Columns, means of five individual measurements; bars, SE.

D1 expression decreased. Expression of cyclin E, on the other hand, was not affected by changes in p300 expression. Following the effects on cyclin D1, expression levels of cyclins A and B also declined, but this effect took an extra 24 h to occur (Fig. 6C). Taken together, these data suggest that p300 plays an important role in regulating both G₁-S and G₂-M transition in prostate cancer cells. To confirm that the primary effect of p300 on cyclin D1 expression could be important for transition through S phase, 5-bromo-2'-deoxyuridine (BrdUrd) incorporation experiments were set up. LNCaP-Rf cells were transfected with siRNAs targeting p300 expression or control siRNA. At the indicated times after transfection, BrdUrd incorporation was assessed by an ELISA assay. As shown in Fig. 6D, p300 knock-down resulted in a decrease of DNA synthesis in a time course that corresponded well with the one observed for the decrease in cyclin D1 expression (see Fig. 6C). These findings are consistent with our previous observation that increased expression of p300 expression in prostate cancer tissue specimens correlates with Ki-67 expression, a marker for cell proliferation (10). Overexpression of cyclins is a hallmark of cancer progression, and expression of cyclin D1, cyclin A, and cyclin B has been reported to increase during prostate cancer progression (28–31). Moreover, multiple literature reports have linked p300 to several aspects of cell proliferation (32, 33). Our data indicate that expression of p300 is critical not only for G₁-S transition but also for progression from G₂ phase of the cell cycle to mitosis. In line with our observations, p300 has been shown to be involved in cyclin D1 gene activation, to bind to the cyclin B1 promoter and

enhance cyclin B1 transcription. Similarly, CHIP assays have shown p300 to bind to the cyclin B2 promoter and revealed this binding to be regulated during cell cycle positively correlating with promoter function (34–36).

In conclusion, our data provide insights into the molecular mechanism that governs the expression of p300 in the progression of prostate cancer. Androgen deprivation strategies, although initially successful, almost invariably fail, resulting in the emergence of more aggressive disease. Here, we present evidence that an increase in p300 expression, fostered by androgen deprivation, offers growth advantage to ADI prostate cancer cells. As the underlying cause of the increases in AR coactivator expression in prostate cancer cells is largely unknown, follow-up studies are necessary to determine whether the effects of androgen deprivation can be generalized to AR coregulators other than p300. Preliminary work in our laboratory and others suggests that changes in androgen concentrations in prostate cancer cells may affect not only the expression status of p300 but could also affect the expression and even the subcellular localization of multiple AR coregulatory proteins (7, 37).⁵ Characterizing and dissecting the molecular machinery underlying these changes in coactivator expression may lead to novel avenues for therapeutic intervention.

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⁵ Our unpublished results.

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