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Androgen regulates Cdc6 transcription through interactions between androgen receptor and E2F transcription factor in prostate cancer cells

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ABSTRACT

Androgen receptor plays a critical role in the development and maintenance of cancers in the prostate. Earlier, we have shown that Cdc6, a regulatory protein for initiation of DNA replication, is down regulated in androgen-insensitive prostate cancer cells. In this report, we studied the involvement of androgen, mediated through androgen receptor (AR) in regulation of Cdc6 expression. Our results demonstrated that androgen treatment stimulated Cdc6 expression in xenograft tumors and androgen-sensitive prostate cancer cells. We also showed that androgen treatment stimulated Cdc6 transcription through possible interaction of AR with the ARE sequence in the Cdc6 promoter and that the stimulatory effect of androgen required intact E2F binding sites in the promoter. Androgen treatment differentially altered nuclear availability of E2F1 and E2F3, and increased the amount of hypophosphorylated retinoblastoma protein (pRb) in the nucleus in a time dependent fashion. We further showed that AR interacted with E2F transcription factors in a ligand-independent manner and that ligand-bound AR was less efficient in interacting with E2F proteins. DNA–protein interaction assays indicated that androgen treatment altered binding of E2F1 to the Cdc6 promoter in prostate cancer cells. We conclude that AR regulates Cdc6 transcription through interaction with the Cdc6 promoter, and complex formation with E2F1 and E2F3 in a differential manner.

1. Introduction

Androgens and androgen receptor (AR) play a pivotal role in maintenance and progression of cancers in the prostate [1]. Because neoplastic prostates depend on circulating androgens for their growth and survival androgen withdrawal is one of the current treatments for prostate cancer. Androgen receptor requires androgen for proper functioning as a transcription factor and regulates a wide variety of genes upon ligand binding [2,3]. In mature prostate, activated AR maintains a constant cell population by stimulating cell proliferation and apoptosis [4]. It has been documented that AR mediates its cell proliferating effect through activation of cell cycle regulatory genes, such as CDK2 and CDK4 [5,6]. In the prostate cancer CWR22 xenograft models, levels of CDK2, CDK4, cyclin B and cyclin E decreased after castration but were restored following treatment with testosterone propionate [7].

An important aspect of the AR mediated cell cycle regulation is its cyclical activation during cell cycle phases, which triggers transcription of androgen-sensitive genes. The transcriptional activity of AR is abolished at the G1/S transition but reappeared in the S phase. AR is predicted to be transcriptionally active at G0 through its interaction with the hypophosphorylated Rb [8]. Transcription activation of the AR-responsive genes is mediated through binding of the activated AR with androgen response elements (ARE) in the promoters. In the absence of ligands, cytoplasmic AR remains as an inactive complex with the heat shock protein 90. Upon binding to androgens, AR is released from the inhibitory complex, translocates to the nucleus and binds to the ARE as a dimer [6,9]. In addition, transcriptional activity of AR is modulated by a number of coactivators or corepressors including cyclin D1 and cyclin E [10–13]. A number of transcription/transacting factors such as Oct1, NF-kB and AP1 also act as coactivators or corepressors of AR [14–16]. Indirect evidence indicated that AR also regulates activation of E2F transcription factor through modulation of Rb phosphorylation in a ligand-dependent manner [17]. However, the exact mechanism of AR mediated regulation of E2F function and the roles of Rb and androgen are unclear as earlier studies have shown that AR physically interacts with hypophosphorylated Rb in a ligand-independent manner [18].

Our earlier studies have indicated that CDC6, a highly conserved cell cycle regulatory protein is down-regulated in androgen-insensitive prostate cancer cells and that the reduced expression of CDC6 is due to inefficient transcription [19]. Recent studies also have shown that treatment with anti-androgen bicalutamide inhibited progression of cells through G1/S phase and inhibited expression of CDC6, in LNCaP cells [20]. CDC6 plays a critical role in restricting DNA replication to once per cell cycle [21] and helps to establish prereplicative

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complex (Pre-RC) on chromatin-bound origin recognition complex (ORC) [22] by recruiting minichromosome maintenance (MCM) proteins and other factors [23–25]. In normally proliferating cells, the concentration of CDC6 is tightly regulated for proper progression of cells through the phases of cell cycle.

Regulation of *Cdc6* gene expression follows the cyclical pattern of G1, S, G2 and M phase. In human cells, *Cdc6* is transcribed in mid to late G1 in E2F-regulatory manner [21,26,27]. *Cdc6* promoter contains three E2F binding sites and ectopic expression of E2F stimulates *Cdc6* promoter activity following serum stimulation. G1 cyclins and CDKs in turn regulate transcriptional activity of E2F through phosphorylation of Rb family of proteins. Importantly, E2F acts as a positive and negative regulator of *Cdc6* transcription depending on the cell cycle stages. In early phases of the cell cycle, E2F sites are occupied by pRb, p107, and/or p130 bound to E2F-DP inhibitory complexes. It has been proposed that *Cdc6* transcription in mid to late G1 phase is achieved by possible displacement of the inhibitory complex by other transcription factors [28].

In this report, we provide evidence that androgen and AR regulates *Cdc6* transcription and that the effect of androgen is mediated through modulation of E2F dependent transcription of *Cdc6* gene. Our study also demonstrates that androgen modulates binding of E2F transcription factors to Cdc6 promoter.

2. Materials and methods

2.1. Cell lines and tumors

Two commercially available cells lines PC3 and LNCaP (ATCC) were maintained in Ham's F-12 (PC3) and RPMI1640 (LNCaP) media, containing 10% fetal bovine serum (FBS) and penicillin/streptomycin. PC3AR and PC3Neo (a gift from Kerry, Burnstein, University of Miami) [29] were maintained in RPMI 1640 containing 10% FBS, 1% antibiotic/antimycotic (Invitrogen) and G418 (350 πg/ml). CWR22 xenograft tumors (a gift from William Grizzle, University of Alabama at Birmingham) [30,31] were obtained from untreated mice, from 28 days post castrated mice and from 28 days post castrated mice with slow releasing testosterone pellets implanted after 21 days.

2.2. Semiquantitative RT-PCR

Total RNA from tumor tissues was used for RT reaction using oligo d(T) primer, 18S RNA primer pairs and their compertimers (Quantum RNA, Ambion), and Superscript II reverse transcriptase (Invitrogen). The 18S primers were used to generate an internal control transcript of 489 bp. A *Cdc6* gene specific primer pairs (F 5′CTGGAGTTTGCTGCTGCCGCT 3′ and R 5′GAGCACCAGAAAGGTA AAGGC) was used to amplify an

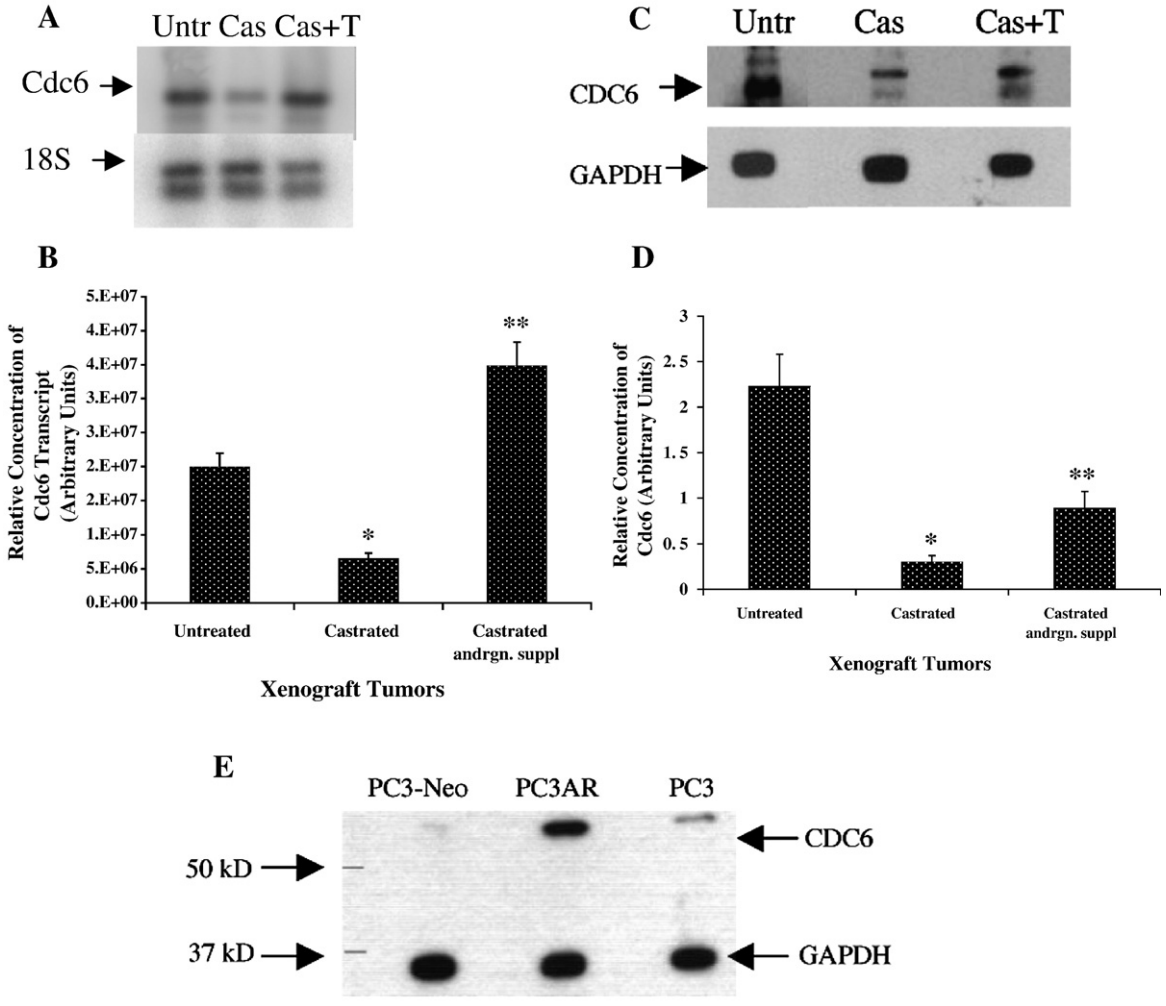

Fig. 1. Differential expression of CDC6 in mouse tumors and transfected cells. A and B: Semiquantitative RT-PCR of Cdc6 transcripts in CWR22 mice tumors. A: Autoradiogram, B: Densitometric analysis of the relative concentration of *Cdc6* mRNA. Data represents mean ± SD of three separate analyses (*p* values: *0.05 castrated vs. untreated; **0.04 castrated vs. androgen supplemented). C and D: Immunoblot (C) and densitometric (D) analysis of CDC6 concentration in crude extracts of CWR22 tumors. Data represents mean ± SD of two sets of tumors (*p* values: *0.014 castrated vs. untreated; **0.02 castrated vs. androgen supplemented). E: Immunoblot analysis of CDC6 concentration in PC3Neo, PC3AR and PC3 cells. 18S rRNA and GAPDH were used as the loading controls.

811 bp fragment. Amplified fragments were visualized by Southern blot using *Cdc6* and 18S ribosomal RNA cDNAs as probes. Positive signals were quantified in a Phosphorimager (Amersham Biosciences) and normalized with the relative values of 18S rRNA signals.

2.3. Preparation of crude cell and nuclear extract

PC3AR, PC3Neo and LNCaP cells were grown to 80% confluency and harvested using freeze—thaw cycle in a lysis buffer (50 mM Tris—HCl, pH 8.0, 120 mM NaCl, 2.5 mM EDTA, pH 8.0, 1 mM phenylmethylsulfonyl fluoride, 0.4% Nonidet P-40, and 2 ml/ml of the protease inhibitor mixture Set III (Calbiochem)). Crude cell lysates were centrifuged at 16,000 *g* for 10 min at 4 °C to remove cellular debris. For nuclear extract, 100 πl of packed cell volume of each cell and nuclear extraction reagent (NE-per Pierce) were used according to the manufacturer's protocol. Nuclear extracts from mouse tumors were prepared using a nuclear extraction kit from Panomics according to the manufacturer's protocol. Nuclear pellets were collected and lysed by vortexing in a lysis buffer NER (Pierce), and soluble proteins were separated in the supernatant by centrifugation at 14,000 *g* for 10 min at 4 °C. Supernatants were stored at 80 °C until use for Western blot analysis.

2.4. Immunoblot and Immunoprecipitation

Total cell extracts from asynchronous cultures of PC3AR, PC3Neo and LNCaP cells were used for immunoblot analysis using a standard procedure. Total protein (50 πg) or nuclear extracts (20 πg) and antibodies against CDC6 (Oncogene Research Products, La Jolla, CA), E2F1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), E2F3 (Santa Cruz Biotechnology, Inc.), pRb (Cell Signaling), and ppRb (Cell signaling) were used to monitor expression of specific proteins. A chemiluminescence detection kit (Pierce) was used to detect positive signals recognized by specific antibodies using either a goat anti-mouse or a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody. When indicated, PC3AR or LNCaP cells were maintained in charcoal-stripped serum (CSS) containing medium for 48 h and treated with DHT (5 nM) or R1881 (1 nM). Cells were harvested at different time points for preparation of crude cell or nuclear extracts as described earlier. For immunoprecipitation, nuclear extracts from freshly harvested cells were diluted in RIPA buffer containing protease inhibitor mixture Set III (Calbiochem) and treated with anti-AR antibodies (US Biologicals) using standard protocol. Antigen–antibody complexes were immunoprecipitated using protein A/G PLUS agarose beads and immunoblotted using specific antibodies.

2.5. Androgen treatment and expression of luciferase gene

The *Cdc6* promoter-luciferase construct, containing *Cdc6* promoter sequence spanning from positions −1436 to +218 was cloned into pGL3 basic firefly luciferase vector (Promega) (*Cdc6*WT) as described [19] and used for transient transfection and luciferase assays. A *Cdc6*-luciferase construct (*Cdc6*AREM) containing deleted putative androgen response element (ARE) (AGAACATAATATTC) (between −748 and −733 bases was prepared using a PCR-based mutagenesis method described in the Quik-Change kit (Stratagene). *Cdc6*-luciferase construct containing point mutations in the two proximal E2F binding sites at positions −43 to −36 and −8 to −1 from the transcription start

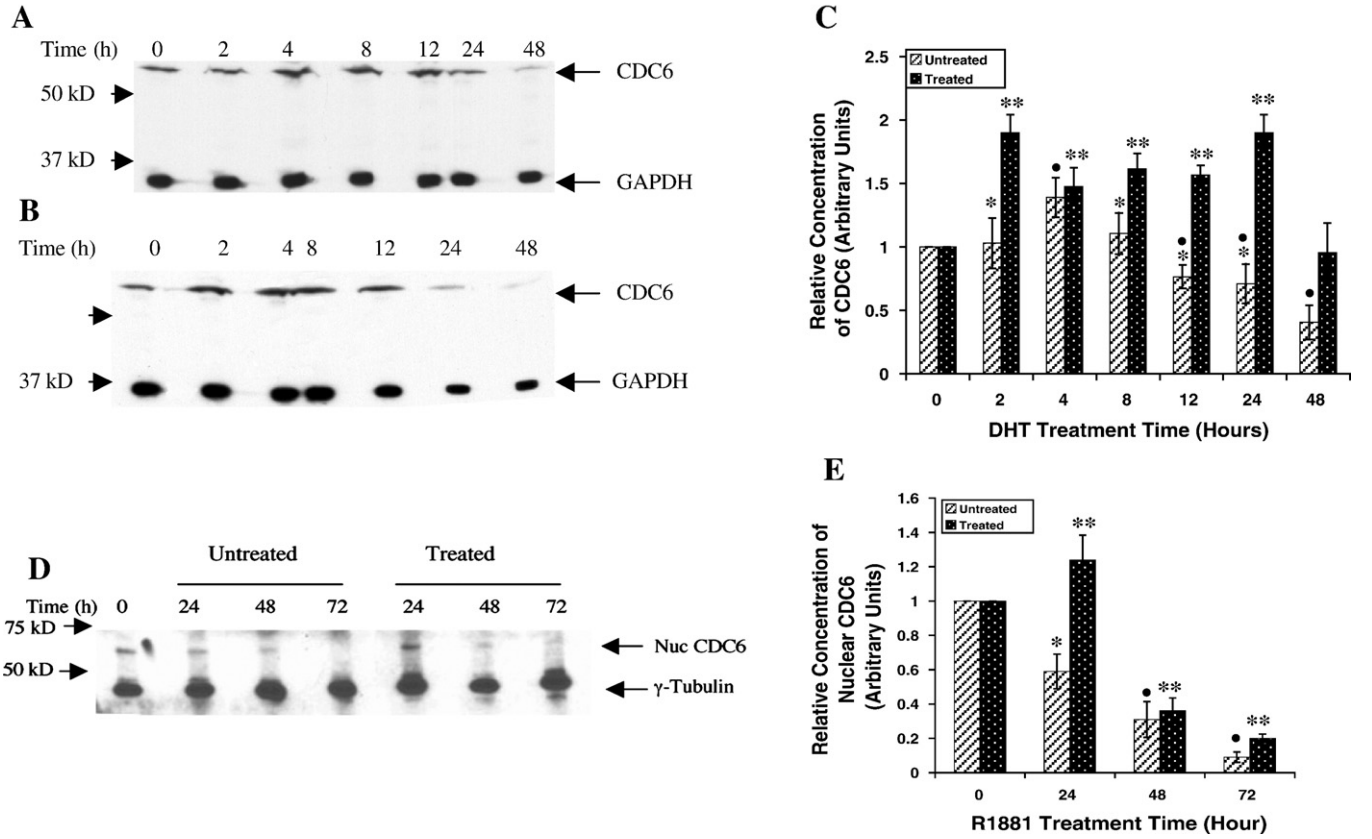

Fig. 2. Treatments of DHT/R1881 increased CDC6 expression in PC3AR and LNCaP cells. Crude cell extracts of PC3AR either left untreated (A) or treated (B) with DHT (5 nM) were immunoblotted with anti-CDC6 and anti-GAPDH antibodies. GAPDH concentration was used as the loading control. C: Densitometric analysis of the relative alteration in CDC6 concentration in untreated and treated cells after normalization with values of GAPDH. Values represent mean ± SD of three different experiments (*p* values: *≤0.0476 untreated vs. treated; **≤0.055 treated vs. 0 h; •<0.0448 untreated vs. 0 h). D: Western blot of CDC6 in the nuclear extracts of LNCaP cells either left untreated or treated with R1881 (1 nM) for different times. E: Densitometric analysis of the relative concentration of CDC6. Gamma tubulin was used as the loading control. Data shows mean ± SD of three different analyses (*p* values: * = 0.005; **≤0.0048; •≤0.0076).

site as described before [19] was used for luciferase assays upon transfection. *Cdc6*-luciferase construct containing deleted putative ARE in addition to point mutations in E2F binding sites (E2F/ARE DM) was also prepared and used for transfection. LNCaP cells were grown to 75% confluence and maintained in CSS medium for 48 h. Next, cells were transfected with the pGL3 constructs and pRL-TK (Promega) as an internal control vector using LipofectAMINE (Life Technologies) according to the manufacturer's specification. Transfected cells were maintained in CSS culture medium and either treated with R1881 (1 nM) or left untreated for 24 h. Cells were harvested at 48 h after transfection and luciferase expressions were monitored using a Dual Luciferase assay kit (Promega) according the supplier's protocol. Relative luminescence from the expressed luciferase was normalized with the expression of *Renilla* luciferase. Transfection efficiencies were calculated as the relative luminescence unit (RLU) ratio of pGL3 constructs over that of pGL3 basic vector.

2.6. Chromatin immunoprecipitation

LNCaP cells were maintained in CSS medium and either treated with R1881 (1 nM) or left untreated for 24 h. Before harvesting, cells were cross-linked with 1% formaldehyde containing culture medium for 10 min with shaking at room temperature. Cells were washed with PBS and treated with Glycine stop-fix buffer (ChIP-IT Express kit, Active Motif, Carlsbad, CA) for 5 min at room temperature. Cells were washed and processed for nuclei isolation followed by chromatin isolation and shearing using a kit (ChIP-IT Express kit, Active Motif, Carlsbad, CA) according to the manufacturer's specification. Chromatin was pre-cleared with salmon sperm DNA/protein G and subjected to immunoprecipitation using anti-E2F1 (SC-193X, Santa Cruz Biotechnology), anti-E2F3 (SC-878x Santa Cruz Biotechnology) or anti-AR (A2281x, US Biologicals) antibodies (2 πg/ChIP reaction) in ChIP buffer (ChIP-IT kit) according to the manufacturer's protocol. Immunoprecipitated DNA was eluted from protein G beads; reverse cross-linked, treated with proteinase K and purified using DNA purification columns. Purified DNA (5 πl) was used for PCR amplification using primers (10 pmol) specific for amplification of E2F (between position −84 and +103 bases) [32] (F: 5′-AAAGGCTCTGTGACTACAGCCAAT-3′, R: 5′-GTGCAG-GATCCTTCTCACGTC TCTCAC-3′) and AR (between positions −831 and −564 bases) (F: 5′-GGCCCTGAAA CCCTAGTGTTTCGCCAT-3′, R: 5′-GGATCCAGATCTCCTGATGGCTGAAC-3′) binding sites on *Cdc6* promoter. Amplified products were analyzed on 2% agarose gel.

3. Results

3.1. CDC6 expression was upregulated in prostate tumor cells in response to androgen treatment or expressing a functional AR

Our earlier studies have shown a differential expression of CDC6 between androgen-sensitive LNCaP and -insensitive PC3 cells. To understand any relation between androgen sensitivity of the prostate tumors and CDC6 expression, we studied the profile of CDC6 expression in CWR22 xenograft tumors with or without androgen deprivation and following supplementation of androgen. *Cdc6* mRNA concentration in the tumors from castrated mice showed a 3-fold reduction ($p=0.05$), which was restored to a level higher than the untreated ones upon implantation of a testosterone pellet (Fig. 1A and B). Western blot analysis of the amounts of CDC6 (62 kDa) in tumors supported the RT-PCR results showing a significant reduction ($p=0.014$) following castration (Fig. 1C and D). CDC6 expression also has been restored partially in PC3AR cells, which express a functional AR and PSA compared to the parental PC3 cells and vector only PC3 cells (PC3-Neo) (Fig. 1E). Next, we studied the effect of treatment of androgen or androgen analog R1881 on CDC6 expression using total extracts of

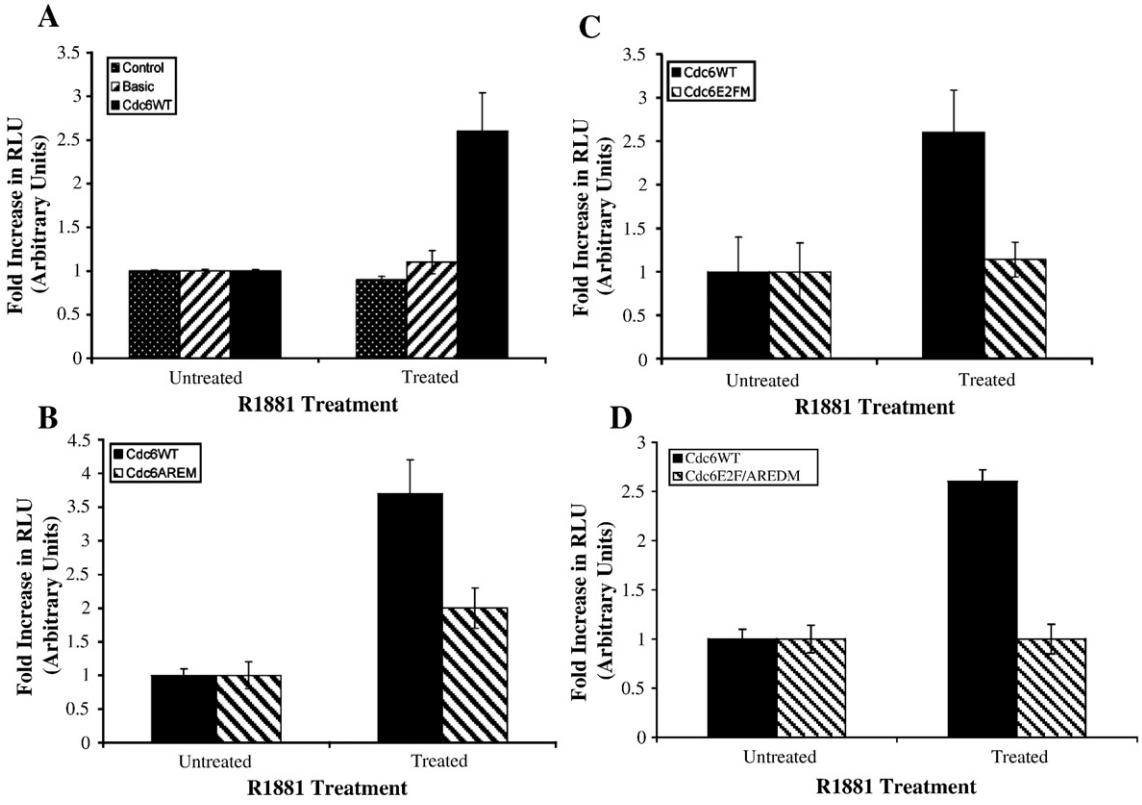

Fig. 3. Effects of androgen, deletion of ARE and mutation in E2F binding sites on *Cdc6* promoter activity. Relative luciferase activity in LNCaP cells transfected with luciferase constructs containing wild type (A and B) or ARE deleted *Cdc6* promoter (AREM) (B) treated with R1881 (1 nM) for 24 h or left untreated. Basic and control plasmids were also used for comparison. Relative luciferase activity in LNCaP cells following transfection of Cdc6 promoter-luciferase constructs containing two point mutations in E2F binding sites without (C) or with deleted ARE sequence (D). Data represents mean±SD of four separate experiments.

PC3AR or nuclear fractions of LNCaP cells. We used physiological concentration of DHT (5 nM) and R1881 (1 nM) for treatment of PC3-AR and LNCaP cells respectively [32,33]. Western blots followed by densitometric analyses of a time course study indicated a biphasic increase in CDC6 expression in total cell lysates following treatment with DHT (Fig. 2A, B and C) at 2 h and 24 h. Treatment with R1881 also increased CDC6 concentration at 24 h ($p=0.055$) in the nuclear extracts of LNCaP cells (Fig. 2D and E). The concentration of CDC6 in both total- and nuclear extracts declined at 48 h after androgen treatment. No increase in CDC6 concentration was observed following treatment with R1881 at 0.1 nM concentration (data not shown).

3.2. Androgen stimulated Cdc6 transcription through interaction of AR with Cdc6 promoter

Sequence analysis of the *Cdc6* promoter indicated the presence of a putative ARE in the 5′ flanking sequence of *Cdc6* promoter between positions −748 and −733, which suggested a possibility of AR mediated regulation of *Cdc6* transcription. To determine whether the effect of androgen treatment is through activation of the *Cdc6* promoter we monitored expression of luciferase reporter gene driven by *Cdc6* promoter in LNCaP cells following treatment with R1881 at different times after transient transfection. We used R1881 at 1 nM concentration as AR is transcriptionally active and stimulates *PSA* transcription in LNCaP cells at this concentration [33,34]. Our results showed a 2.5–3.7-fold increase in RLU following R1881 treatments (Fig. 3A and B). To confirm the involvement of ARE in androgen-induced Cdc6 transcription activation we tested the effect of androgen following deletion of the ARE. A significant loss of induction of *Cdc6* transcription was noted in cells transfected with the construct containing *Cdc6* promoter with deleted ARE (Fig. 3B). A modest 1.4 to 2.0-fold increase in RLU was noted in these cells following R1881 treatment. This result confirms that the putative ARE sequence is indeed utilized by the AR. Interestingly, deletion of the ARE did not abrogate the effect of androgen completely suggesting a cooperative transcriptional activity of AR apart from its DNA binding activity.

3.3. Stimulation of Cdc6 transcription by androgen requires intact E2F binding sites in the Cdc6 promoter

Because androgen has been shown to regulate transcriptional activity of E2F transcription factor [34] and given the fact that *Cdc6* transcription is regulated by E2F [27] we determined whether AR mediated stimulation of *Cdc6* transcription is dependent on DNA–protein interaction between *Cdc6* promoter and E2F. We monitored luciferase expression following transfection of the promoter construct Cdc6E2FM containing point mutations in two proximal E2F binding sites. Our results showed that the construct with mutated E2F binding sites failed to replicate the stimulatory effect of R1881 on *Cdc6* transcription (Fig. 3C). Luciferase expression following transfection of Cdc6E2F and ARE double mutant construct (Cdc6E2F/AREDM) did not indicate the stimulatory effect of R1881 on *Cdc6* transcription either (Fig. 3D). When RLU of Cdc6E2FM and Cdc6E2F/AREDM were compared to the wild type *Cdc6* promoter a 4-fold increase in RLU could be noted in untreated LNCaP cells, which did not change significantly following R1881 treatment (Fig. 4B). These experiments further confirmed the repressor effect of E2F on *Cdc6* promoter and that the increased promoter activity in the absence of the inhibitory effect of E2F binding to the proximal E2F binding sites was not mediated through AR and *Cdc6* promoter interaction or through the indirect effect of R1881 treatment.

3.4. Androgen treatment was associated with altered levels of the E2F proteins in the nucleus

Earlier studies have shown differentiable roles of different E2F family members on transcription of Cdc6 gene [35]. E2F3 has been

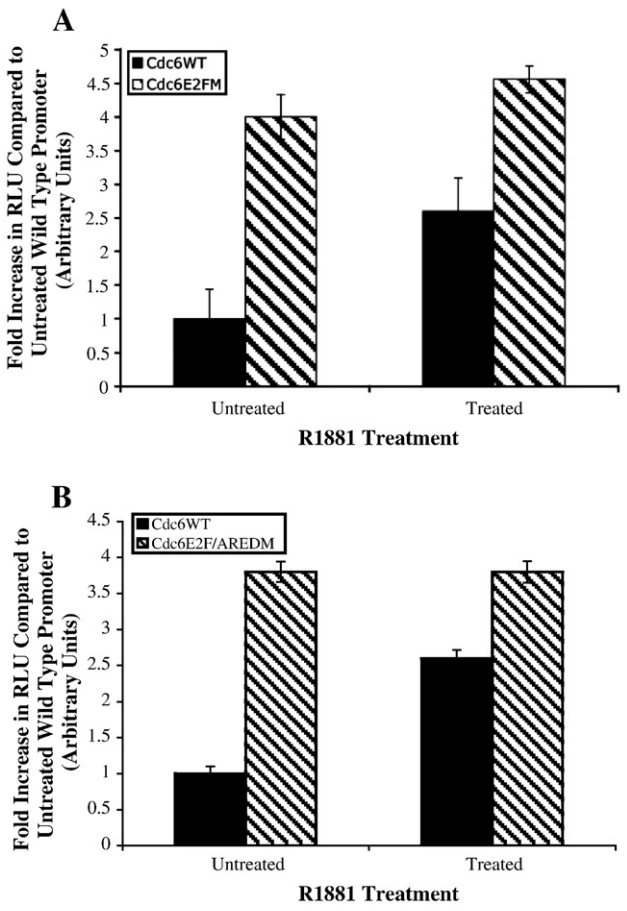

Fig. 4. Mutation in E2F binding sites abrogated repression of *Cdc6* promoter activity with or without an intact ARE. Relative luciferase activity in LNCaP cells transfected with luciferase constructs containing wild type; E2F binding sites mutated; or E2F/ARE doubly mutated *Cdc6* promoters (E2FM and E2F/AREDM) and treated with R1881 (1 nM) for 24 h or left untreated. Data represents mean ± SD of four separate experiments.

shown to bind to the *Cdc6* promoter and stimulate transcription during G/S phase. The other family member E2F1 instead forms a repressor complex with Rb, which occupies *Cdc6* promoter and prevents untimely transcription of *Cdc6* gene at G0 [35–37]. Because androgen has been shown to have influence on expression and transcriptional activities of E2F1 [34] we intended to monitor the effect of R1881 treatment on nuclear concentration of E2F1 and E2F3 in LNCaP cells. Our results showed that in untreated LNCaP cells, E2F1 concentration increased gradually from 0hr reaching a peak at 48h before declining sharply at 72 h. Cells treated with R1881 instead, showed an oscillation in E2F1 concentration between 24 h and 72 h, which varied between 0.8 and 1.3-fold of the E2F1 concentration at the 0 h ($p \leq 0.0445$) (Fig. 5A and B), but no significant difference was noted between treated and untreated cells. When compared with the untreated cells, nuclear E2F1 concentration decreased in the treated cells at 48 h but restored to the initial level at 72 h showing a difference of 6.5-fold ($p=0.0184$) from the untreated cells at 72 h. The concentration of nuclear E2F3 showed a significant increase at 24 h in treated cells compared to the untreated ones ($p=0.013$) but decreased steadily thereafter reaching a nadir at 72 h (Fig. 5C and D). Untreated LNCaP cells whereas, maintained a steady concentration of nuclear E2F3 till 48 h but showed a reduced level of E2F3 at 72 h. No change in E2F1 or E2F3 expressions at 24 h in total cell lysates of LNCaP cells treated with a lower dose of R1881 (0.1 nM) was noted (data not shown).

Analysis of Rb phosphorylation indicated a decline in the hyper-phosphorylated Rb (ppRb) at 24, 48 and 72 h but a sharper decline was noted following R1881 treatment (Fig. 5C and E). Consequently, a

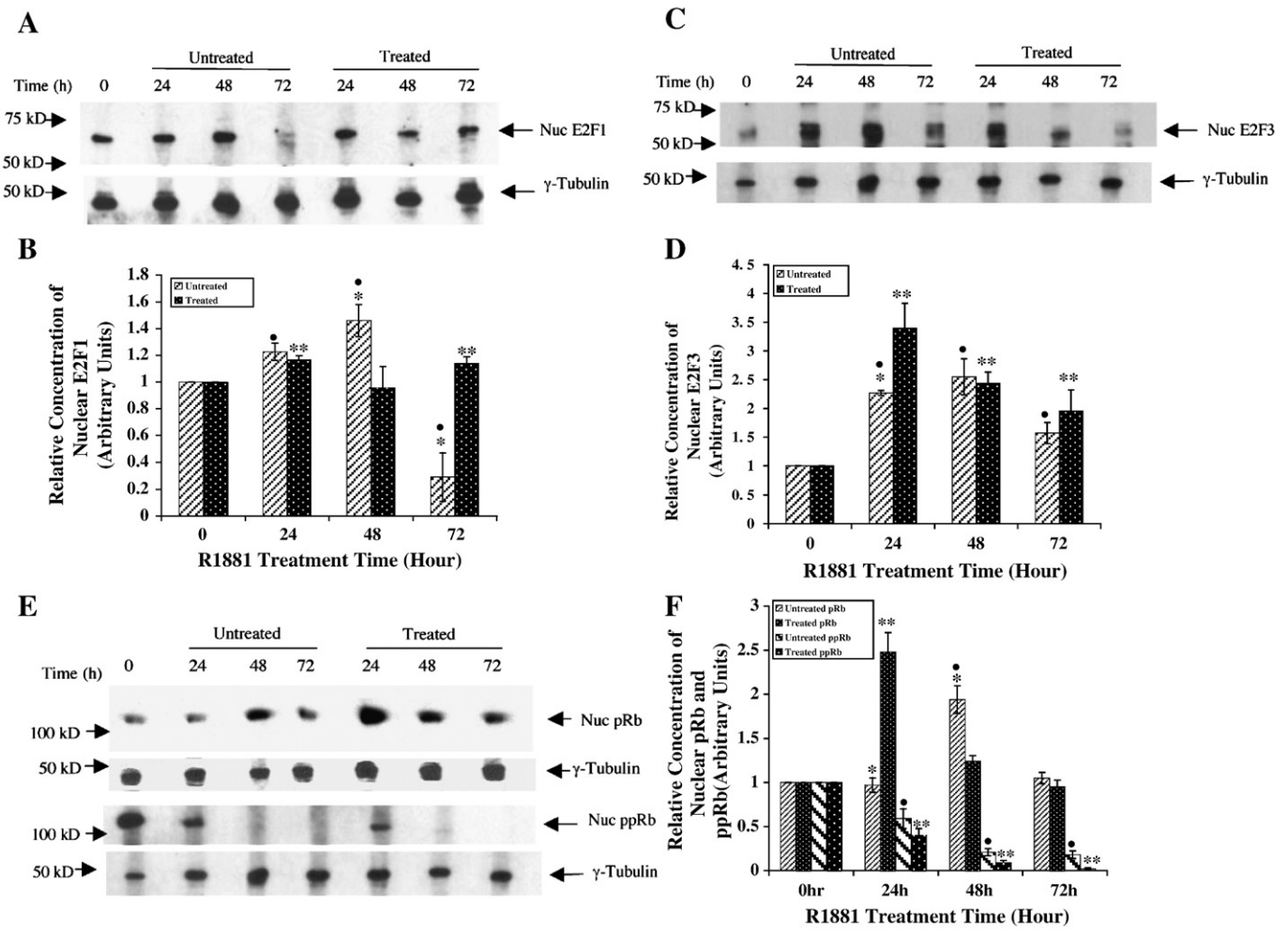

Fig. 5. R1881 treatment modulates levels of E2F transcription factors and Rb in the nucleus of LNCaP Cells. A, C and E: Western blots of nuclear E2F1 (A), E2F3 (C), pRb (E) and ppRb (E) levels in LNCaP cells at different times following R1881 treatment. Gamma tubulin was used as the loading control. B, D, and F: Densitometric analysis of the relative concentration of E2F1 (B), E2F3 (D) and pRb/ppRb (F) after normalization with the values of gamma tubulin. Data represent mean ± SD of three separate analyses for all proteins (p values: *≤0.0346 untreated vs. treated; **≤0.0445 treated vs. 0 h; •≤0.0346 untreated vs. 0 h).

~2.5-fold increase (p=0.007) in the concentration of hypophosphorylated Rb (pRb) was noted in the R1881-treated nuclear extracts at 24 h, which reduced progressively at 48 h and 72 h (Fig. 5F and G). In untreated cells, a delayed increase in pRb concentration was noted at 48 h, which again reduced at 72 h.

3.5. Androgen receptor physically interacts with E2F transcription factor

Retinoblastoma protein has been shown to bind to AR and stimulate its transcription activity through interaction with general transcription factors [18]. Because pRb regulates transcriptional activities of E2F family proteins we intended to monitor whether there is any direct or indirect interaction between AR and E2F proteins using coimmunoprecipitation method (Fig. 6). Western blot analysis showed an increased accumulation of AR in the nucleus following treatment with R1881 (Fig. 6A). Immunoprecipitation of AR from the nuclear extracts using an anti-AR antibody also showed enrichment of AR following R1881 treatment (Fig. 6B). Western blot analysis of the AR immunoprecipitation complex, using an anti E2F3 antibody, showed that E2F3 was pulled down along with AR indicating a distinct association between AR and E2F3 in LNCaP cells (Fig. 6C). Interestingly, unliganded AR is more efficient in enriching E2F3 in immunoprecipitated complex, suggesting that AR–E2F3 interaction is independent of ligand binding. Although treatment with R1881 increased the concentration of E2F3 in nuclear extracts ligand-

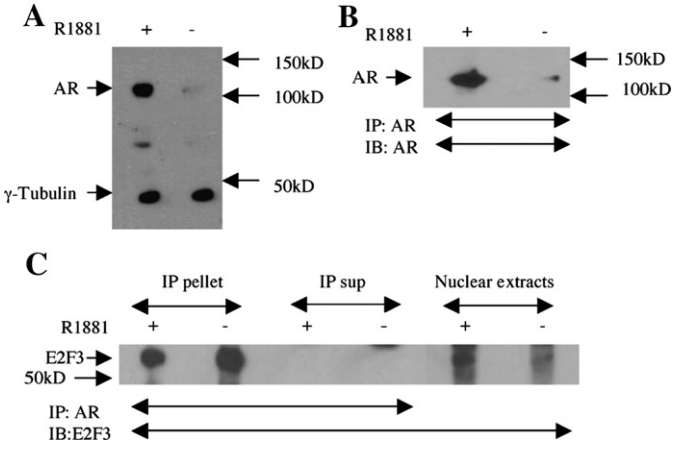

Fig. 6. Complex formation between AR and E2F3. A: Immunoblot analysis of AR in nuclear extracts of LNCaP cells with or without treatment with R1881 for 24 h. Gamma-tubulin was used as the loading control. B: Immunoprecipitation followed by immunoblot analysis of AR using anti-AR antibody from nuclear extracts of LNCaP cell with or without treatment with R1881. C: Immunoprecipitation followed by immunoblot analysis of E2F3 from nuclear extracts of LNCaP cells with or without treatment with R1881. sup: Supernatant.

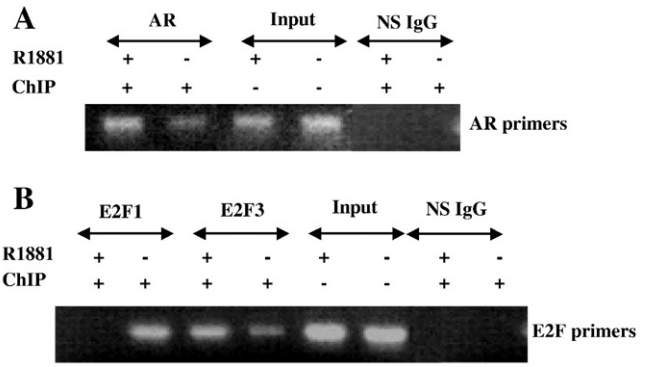

Fig. 7. Binding of AR, E2F1 and E2F3 transcription factors to *Cdc6* promoter changes following R1881 treatment. Chromatin immunoprecipitations using nuclei of LNCaP cells were performed with antibodies specific for AR, E2F1 and E2F3 as indicated and resulting immunoprecipitates or input DNA were amplified using primer pairs corresponding to ARE and E2F binding sites on *Cdc6* promoter. LNCaP cells were treated with R1881 (1 nM) or left untreated for 24 h before harvested for ChIP assay.

bound AR was less competent in associating with E2F3 (Fig. 6C). Similar association between AR and E2F1 was also noted in coimmunoprecipitation experiments (data not shown).

3.6. Androgen facilitates binding of AR and E2F3 to Cdc6 promoter

To determine the promoter occupancy of AR and E2F family transcription factors we used ChIP analyses using R1881 treated and untreated LNCaP cells. We have designed primers to cover 267 bp regions between −732 and −467 bases. Our results showed binding of AR with the *Cdc6* promoter spanning the area containing the putative ARE (Fig. 7A). R1881 treatment facilitated binding of AR to *Cdc6* promoter as the intensity of the AR amplicon from AR ChIP DNA of R1881 treated LNCaP extracts was substantially higher compared to that of untreated extracts. R1881 treatment also enhanced binding of E2F3 but prevented binding of E2F1 to the E2F binding sites in *Cdc6* promoter between −43 and −1 bases [38]. (Fig. 7B) as noted by differential amplification intensities of the amplicons obtained from PCR reactions using E2F1 ChIP and E2F3 ChIP of LNCaP extracts. No amplicon could be detected when E2F1 ChIP DNA from R1881 treated nuclear extracts was used. This observation confirms that the putative ARE in the Cdc6 promoter is indeed a binding site of AR and that the occupancy of the proximal E2F binding site on *Cdc6* promoter by E2F1 and E2F3 is modulated by R1881 treatment.

4. Discussion

In this study, we investigated the involvement of androgen and AR in regulation of *Cdc6* transcription in prostate tumor cells. Our study indicated that androgen supplementation or expression of a functional AR in PC3 cells significantly increased *Cdc6* mRNA and protein concentrations, and androgen withdrawal by castration reduced CDC6 expression. This observation is in support of our earlier finding that CDC6 expression is down regulated in PC3 prostate cancer cells [19], which does not express a functional AR. Treatment of androgen also increased total concentration and nuclear accumulation of CDC6 in both PC3AR and androgen-sensitive LNCaP cells, respectively. Our observations on the effect of androgen on Cdc6 expression are in agreement with studies showing that functional down regulation of AR using genistein or bicalutamide was associated with decreased expression of CDC6 in LNCaP cells [20,39,40]. The present study showed a direct involvement of AR in transcriptional regulation of *Cdc6* in androgen-sensitive prostate cancer cells.

In our earlier studies, we have noted that inclusion of 403 bases (between −391 and −794), which contains a putative ARE sequence in the Cdc6 promoter-luciferase constructs improved luciferase expression 4–5-fold [19]. In this study, we provide evidence of androgen-induced stimulation of *Cdc6* transcription through possible interaction of AR with the putative ARE present in the *Cdc6* promoter as deletion of the ARE sequence significantly attenuated this effect. Occupancy of the ARE sequence by AR is confirmed by our ChIP analysis which showed enriched binding of AR with the region spanning ARE upon R1881 treatment. Interestingly, luciferase reporter assays also indicated a possible functional co-operativity between AR with other transcription factors in regulation of *Cdc6* transcription as the construct with deleted ARE showed a 2.0-fold increase in RLU, although much less than the wild type promoter, following treatment with R1881.

One of the key transcription factors that regulate *Cdc6* transcription is E2F. It has been proposed that in quiescent stages, two proximal E2F sites are occupied by E2F–Rb repressor complexes possibly E2F1–Rb, which upon serum stimulation become dislodged by some unknown mechanism. This allows binding of the other family member, E2F3 as Rb becomes hyperphosphorylated by Cyclin D1/CDK2 and stimulates *Cdc6* transcription [35,36]. In transiently transfected asynchronous cell population, we noticed an increased transcription of *Cdc6* upon point mutations in two proximal E2F binding sites that prevented E2F binding. This suggests abrogation of the repressor effects of Rb–E2F complex on *Cdc6* transcription, which is in agreement with earlier studies done in our laboratory and by others [36,37]. Similar increase in luciferase expression was noted upon removal of the ARE sequence which indicates that the increased *Cdc6* transcription upon mutation of the E2F binding site does not require intact ARE sequence. This is further confirmed by the experiment showing no additional increase in *Cdc6* transcription in Cdc6E2F or Cdc6E2FAREDM cells following treatment with R1881. It is possible that androgen-induced stimulation of *Cdc6* transcription is mediated through removal of the E2F1–Rb inhibitory complex, which in turn indirectly facilitates binding of free E2F3 that acts as a transcription activator. Furthermore, our immunoprecipitation data indicated a ligand-independent interaction of AR with E2F proteins, which could be through a direct binding or through the binding of Rb–E2F complex as AR has been shown to binds to pRb also in a ligand-independent manner [18].

Our DNA/protein immunoprecipitation assays showed that androgen treatment reduced DNA binding of E2F1 transcription factor, which is in support of the earlier studies showing decreased E2F1 transcriptional activity following treatment of LNCaP cells with R1881 [34]. Based on our observation and published reports, we propose a model of androgen-induced *Cdc6* transcription (Fig. 8). In untreated LNCaP cells, pRb forms a complex with E2F and acts as a repressor of *Cdc6* transcription through the occupancy of E2F binding sites. Androgen treatment increases nuclear accumulation of the ligand-

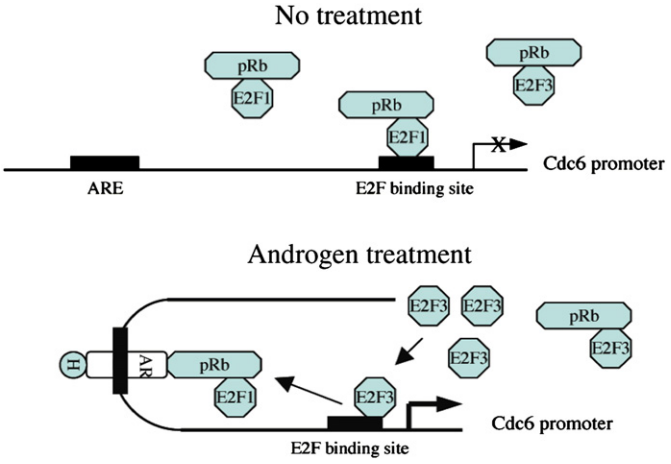

Fig. 8. Proposed model of AR mediated Cdc6 transcription following treatment with androgen (R1881). The diagram is not to the scale and does not show dimerization of the transcription factors.

bound AR, which then binds to the ARE sequence of the *Cdc6* promoter. DNA-bound AR then dislodges Rb–E2F1 repressor complex through binding of the pRb–E2F complex, which possibly occurs through its N-terminal domain close to its DNA binding domain as suggested earlier [18]. Removal of the repressor complex then allows free E2F family proteins, possibly E2F3, to bind to the E2F binding sites in the Cdc6 promoter and thereby, activates its transcription. This model is supported by the data obtained by ChIP analysis showing that R1881 treatment prevented binding of E2F1, while enhancing binding of E2F3 to the *Cdc6* promoter. A significant increase in nuclear accumulation of hypophosphorylated Rb (pRb) at 24 h and subsequent decrease in phosphorylated Rb (ppRb) concentration at 24, 48 and 72 h in R1881 treated cells increases the availability of pRb. Given that the binding of pRb with AR stimulates its transcriptional activity the availability of unphosphorylated Rb aids in binding of co-activators to AR for transcription activation, which includes TFIIF, TBP [41], and TAFII250 [42].

Although AR binds to Rb through its N-terminal domain it is unclear whether the endogenous AR–Rb interaction depends on binding of AR to ARE upon ligand binding. Our results indicated a reduced interaction of AR with E2F3 following androgen treatment suggesting a possible enrichment of free E2F3 proteins in the nucleus. Furthermore, androgen treatment showed an increased nuclear concentration of E2F3 at 24 h. Because androgen treatment increased nuclear accumulation of ligand-bound AR it could be speculated that liganded AR is responsible for sequestration of hypophosphorylated Rb [18] and increased availability of free E2F3, which facilitates its binding to the *Cdc6* promoter. Although we noted an increase in LNCaP cell growth at 24 h following R1881 treatment using MTT assays (data not shown), which is in support of the published study [33], in-depth understanding of the specific function of AR in cell cycle regulation with the context of *Cdc6* transcription requires further study.

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