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# Inhibition of cyclin D1 expression by androgen receptor in breast cancer cells-identification of a novel androgen response element

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**Scientific Publication**

- *Inhibition of cyclin D1 expression by androgen receptor in breast cancer cells-identification of a novel androgen response element*

# SUMMARY

Cyclin D1 gene (CCND1) is a critical mitogen-regulated cell-cycle control element whose transcriptional modulation plays a crucial role in breast cancer growth and progression. Here we demonstrate that the non-aromatizable androgen 5-α-dihydrotestosterone (DHT) inhibits endogenous cyclin D1 expression, as evidenced by reduction of cyclin D1 mRNA and protein levels, and decrease of CCND1-promoter activity, in MCF-7 cells. The DHT-dependent inhibition of CCND1 gene activity requires the involvement and the integrity of the androgen receptor (AR) DNA-binding domain. Site directed mutagenesis, DNA affinity precipitation assay, electrophoretic mobility shift assay and chromatin immunoprecipitation analyses indicate that this inhibitory effect is ligand dependent and it is mediated by direct binding of AR to an androgen response element (CCND1-ARE) located at -570 to -556-bp upstream of the transcription start site, in the cyclin D1 proximal promoter. Moreover, AR-mediated repression of the CCND1 involves the recruitment of the atypical orphan nuclear receptor DAX1 as a component of a multiprotein repressor complex also embracing the participation of Histone Deacetylase 1. In conclusion, identification of the CCND1-ARE allows defining cyclin D1 as a specific androgen target gene in breast and might contribute to explain the molecular basis of the inhibitory role of androgens on breast cancer cells proliferation.

# INTRODUCTION

The progression from a normal mammary epithelial cell to invasive breast carcinoma is driven by the accumulation of genetic damage. This allows the emergence of cell populations harboring genetic alterations which confer the deleterious phenotypes of uncontrolled cell proliferation, increased cell motility, the ability to degrade basement membranes, invade local tissues and metastasize to distant tissue sites. Genetic damage is revealed in dramatic alterations in the karyotypes of breast cancer cells. Mechanistically, amplifications are presumed to contain protooncogenes while deletions contain tumor suppressor genes, these events conferring a selective advantage to their hosts. Thus the subset of genetic alterations found in an individual cancer confer its phenotype, and identification of the genes involved in these processes has the potential to provide better phenotypic markers for use in prognosis and treatment decisions, as well as providing potential new targets for therapeutic intervention.

Cyclin D1 is a cell cycle regulator with a well-characterized role in normal mammary development and mammary carcinogenesis. It plays a pivotal role in the regulation of progression from G1 to S phase of the cell cycle through the formation of active enzyme complexes with the cyclin-dependent kinases Cdk4 and Cdk6. As the name suggests, the activity of cyclin-dependent kinases requires cyclin binding and consequently cyclin abundance is a critical determinant of kinase activity. These kinases phosphorylate substrates including the retinoblastoma tumor suppressor gene product, pRb, relieving its inhibitory function and promoting cell cycle progression. Induction of cyclin D1 gene expression by steroids and growth factors is a key event in mediating their mitogenic responses in

breast cancer cells, since cyclin D1 is rate-limiting for progress through G1 phase. Overexpression of cyclin D1 renders breast cancer cells less dependent on growth factors and steroids, suggesting that its increased expression may lead to loss of normal regulatory constraints and confer a growth advantage. For these reasons, over-expression of cyclin D1 has been linked to breast cancer growth and progression (1–4), as well as development of resistance to hormone therapy (5–8).

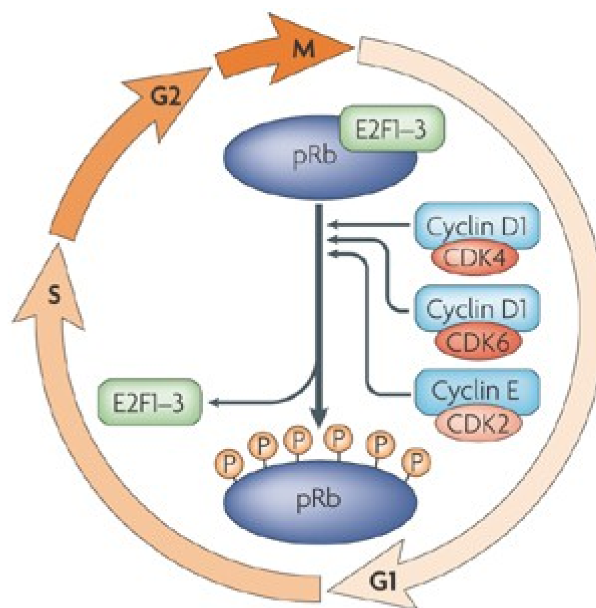


**Figure 1. The central role of the cycline D1 in cell-cycle progression**. Entrance into S phase is mediated by cycline D1 through the formation of active enzyme complexes with the cyclin D1–cyclin-dependent kinase CDK-4 and cyclin D1–CDK6. These coplexes phosphorylate pRb inducing the release of the E2F1–3 transcription factors, which then activate genes that are required for cell-cycle progression.

Strong evidence implicates cyclin D1 amplification and overexpression as a driving force in human breast cancer. Approximately 13% to 20% of breast

cancers possess three to more than 10-fold amplification of DNA encoding for cyclin D1. Cyclin D1 protein overexpression is found in up to 50% of human breast cancers. In many of these tumors the overexpression cannot be explained by increased gene copy number, suggesting that pathogenic activation of cyclin D1 can occur via additional mechanisms, including transcriptional and post-transcriptional dysregulation. The pattern of cyclin D1 overexpression in tissues along the spectrum from normal epithelium to invasive breast cancer also suggests the involvement of cyclin D1 in the earliest stages of mammary carcinogenesis, and in both ductal and lobular subtypes (1). The biologically relevant role of cyclin D1 in breast tumourigenesis has been evidenced by several findings: mammary gland-targeted cyclin D1 over-expression resulted in mammary hyperplasia and adenocarcinoma in transgenic mice (9); cyclin D1 antisense blocked ErbB2-induced mammary tumour growth in vivo (10), and cyclin D1-deficient mice were resistant to ErbB2- or Ras-induced mammary tumourigenesis (11). In addition, the correlation between cyclin D1 expression levels and cellular proliferation in breast cancer cells has been further confirmed by silencing experiments (1,12). Several hormones are involved in breast cancer cells proliferation, so that cyclin D1 represents an important target of their intracellular-signalling pathways (13–16).

Androgens have important physiological effects in women. Not only they are the precursor hormones for estrogen biosynthesis in the ovaries and extragonadal tissues, but androgens act directly via androgen receptors (ARs) throughout the body.

Although the androgen receptor is often co-expressed with the estrogen receptor (ER) and progesterone receptor (PR) in human breast tumors, its role in breast cancer is poorly understood. Specific growth stimulatory and

inhibitory actions of androgens have been described in human breast cancer cell lines. The mechanisms by which androgens exert these contrasting growth effects are unknown.

AR is a member of the steroid hormone receptor which belongs to the superfamily of nuclear receptor that are ligand-inducible transcription factors and, as mediator of androgen signaling, it plays important roles for the coordinated gene expression in male reproductive tissues. The nuclear receptor superfamily describes a related but diverse array of transcription factors, which include nuclear hormone receptors (NHRs) and orphan nuclear receptors. NHRs are receptors for which hormonal ligands have been identified, whereas orphan receptors are so named because their ligands are unknown, at least at the time the receptor is identified.

The AR in the absence of ligand, it is cytoplasmic, existing in a complex containing heat-shock proteins, believed to hold the AR in a ligand binding-competent, inactive conformation. On ligand binding, these proteins were thought to dissociate, although recent evidence indicates that even nuclear steroid receptors are associated with heat-shock proteins. The receptor dimerizes and translocates into the nucleus where it bind to AREs (androgen-response elements) in the promoters of target genes and alters the rate of transcription from these promoters. Increasing transcription requires the recruitment of accessory proteins called co-activators, while inhibiting transcription may require the recruitment of co-repressor proteins (17).

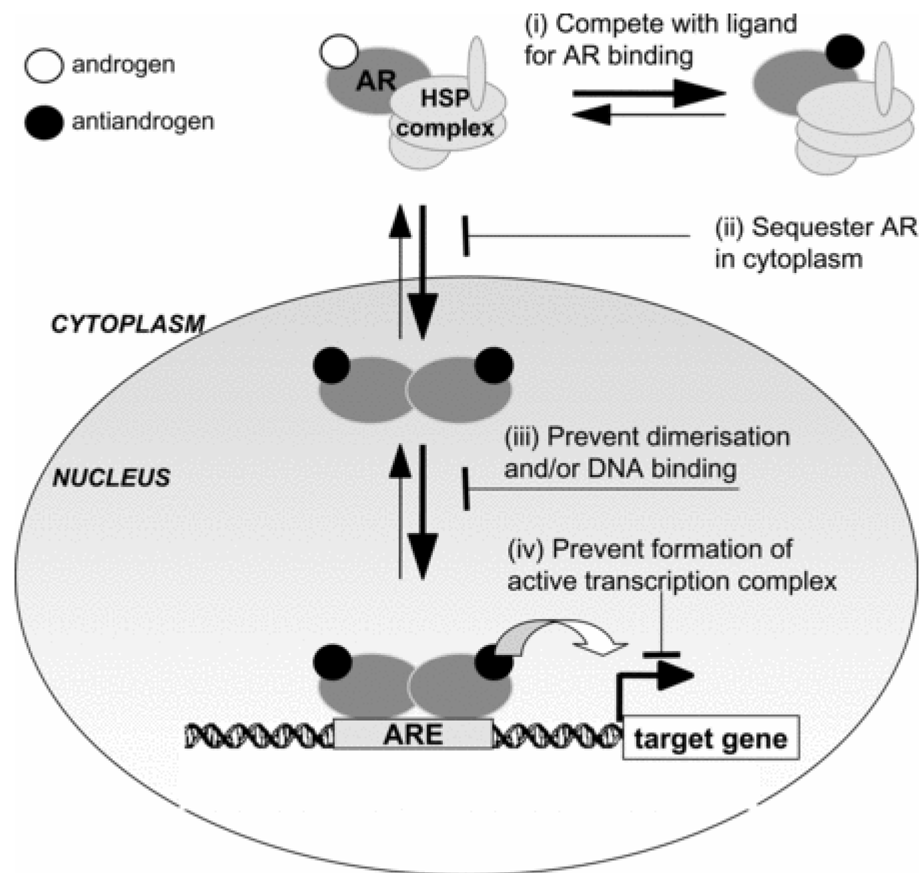


**Figure 2. The AR activation pathway.**

All of the nuclear receptors have common structural features, which include a central DNA binding domain (DBD) responsible for targeting the receptor to highly specific DNA sequences comprising a response element. The ligand binding domain (LBD) is contained in the C-terminal half of the receptor and recognizes specific hormonal and nonhormonal ligands directing specificity to the biologic response. These receptors contain variable N-terminal and C-terminal domains, as well as a variable length hinge region between the DBD and LBD. The androgen receptor in several species (human, rat, calf) is a monomeric protein with a molecular mass of 100–110kDa. The steroid binding domain is confined to a region of 30

kDa, while the DNA-binding domain has the size of approx. 10 kDa. A 40 kDa fragment containing both the DNA and steroid binding domain displayed a higher DNA binding activity than did the intact 100 kDa molecule.(18)

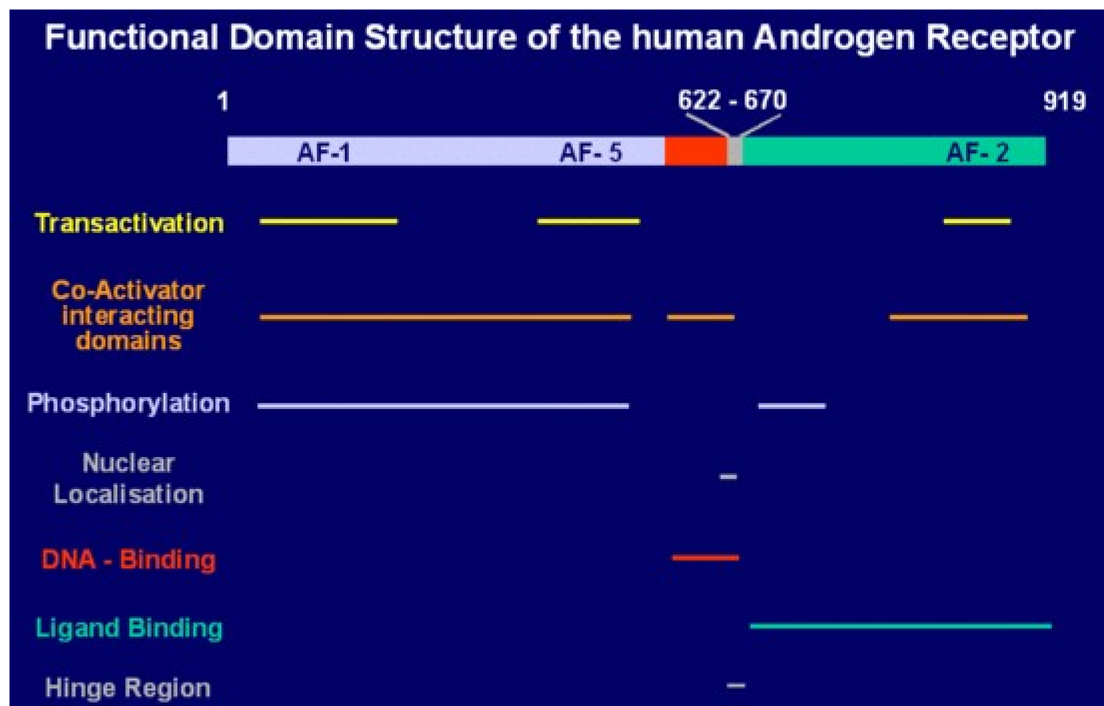


**Figure 3. Human androgen receptor structure.**

Emerging evidences indicate that the androgen signalling pathway mainly exerts inhibitory effects on the growth of normal mammary epithelial cells and plays a protective role in the pathogenesis of breast cancer (19–21). Nonetheless, there are also some epidemiologic reports supporting the concept that androgens, in certain settings, can contribute to breast cancer growth (22–23). Androgens excess (e.g. in congenital adrenal hyperplasia) suppresses breast development (20), while mice lacking a functional androgen receptor (AR) display defective mammary gland development and morphogenesis (21). Furthermore, in vivo studies evidenced that

blocking the action of endogenous androgens results in a significant increase in mammary epithelial cell proliferation (24–25). In vitro, androgen signalling may counteract the proliferative effect of estrogens in AR-positive breast cancer cells, (26), while over-expression of the AR in MCF-7 cells markedly decreases oestrogen receptor α (ERα) transcriptional activity (27- 28). Furthermore, the non-aromatizable androgen 5-α-dihydrotestosterone (DHT) is able to inhibit serum as well as estradiol-induced proliferation in ERα positive breast cancer cell lines (19,28–31), through a mechanism involving an increase in AR protein cell content (28) concomitantly with the down-regulation of the G1/S transition of the cell cycle (28,30). The AR is present in both primary breast tumours (70–90%) and metastases (75%) (32–34) and shows significant association with important clinical and pathologic prognostic factors (35), since AR expression and functional activity correlate with a low tumour grade, smaller tumour size, improved response to hormone therapy and longer patient survival (33,35–41). Conversely, reduced or impaired AR signalling has been demonstrated in hereditary male breast cancer (42) as well as in HER2-positive breast cancers, generally associated with a worse outcome (43). Androgens have been previously used in the adjuvant therapy of breast cancer in both pre-menopausal and post-menopausal women, with an efficacy comparable to that of current hormonal treatment (44–45), and combined hormonal therapy using tamoxifen plus the androgen fluoxymesterone offered some therapeutic advantage over tamoxifen alone in metastatic breast carcinoma (45). Furthermore, it has been suggested that in AR positive breast cancer cells and breast carcinomas, the anti-proliferative effect of aromatase inhibitors seems to be due not only to the reduction in estrogens biosynthesis but also to the unmasking of the

inhibitory effect of androgens acting via the AR (46–47).

Thus, AR is not only frequently expressed in breast tumours and related to prognosis (48), but it also may serve as a predictive marker for adjuvant hormonal therapy (49). However, events following AR activation and leading to inhibition of cell growth are not clearly identified in breast cancer cells.

DAX1 (NROB1; dosage-sensitive sex reversal, adrenal hypoplasia (AHC) critical region on the X chromosome, gene 1) is an atypical member of the nuclear receptor family of transcription factors. Previous work has identified DAX-1 as an inhibitory coregulator for AR (50). DAX1 can sequester AR in the cytoplasm, indicating a possible function of DAX1 as a cytoplasmic retention factor. Furthermore, DAX1 could also inhibits AR function as a corepressor in the nucleus, probably interfering with a crucial event for AR activation in the nucleus (50).

Here we investigated whether DHT-dependent inhibition of breast cancer cell proliferation might be due to the modulation of cyclin D1, whose induction represents a key rate-limiting event in mitogenic signalling leading to S-phase entry. We report the identification of a novel androgen-mediated mechanism that controls the expression of cyclin D1 in MCF-7 breast cancer cells by negatively regulating cyclin D1 transcript and protein levels. Indeed, we identified, in the human cyclin D1 promoter a functional androgen responsive element (ARE), which binds the AR in response to DHT stimulation. Transcriptional repression of CCND1 by AR appears to be consequent to the recruitment of a multiprotein repressor complex involving the participation of the AR corepressor DAX1 and containing histone deacetylase activity.

# MATERIALS AND METHODS

## Cell culture and treatments

Breast cancer epithelial cell line MCF-7 and human embryonic kidney cell line HEK-293 were grown in DMEM/F12 (Gibco, USA) supplemented with 5% calf serum (CS; Gibco) and in DMEM plus 10% foetal calf serum, respectively. 5α-DHT (Sigma, USA) and hydroxyflutamide (OHFl;Sigma) were used at a concentration of $10^{-7}$ M and $10^{-6}$ M, respectively. Before each experiment, cells were grown in phenol red-free (PRF) DMEM, containing 5% charcoal-treated foetal calf serum (PRF–CT) for 3 days and then serum starved in PRF for 24 h to synchronize the cells. All the experiments were performed in 2.5% PRF–CT.

## Cell proliferation assays

MCF-7 cells were seeded on six-well plates ( $10^5$ cells/well) in 2.5% PRF–CT. After 24 h, cells were exposed for 3 days to $10^{-7}$ M DHT and/or $10^{-6}$ M OHFl or left untreated. Media were renewed daily. The effects of the various drugs on cell proliferation were measured 0, 24, 48 and 72 h following initial exposure to treatments by counting MCF-7 cells using a Burker's chamber, with cell viability determined by trypan blue dye exclusion.

In the same experimental conditions, cell viability was also examined using the method of Transcriptional and translational (MTT) colorimetric assay. At the above indicated time points, 100 μl of MTT (5 mg/ml) were added to each well, and the plates were incubated for 4 h at 37°C. Then, 1 ml

0.04N HCl in isopropanol was added to solubilise the cells. The absorbance was measured with the Ultrospec 2100 Prospectrophotometer (Amersham-Biosciences, Italy) at a test wavelength of 570 nm.

**Cell-cycle analysis**

Cell cycle kinetics were studied by flow cytometry (28). MCF-7 cells were seeded on six-well plates ($10^5$ cells/well) in 2.5% PRF–CT. After 24 h, cells were exposed to $10^{-7}$M DHT or left untreated. Cell-cycle analysis was performed 72 h following initial exposure. Cells were washed with PBS, trypsinized, pelletted, resuspended in 2.5% PRF–CT and washed twice with PBS. Cells were then resuspended in 1 ml of PBS, fixed by adding 5 ml of 70% cold ethanol, incubated at 4 °C for 15 min and pelletted. Harvested cells were resuspended in PBS containing 75 M propidium iodide at a density of $10^6$ cells/ml and treated for 30 min at 4 °C in the dark with 2.5 U/ml Rnase A. Finally the cells were filtered to remove aggregates and analysed for DNA content by quantitating the red fluorescence in a FACSCAN apparatus (Becton & Dickson). The percentage of cells in G0/G1, S or G2/M phases of cell cycle was determined by analysis of the results by use of the CELLFIT computer program (Becton & Dickson).

**Plasmids, transfections and luciferase reporter assays**

The following plasmids were used: pcDNA3-AR (AR) encoding full-length AR (27); CMV-P881 (AR(Cys574→Arg)) encoding the full-length AR carrying a mutation in the DNA-binding domain (DBD; (Cys574→Arg)) (51); D1Δ-2960, D1Δ-944, D1Δ-848, D1Δ-254, D1Δ-136 and D1Δ-96,

carrying fragments from the human CCND1 promoter and inserted into the luciferase vector pXP2 (a gift from Dr A. Weitz, University of Naples, Italy); the vector-based pSiAR plasmid, coding for small interfering RNA targeting the 5'-untranslated region of AR mRNA, and the scrambled control construct pSiCon (52); The Renilla reniformis luciferase expression vector used was pRL-Tk (Promega, USA). MCF-7 cells were transfected using Fugene 6 (Roche, CH, USA) according to the manufacturer's instructions. pRL-Tk was used to assess transfection efficiency. Luciferase activity was measured using dual luciferase assay System (Promega), normalized to renilla luciferase activity and expressed as relative luciferase units. For western blotting (WB) assays, MCF-7 cells were plated on 60-mm dishes and transfected with 0.5 μg pSiAR or 0.5 μg pSiCon scrambled control and treated for 24, 48 and 72 h with $10^{-7}$ M DHT or left untreated in PRF–CT. Total proteins were isolated from MCF-7 cells transfected and 50 μg of protein lysates were analysed by WB to evaluate the expression of cyclin D1 and AR. The expression of β-actin was assessed as control of protein loading. AR negative HEK 293 cells were co-transfected with D1Δ-2960 (0.25 μg/well) plus increasing quantities (0,1; 0,25; 0,5 μg/well) of pcDNA3-AR (AR) or CMVP881 (AR Cys574→Arg) (0.5 μg/well). Upon transfection, cells were treated with $10^{-7}$ M DHT, or left untreated in PRF–CT for 24 h. Firefly luciferase activity was detected and expressed as percentage of relative luciferase activity. Activation of the reporter gene D1 Δ-2960 in the presence of PRF–CT is arbitrarily set at 100%. Linear relation between transfected AR plasmid and expressed AR protein quantity was evaluated by WB.

**Immunoprecipitation and WB**

Total cell proteins and the cytoplasmic and nuclear fractions were obtained from 70% confluent cell cultures. The cytoplasmic lysis buffer contained the following: 50mM HEPES pH 7.5, 150mM NaCl, 1% Triton X-100, 1.5mM $MgCl_2$, EGTA 10mM pH 7.5, glycerol 10%, inhibitors (0.1mM $Na_3VO_4$, 1% PMSF, 20 mg/ml aprotinin). Following the collection of cytoplasmic proteins, the nuclei were lysed with the buffer containing 20mM HEPES pH 8, 0.1mM EDTA, 5mM $MgCl_2$, 0.5M NaCl, 20% glycerol, 1% NP-40, inhibitors (as above). A 50 μg of protein lysates was used for WB, while 500 μg was used for IP (53). The following monoclonal (m) and polyclonal (p) antibodies (Ab) were used: anti-AR mAb (441), anti-DAX1 pAb (K-17), anti-Lamin B pAb (C-20), anti-GAPDH pAb (FL-335) and normal mouse immunoglobulin G (Ig) (Santa Cruz Biotechnology, USA). In all IPs, protein lysates were first incubated with primary Abs at 4°C for 4 h in HNTG buffer (20mM HEPES pH 7.5, 150mM NaCl, 0.1% Triton X-100, 10% glycerol, 0.1mM $Na_3VO_4$), and then immune complexes were precipitated for 1 h with appropriate beads, specifically with protein A agarose (Calbiochem) for IPs with polyclonal Abs and anti-mouse IgG agarose (Sigma) for IPs with mouse mAbs. In control samples, the primary Abs were substituted with nonimmune IgGs (rabbit or mouse, depending on the source of the primary Abs). The immunoprecipitated proteins were washed three times with HNTG buffer, separated on a 11% polyacrylamide denaturing gel, analysed by WB, and visualized by ECL chemiluminescence (Amersham).

**Real-time reverse transcription–PCR**

Total RNA was isolated using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions and treated with DNase I (Ambion, Austin, TX, USA). Two micrograms of total RNA were reverse transcribed with the ImProm-II Reverse transcription system kit (Promega); cDNA was diluted 1 : 3 in nuclease-free water and 5 μl were analysed in triplicates by real-time PCR in an iCycler iQ Detection System (Bio-Rad, USA) using SYBR Green. Universal PCR Master Mix (Bio-Rad) with 0.1 μmol/l of each primer in a total volume of 30 μl reaction mixture. Primers used for the amplification were:

**forward:** 5'-CGTGGCCTCTAAGATGAAGGA-3'

**reverse:** 5'-CGGTGTAGATGCACAGCTTCTC-3'

Negative controls contained water instead of first strand cDNA. Each sample was normalized on its 18S rRNA content. The 18S quantification was done using a TaqMan rRNA Reagent kit (Applied Biosystems, USA) following the manufacturer instructions. The relative gene expression levels were normalized to a calibrator that was chosen to be the basal, untreated sample. Final results were expressed as n-fold differences in gene expression relative to 18S rRNA and calibrator, calculated using the $\Delta\Delta C_t$ method as follows: n-fold= $2^{-(\Delta Ctsample - \Delta Ctcalibrator)}$, where $\Delta C_t$ values of the sample and calibrator were determined by subtracting the average $C_t$ value of the 18S rRNA reference gene from the average $C_t$ value of the different genes analysed.

**Site-directed mutagenesis**

The cyclin D1 promoter plasmids bearing the AR responsive (CCND1-ARE) mutated sites (D1Δ-944mARE) were created by site-directed mutagenesis using Quick Change kit (Stratagene, La Jolla, CA, USA), (54).

Briefly, this was based on a PCR reaction with two complementary oligonucleotide primers containing the mutation. The PCR was performedikio454 with the Pfu DNA polymerase during 16 cycles (30 sec at 95°C, 30 sec at 55°C, and 8 min at 68°C), using the following mutagenic primers (mutations are shown as lowercase letters):

- **CCND1 ARE forward**:

5'TTGTGTGCCCGGTCCTCCCCGTCCTTGCATaaaAAATTAGggtTTGCAATTTACA CGTGTTAATGAAA-3'

- **CCND1-ARE reverse:**

5'TTTCATTAACACGTGTAAATTGCAAaccCTAATTTtttATGCAAGGACGGGGAGGACCGGGCACACAA-3';

- **Sp1 forward:**
  5'GCCCCCTCCCCCTGCaaCCaaCCCCaaCaCCCTCCCGCTCCCAT-3'
- **Sp1 reverse:**
  5'ATGGGAGCGGGAGGGtGttGGGGttGGttGCAGGGGGAGGGGGC-3'.

The PCR products were then incubated with DpnI which only digests the parental methylated cDNA and the constructed mutated expression vectors were confirmed by DNA sequencing.

**DNA affinity precipitation assay**

DNA affinity precipitation assay was performed using DAPA protocol (55). Briefly, Nuclear extracts were obtained from MCF-7 cells treated with $10^{-7}$ M DHT or left untreated in PRF–CT for 2 h, 70 μg of nuclear proteins were mixed with 2 μg of specific biotinylated DNA probes (see below) in 400 μl of Buffer D (20mM HEPES, pH 7.9, 10% glycerol, 50mM KCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 10 mM $ZnCl_2$, 1 mM dithiothreitol, and 0.25% Triton X-100) and then incubated on ice for 45 min. After that, 50 μl of streptavidin-agarose beads (Invitrogen) were added, and the samples were agitated for 2 h at 4 °C. Next, the agarose beads-protein complexes were collected by brief centrifugation and washed twice in Buffer D. Proteins were uncoupled from DNA probes by the addition of 40 μl of SDS loading buffer and heating at 96 °C for 10 min. After removal of the beads, the supernatants were analyzed by WB for the presence of AR. The DNA motif probes were prepared by annealing:

- for **CCND1-ARE:**

5'-[Bio]-GCTAAATTAGTTCTTGCAATTTAC-3';

- for **CCND1-mutated ARE:**

5'-[Bio]-CATAAAAAATTAGGGTTTGCAAT-3';

- for **Sp1:**

5'-[Bio]-TGCCCGCGCCCCCTCCCCCTGCGCCCGCCCCCGCCC

CCCT-3'

with the respective unbiotinylated complementary oligonucleotide

- for **CCND1-ARE:**

5'-GTAAATTGCAAGAACTAATTTAGC-3'

- for **CCND1- mutated ARE:**

5'-ATTGCAAACCCTAATTTTTTATG -3'

- for **Sp1:**

5'-AGGGGGGCGGGGCGGGCGCAGGGG-GAGGGGGCGCG GCA-3'.

The specificity of the binding was tested by loading the unbound fraction (Negative control). MCF-7 cell nuclear extracts were used as positive control.

**Electrophoretic mobility shift assay**

Nuclear protein extracts were obtained from MCF-7 cells left untreated or treated with $10^{-7}$ M DHT for 2 h (54). The cytoplasmic lysis Buffer A contained the following :10 mM HEPES-KOH pH 7.9, 1.5 mm $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM PMSF. Following the collection of cytoplasmic proteins, the nuclei were lysed with the Buffer B containing: 20 mM HEPES-KOH pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF).

The double-stranded oligonucleotides used as probes were end labelled with [$\gamma$–$^{32P}$]ATP and T4 polynucleotide kinase and purified using Sephadex G50 spin columns. The oligonucleotides used as probes and as cold competitors (Sigma Genosys, UK) were: (nucleotide motifs of interest are underlined)

- **Probe**: 5'-TGCATGCTAAAATAGTTCTTGCAA-3'
- **Mutated probe**: 5'-TGCATaaaAAAATAGggtTTG-CAA-3'

The protein binding reactions were carried out in 20 µl of buffer (20 mM

HEPES, pH 8, 1 mM EDTA, 50 mM KCl, 10 mM DTT, 10% glycerol, 1mg/ml bovine serum albumin, 50 μg/ml poly(dI-dC)) with 50,000 cpm of labeled probe, 20 μg of MCF-7 nuclear protein, and 5 μg of poly(dI-dC).

The mixture was incubated at room temperature for 20 min in the presence or absence of the unlabelled competitor oligonucleotide. Mouse anti-AR monoclonal antibody (441), or rabbit anti-AR polyclonal antibody (C-19) or normal rabbit IgG (Santa Cruz Biotechnology), were included in some of the reaction mixtures with an additional 12-h incubation at 4 °C before addition of labelled probe. The entire reaction mixture was electrophoresed through a 6% polyacrylamide in 0.25 Tris-borate-EDTA gel for 3h at 150V.

**Chromatin IP**

Chromatin IPs were performed on serum starved MCF-7 cells (56). Cells were treated with $10^{-7}$ M DHT or left untreated in PRF–CT for 2 h. Following treatment, the cells were washed twice with PBS and crosslinked with 1% formaldehyde at 37°C for 10 min. Next, the cells were washed twice with PBS at 4°C, collected and resuspended in 200 μl of lysis buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl pH 8.1) and left on ice for 10 min. Then, the cells were sonicated four times for 10 s at 30% of maximal power (Fisher Sonic Dismembrator) and collected by centrifugation at 4°C for 10 min at 14 000 rpm. The supernatants were collected and diluted in 1.3 ml of IP buffer (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl pH 8.1, 16.7mM NaCl) followed by immunoclearing with 80μl of sonicated salmon sperm DNA/protein A agarose (UBI) for 1 h at 4°C. The immuno-cleared chromatin was precipitated for 12h with specific antybody: anti-AR mAb, anti-DAX1 pAb, anti- Polymerase II

pAb, anti-HDAC1 mAb, anti-HDAC3 pAb and anti-AIB1 mAb (Santa Cruz, USA). After this, 60 μl of salmon sperm DNA/protein A agarose was added and precipitation was continue for 2 h at 4°C. After pelleting, precipitates were washed sequentially for 5 min with the following buffers: Wash A (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH 8.1, 150mM NaCl), Wash B (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH 8.1, 500mM NaCl), and Wash C (0.25M LiCl, 1% NP-40, 1% sodium deoxycholate, 1mM EDTA, 10mM Tris-HCl pH 8.1), and then twice with TE buffer (10mM Tris, 1mM EDTA). The immune complexes were eluted with elution buffer (1% SDS, 0.1M $NaHCO_3$). The eluates were reverse crosslinked by heating at 65°C for 12 h and digested with proteinase K (0.5 mg/ml) at 45°C for 1 h.DNA was obtained by phenol and phenol/chloroform extractions. A 2 μl portion of 10 mg/ml of yeast tRNA was added to each sample and DNA was precipitated with EtOH for 12 h at 4°C and then resuspended in 20 μl of TE buffer. A 4 μl volume of each sample was used as template for PCR with specific primers. The following specific primer pairs were used to amplify 168 bp of the ARE-containing cyclin D1 promoter fragment:

- **forward:** 5'-TACCCCTTGGGCATTTGCAACGA-3'
- **reverse:** 5'-ACAGACGGCCAAAGAATCTCA-3'

and 228 bp of the Sp1-sites containing cyclin D1 promoter fragment:

- **forward:** 5'-GGCGATTTGCATTTCTATGA-3'
- **reverse:** 5'-CAAAACTCCCCTGTAGTCCGT-3'

The amplification products obtained were analysed in a 2% agarose gel and visualized by ethidium bromide staining.

Immunoprecipitated DNA was also analysed in triplicates by real-time PCR by using 5 μl of the diluted (1 : 3) template DNA as described earlier. The

following primer pairs were used:

- **forward:** 5'-GCGCCGGAATGAAACTTG-3'
- **reverse:** 5'-CTGCATCTTCTTTCATTTCATTAACAC-3'

to amplify the ARE-containing cylin D1 promoter fragment. Real-time PCR data were normalized with respect to unprocessed lysates (input DNA). Inputs DNA quantification was performed by using 5 μl of the diluted (1/50) template DNA. The relative antibody-bound fractions were normalized to a calibrator that was chosen to be the basal, untreated sample. Final results were expressed as fold differences with respect to the relative inputs.

**RNA silencing**

For AR gene silencing experiments, MCF-7 cells were transfected using the vector-based pSiAR plasmid or the scrambled control construct pSiCon (52), as described in the 'Plasmids, transfections and luciferase reporter assays' paragraph. For DAX1 gene silencing experiments, custom synthesized siRNA (Ambion) annealed duplexes were used for effective depletion of DAX1 mRNA. A scrambled siRNA that does not match with any human mRNA was used as a control for non-sequence-specific effects (Ambion). Growing cells were switched to PRF for 24 h and then switched to PRF–CT medium for 48 h. After that, cells were trypsinized and transfected in suspension with 5nM siRNA (siDAX1 or scrambled siRNA) in 35-mm dishes, using Lipofectamine 2000 (Invitrogen), following the manufactuer's instructions. Cells were incubated with the siRNA-Lipofectamine 2000 complex at 37°C for 4 h and then switched to fresh PRF and treated or not with DHT ($10^{-7}$ M) for 72 h before analysis. For ChIP assay 100nM siDAX1 was used to silence 60% confluent cells plated

in 150-mm dishes.

**Statistical analysis**

All data were expressed as the mean±SD of at least three independent experiments. Statistical significances were tested using Student's t-test.

# RESULTS

## DHT administration inhibits serum-induced MCF-7 cells proliferation

We previously demonstrated that MCF-7 cells are androgen responsive and that DHT treatment induces a transient increase in AR protein levels (27), similar to those seen in other cell types (57). To investigate the role of activated AR on breast cancer cell proliferation, the response of MCF-7 cells to the non aromatizable androgen DHT and/or the AR antagonist OHFl was measured after 24, 48 and 72 h of treatment in PRF DMEM implemented with 2.5% of steroid-depleted serum (PRF–CT). DHT concentration was chosen based on previous studies demonstrating dose-dependent inhibitory effects of DHT on MCF-7 cells proliferation (28,30). As expected, cells grown in presence of PRF-CT proliferated; DHT treatment instead inhibited serum-induced MCF-7 cells proliferation and, by the end of the treatment, the mean number of DHT-treated cells was ~30% of respective controls. Addition of the AR antagonist OHFl (or, bicalutamide, data not shown) effectively reversed the inhibitory effect of DHT on MCF-7 cells proliferation, suggesting that it was mediated by the AR (Figure 1A). A similar pattern of DHT-dependent effect on MCF-7 cells proliferation was obtained by simultaneously performed MTT colorimetric assay (Figure 1B). These data well correlated with cell-cycle analysis showing an increase of the percentage of cells in G0/G1 phase and a concurrent decrease in the S phase, following 72 h of DHT treatment. At this time point the presence of sub-G1 apoptotic cells was undetectable (Figure 1C).

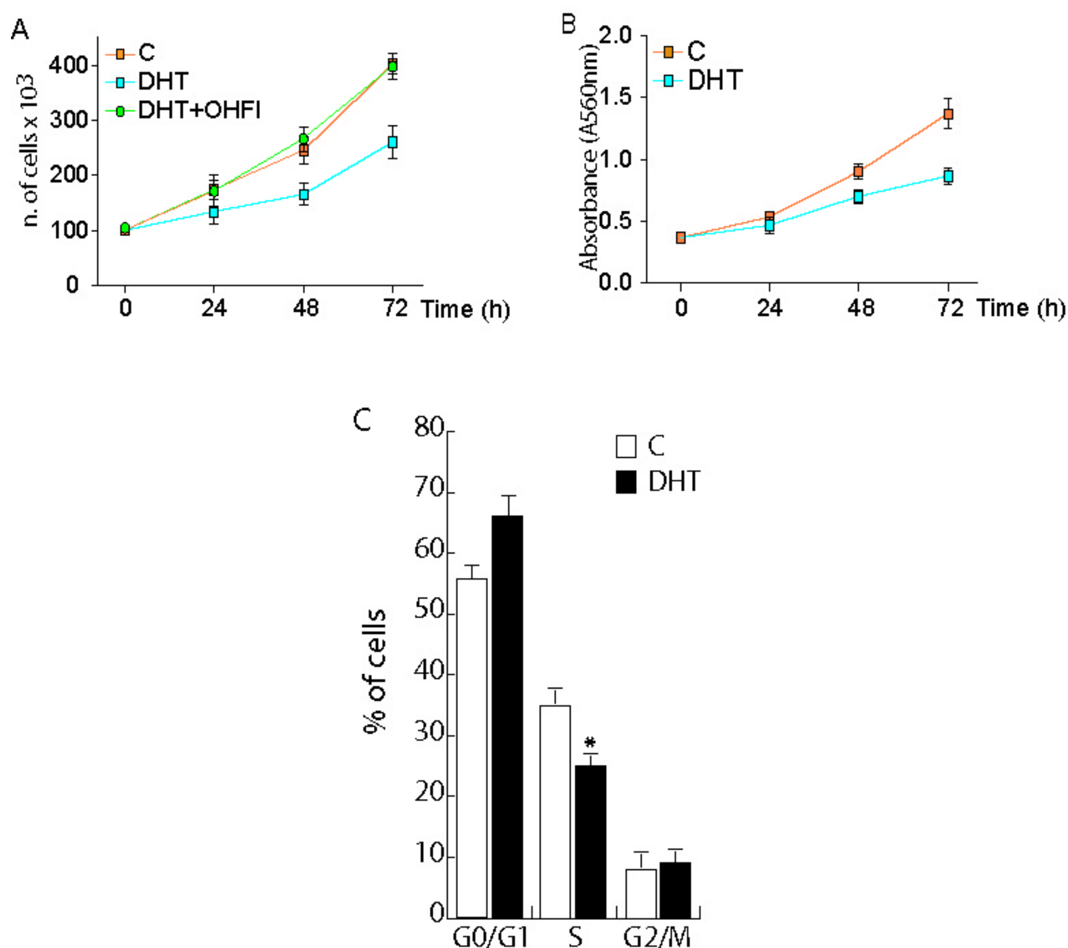


**Figure 1. Proliferative response of MCF-7 cells to DHT**. Serum starved MCF-7 cells were grown in PRF–CT in absence or presence of $10^{-7}$ M DHT and/or $10^{-6}$ M OH-Fl. The effects of the various drugs on cell proliferation were measured 0, 24, 48 and 72 h following initial exposure to treatments by: (A) cell counting using a Burker's Chamber, with cell viability determined by trypan blue dye exclusion or (B) the MTT assay as described in 'Materials and Methods' section. (B) Serum starved MCF-7 cells were grown in PRF–CT in absence or presence of $10^{-7}$ M DHT for 72 h and then subjected to cell-cycle analysis as described in 'Materials and Methods' section. Data, representing a mean±SD of three independent experiments, each in triplicate, were statistically analysed by Student's t-test, *P<0.05 versus untreated.

**Activated AR decreases cyclin D1 expression and promoter activity'**

Since DHT administration reduces the G1/S phase transition in MCF-7 cells (28,30), we inquired whether DHT-induced decrease of MCF-7 cells proliferation might be consequent to the modulation of cyclin D1 expression, whose induction represents a key rate-limiting event in mitogenic signalling leading to S-phase entry. To this aim, serum starved MCF-7 cells were left untreated or treated with $10^{-7}$ M DHT for 24 h, 48 h or 72 h and cyclin D1 expression was assessed by real- time RT–PCR and WB analysis. As shown in Figure 2A, MCF-7 cells exhibited a decrease in the serum-dependent levels of cyclin D1 mRNA (Figure 2A) and protein (Figure 2B) following 48 and 72 h of DHT treatment. The involvement of the AR in the negative regulation of cyclin D1 expression was ascertained by silencing AR expression in MCF-7 cells (Figure 2B).

To test whether activated AR might negatively modulate cyclin D1 promoter activity, MCF-7 cells were transiently transfected with a cyclin D1 promoter reporter plasmid (D1Δ-2996) and left untreated or treated with $10^{-7}$ M DHT. The cyclin D1 promoter was induced by serum, while DHT treatment inhibited basal cyclin D1 promoter activity, decreasing the serum-induced signal by ~50% OHFl addition reversed this effect, suggesting that it was due to AR activation (Figure 2C).

To substantiate the AR involvement in the modulation of CCND1 expression, we evaluated the effects of ectopic AR expression on the transcriptional activity of the cyclin D1 promoter. HEK-293 cells, which do not express AR, were co-transfected with the D1Δ-2996 plasmid and increasing amounts of a full length AR encoding plasmid. In the absence of exogenous AR expression, DHT treatment cannot influence cyclin D1

promoter activity. On the contrary, in the presence of ectopic AR, a dose-dependent decrease in the serum-induced cyclin D1 promoter activity was observed upon DHT administration (Figure 2D).

To investigate whether the effect of AR on cyclin D1 promoter activity is dependent on its transactivation properties, luciferase assay was performed in AR-negative HEK-293 cells transfected with an expression plasmid encoding an AR carrying a mutation (AR(Cys-574→Arg)) in the DBD, which disrupts its ability to bind target DNA sequences (27,51). In these circumstances, no decrease in cyclin D1 promoter activity could be detected (Figure 2D), suggesting the existence, in the cyclin D1 promoter, of putative androgen responsive region(s).

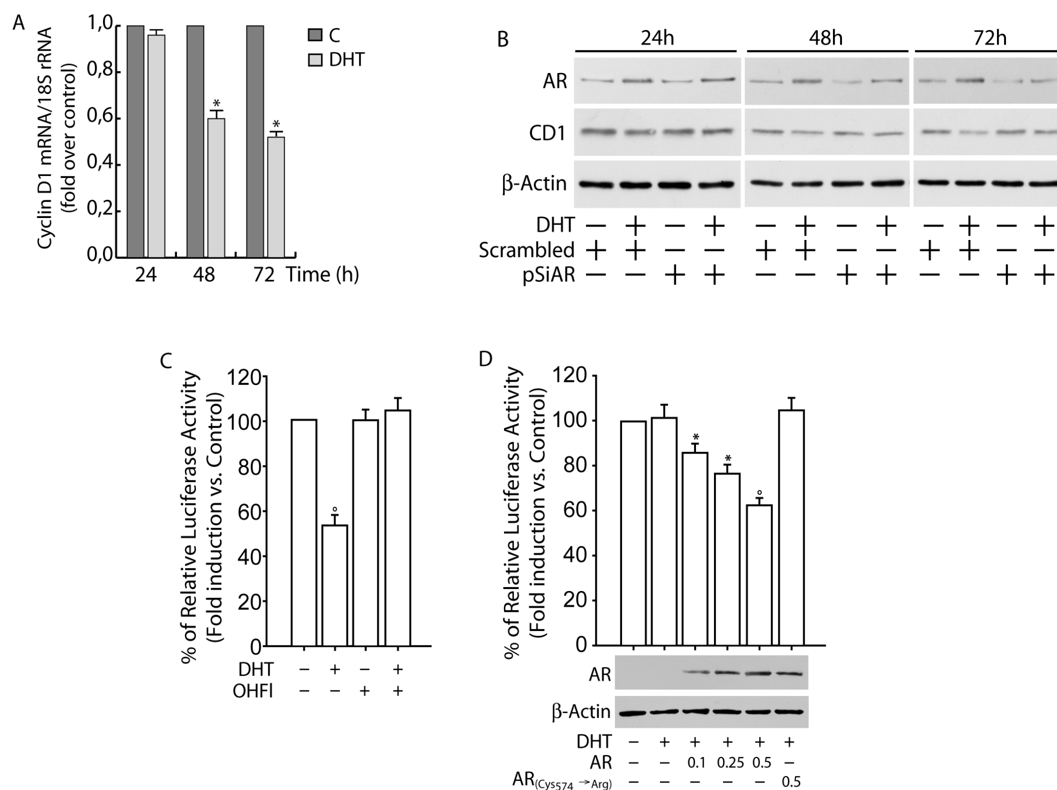


**Figure 2. CCND1 expression and promoter activity are inhibited by DHT-activated AR.** (A) Serum-starved MCF-7 cells were treated or left untreated with $10^{-7}$ M DHT for 24, 48 or 72 h in PRF–CT and total RNA was extracted. The abundance of cyclin D1 mRNA was detected by real-time reverse transcription-PCR, as described in 'Materials and Methods' section. (B) Total proteins were isolated from MCF-7 cells transfected with 0.5 μg pSiAR or 0.5 μg pSiCon scrambled control and treated for 24, 48 and 72 h with $10^{-7}$ M DHT or left untreated in PRF–CT. A 50 μg of protein lysates were analysed by WB to evaluate the expression of cyclin D1 and AR. The expression of β-actin was assessed as control of protein loading. Results are representative of three independent experiments. (C) Serum-starved MCF-7 cells were transiently transfected with D1Δ-2960 reporter plasmid (0.25 μg/well). Upon transfection, cells were treated with $10^{-7}$ M DHT and/or $10^{-6}$ M OH-Fl, or left untreated in PRF–CT for 24 h. Firefly luciferase activity was detected and expressed as percentage of Relative Luciferase Activity. Activation of the reporter gene D1Δ-2960 in the presence of PRF–CT is arbitrarily set at

100%. Results represent the means±SD of five separate experiments each in triplicate. Data were statistically analysed by student's t-test, *P<0.01 versus untreated. (D) AR negative HEK 293 cells were co-transfected with D1Δ-2960 (0.25 μg/well) plus increasing quantities (μg) of pcDNA3-AR (AR) or CMVP881 ($AR_{(Cys\text{-}574 \rightarrow Arg)}$ 0.5 μg/well) as indicated. Upon transfection, cells were treated with $10^{-7}$M DHT, or left untreated in PRF–CT for 24 h. Firefly luciferase activity was detected and expressed as percentage of relative luciferase activity. Activation of the reporter gene D1 Δ-2960 in the presence of PRF–CT is arbitrarily set at 100%. Linear relation between transfected AR plasmid and expressed AR protein quantity was evaluated by WB. Results represent the mean±SD from five independent experiments. Data were statistically analysed by Student's t-test, *P<0.05 and °P<0.01 versus untreated.

**Characterization of functional androgen responsive region(s) in the cyclin D1 promoter**

To define the AR responsive region(s) of the cyclin D1 promoter, a series of 5'-promoter-deleted mutants were used and tested for both androgen sensitivity and promoter activity, MCF-7 cells. The constructs D1 Δ-2996, D1Δ-944 and D1Δ-848, which include 2.960 kb, 0.944 kb and 0.848 kb of the cyclin D1 promoter fragment, respectively, showed a decreased transcriptional activity upon DHT stimulation with respect to untreated controls. A weaker inhibition of the cyclin D1 promoter signal was evidenced using the D1Δ-254 and D1Δ-136 constructs, while the D1Δ-96 plasmid, failed to respond to DHT (Figure 3).

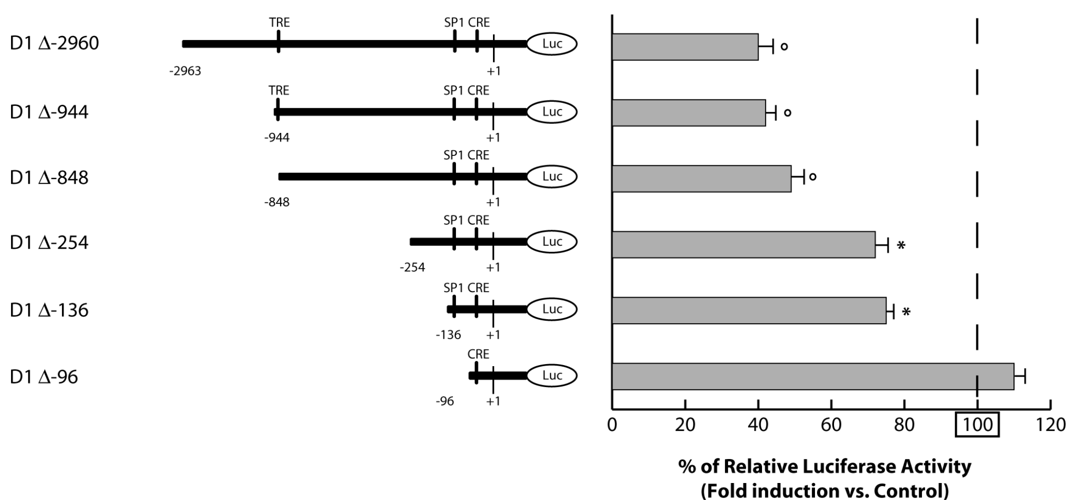


**Figure 3. Functional analysis of the promoter regions involved in AR-mediated modulation of the cyclin D1 promoter.** (Left panel) Schematic representation of human cyclinD1 promoter fragments used in this study. All of the promoter constructs contain the same 3'-boundary (+142). The 5'-boundaries of the promoter fragments varied from 2960 to 96. (Right panel) Transcriptional activity of cyclin D1 promoter constructs following DHT stimulation is shown. Serum-starved MCF-7 cells were transiently transfected with the indicated mutated plasmid (0.25 μg/well), as reported in 'Materials and Methods' section. Upon transfection, cells were treated with $10^{-7}$ M DHT or left untreated in PRF–CT for 24 h. Firefly luciferase activity was detected and expressed as percentage of Relative Luciferase Activity. Activation of reporter plasmids in the presence of PRF–CT only is arbitrarily set at 100. Results represent the means±SD of three separate experiments each in triplicate. Data were statistically analysed by Student's t-test, *P<0.05 and °P<0.01 versus untreated.

Sequence analysis of the -848 bp to -254 bp cyclin D1 promoter DNA fragment revealed a likely ARE sequence (TGCTAAattAGTTCT) located at the -570 bp position of the promoter. This putative ARE (CCND1-ARE) is homologous to the AREs found in the promoters of the *sc* (*sc*-ARE1.2)

and *slp* (*slp*-HRE2) genes (58) (Figure 4A). To assess the relative importance of this sequence we used site-directed mutagenesis to alter it. Nucleotide substitutions were introduced into the 570-bp to 556-bp fragment of D1Δ-944 and the mutant promoter D1Δ-944-mARE was assayed in parallel with the unmutated D1Δ-944 promoter. Disruption of the putative ARE consensus site resulted in a significant loss of the inhibitory effect of DHT on cyclin D1 promoter activity (Figure 4B). These results address the CCND1- ARE as a crucial sequence in mediating cyclin D1 promoter inhibition upon DHT exposure.

Nevertheless, mutations at the putative ARE did not completely abolish cyclin D1 promoter responsiveness to DHT, as indicated by the persistence of a weak DHT-induced decrease in the luciferase activity of the D1Δ-136 construct, containing two Sp1 sites. To investigate whether this additional *cis*-element might be involved in the androgen response, as previously demonstrated for other AR target genes (57,58), site-directed mutagenesis was used to introduce nucleotide substitutions into the -117-bp to -101-bp fragment of D1Δ-944 (D1Δ-944-mSp1) or D1Δ-944-mARE (D1Δ-944-mARE/ Sp1) and the mutant promoters were assayed in functional analysis. As depicted in Figure 4B, DHT treatment is able to induce only an about 30% inhibition of D1Δ-944-mSp1 promoter activity, indicating that Sp1 sites are important for an AR full inhibitory effect on the cyclin D1 promoter. Consistent with these observations, mutation of both ARE and Sp1 motifs completely abolishes DHT responsiveness of the cyclin D1 promoter further suggesting that its regulation occurs through a functional interaction of the AR with the CCND1 ARE and Sp1 responsive sequences.

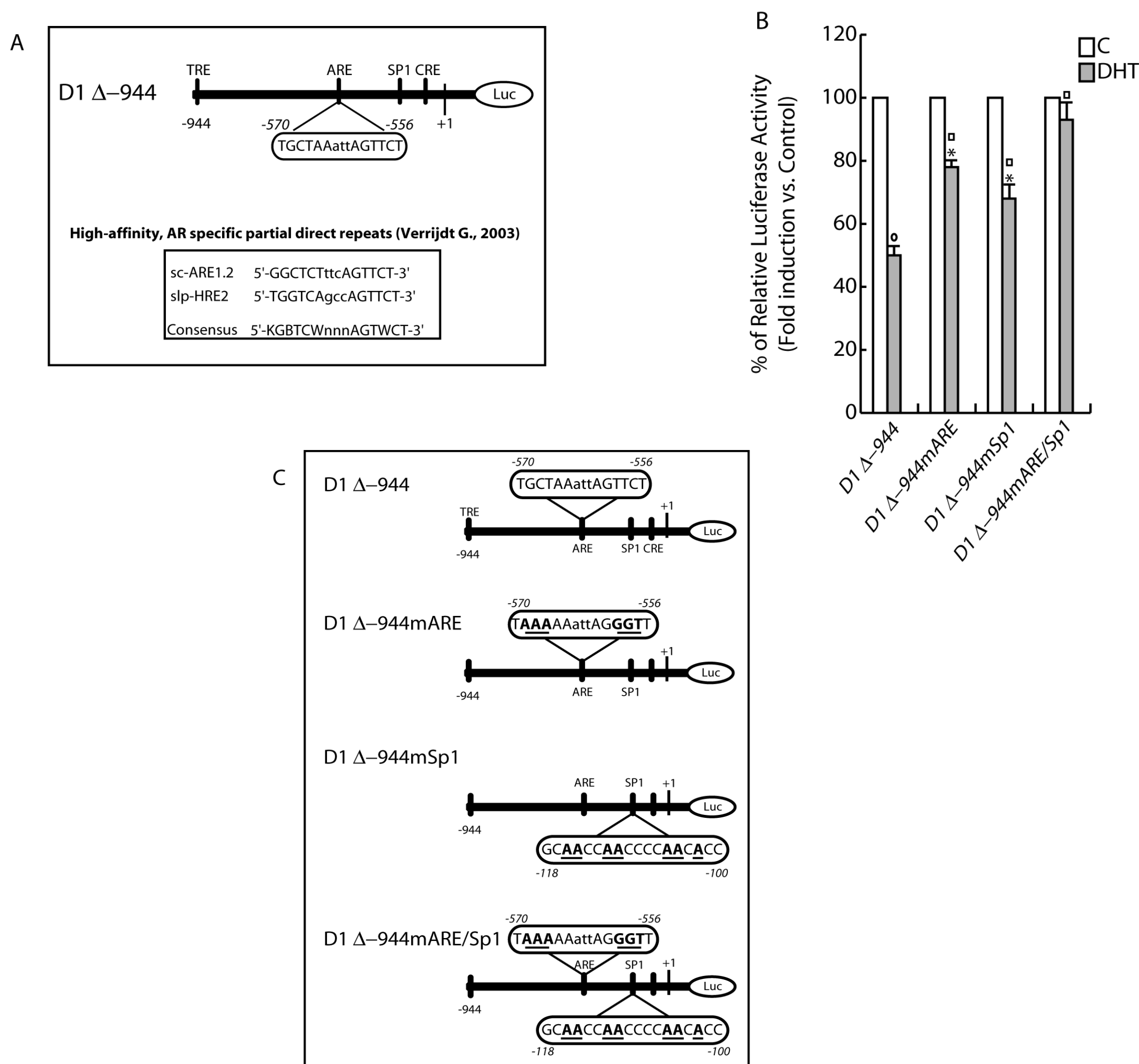


**Figure 4. Mutations of the putative ARE site or the Sp1 sites reduce the ability of AR to down-regulate cyclin D1 transcriptional activity**. (A) Representation of the putative ARE site location within the cyclin D1 promoter. A list of known high affinity AR-specific ARE sequences is listed in the lower panel. (B) Site-directed mutagenesis was performed as described in 'Materials and Methods' section. MCF-7 cells were co-transfected, as reported in 'Materials and Methods' section, by using the mutated promoter constructs, schematically represented in (C). Upon transfection, cells were treated with $10^{-7}$ M DHT or left untreated in PRF–CT for 24 h. Firefly luciferase activity was detected and expressed as percentage of Relative Luciferase Activity. Activation of reporter gene in the presence of PRF–CT is arbitrarily set at 100. Results

represent the means±SD of three separate experiments each in triplicate. Data were statistically analysed by Student's t-test, *P<0.05 and °P<0.01 versus control (C); ▫P<0.01 versus DHT treated D1Δ-944.

**AR interacts with the ARE containing region of the cyclin D1 promoter**

To support the functional importance of the identified ARE sequence, a double stranded oligonucleotide containing the putative ARE sequence was used in a DNA affinity precipitation assay (DAPA) to determine whether AR can bind to the CCND1-ARE consensus sequence. Endogenous AR was found associated with the putative consensus oligonucleotide following DHT administration only, while AR binding was undetectable in nuclear cell lysates from untreated cells (Figure 5A). Moreover, AR from DHT treated MCF-7 lysates was unable to bind to a cyclin D1 promoter oligo in which the putative ARE consensus site was mutated. To further determine the specificity of the putative ARE site we performed electrophoretic mobility shift assay (EMSA). As shown in Figure 5B, factors present in MCF-7 cells nuclear extracts retarded the mobility of the putative ARE domain in a specific manner, since the formation of a protein–DNA complex was found (Figure 5B, lane 1). Of note, DHT treatment induced a strong increase in the protein–DNA association (Figure 5B, lane 2) whose appearance was competed effectively by a 100-fold molar excess of unlabelled probe (Figure 5B, lane 3), demonstrating the specificity of the DNA-binding complex. This inhibition was no longer observed using a mutated putative ARE oligonucleotide as competitor (Figure 5B, lane 4). Contemporary administration of the anti-androgen bicalutamide caused a dramatic decrease in the DNA-binding complex

induced by DHT (Figure 5B, lane 6) suggesting that the AR is involved in the binding to the CCND1-ARE. Finally the specificity of these bands was proved by the drastic attenuation of the complex in the presence of a mouse anti-AR monoclonal antibody, indicating that this antibody recognizes AR epitopes that interact or interfere with AR association to ARE (Figure 5B, lane 7). Moreover, the addition of a rabbit anti-AR polyclonal antibody to the EMSA generated a molecular weight super shift (Figure 5B, lane 8), further indicating the involvement of the AR. Normal rabbit IgG addition did not affect protein–DNA complex formation (Figure 5B, lane 9). To better determine the physiological relevance of the CCND1-ARE, we investigated whether AR interacts with this region of the CCND1 as it exist in native chromatin, performing ChIP in AR-positive MCF-7 and T47D breast cancer cells, using anti-AR or anti-RNA Polymerase (Pol) II antibodies. AR occupancy of the CCND1-ARE containing region of cyclin D1 promoter was induced in a ligand-dependent manner, since AR recruitment was enhanced by DHT (Figure 5C). The inhibitory role of AR on cyclin D1 promoter, is further evidenced by the dynamic of RNA Pol II recruitment onto the cyclin D1 promoter, that appears to be drastically reduced upon DHT treatment (Figure 5C).

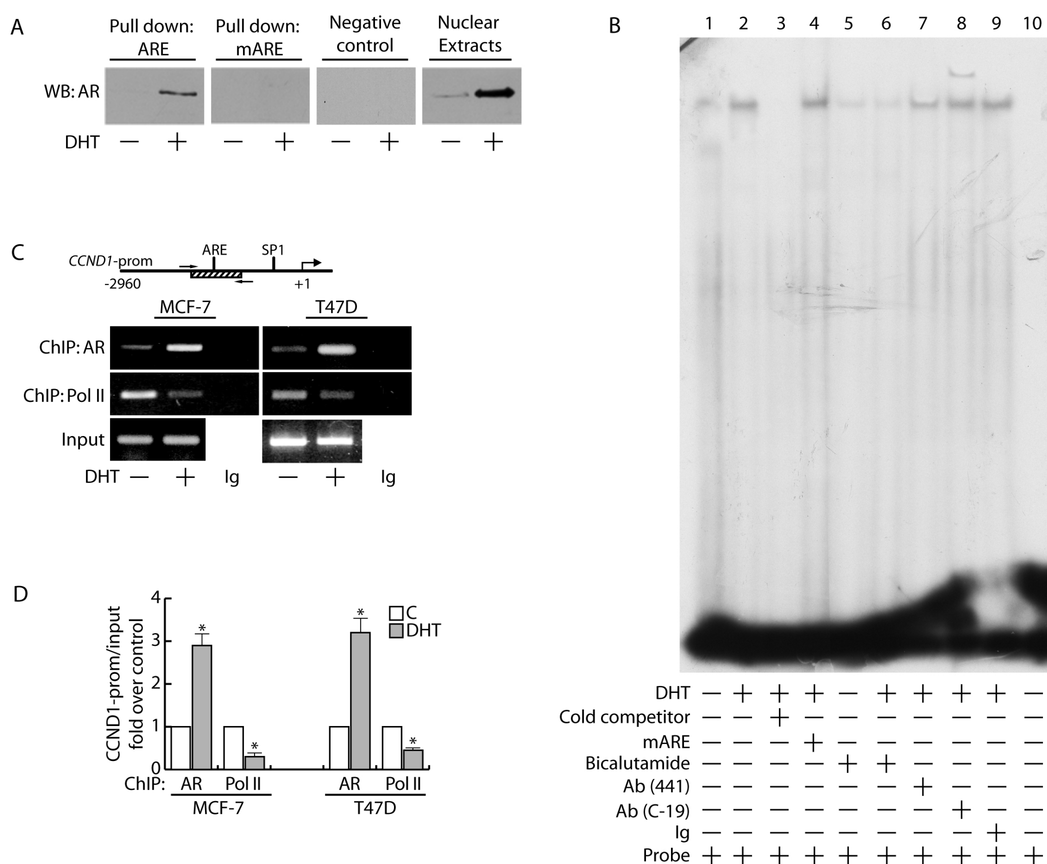


**Figure 5. DHT induces activation of AR-DNA-binding activity in MCF-7 cells and AR recruitment to the ARE containing region of the cyclin D1 promoter**. (A) Nuclear extracts from MCF-7 cells treated with $10^{-7}$ M DHT or left untreated in PRF–CT for 2 h, were incubated with either a biotinylated oligonucleotide containing the CCND1-ARE site or a biotinylated oligonucleotide mutated in the CCND1-ARE (mARE) consensus site and subjected to DNA affinity precipitation assay as described in 'Materials and Methods' section. Specifically bound proteins were subjected to WB analysis using an antibody specific to AR. The specificity of the binding was tested by loading the unbound fraction (Negative control). MCF-7 cell nuclear extracts were used as positive control. (B) Nuclear extracts from MCF-7 cells left untreated (lane 1) or treated with $10^{-7}$ M DHT for 2 h (lanes 2–4 and 6–9) were incubated with a double-stranded CCND1-ARE-specific consensus sequence probe labelled with [$\gamma^{32P}$]ATP and subjected to electrophoresis in a 6% polyacrylamide gel. Competition experiments were

done by adding as competitor a 100-fold molar excess of unlabelled probe (lane 3) or a 100-fold molar excess of unlabelled oligonucleotide containing a mutated CCND1-ARE (lane 4). The formation of the DNA–AR complex was blocked by the addition of $10^{-5}$M Bicalutamide (lanes 5 and 6). The specificity of the binding was tested by adding an anti-AR antibodies to the reaction mixture (lane 7-8) or IgG (lane 9). Lane 10 contains probe alone. (C) Chromatin IPs were carried out on serum starved MCF-7 and T47D cells as described in 'Materials and Methods' section. Cells were treated with $10^{-7}$ M DHT or left untreated in PRF–CT for 2 h, lysed and DNA-associated proteins were precipitated using either anti-AR Ab or anti-RNA Pol II Ab (2 μg/sample each). In control samples (Ig), normal mouse or rabbit IgG, respectively, was used instead of the primary Abs as control of Ab specificity. The 5'-flanking sequence of the CCND1 containing the ARE site was detected by PCR with specific primers listed in 'Materials and Methods' section. Inputs DNA were amplified as loading controls. (D) Five-microlitres volume of each sample and input from ChIP were used for real-time PCR as described in the 'Materials and Methods' section. Data were statistically analysed by Student's t-test, *P<0.05 versus untreated.

## AR associates with the SP1 sites present in the cyclin D1 proximal promoter

To further investigate the functional importance of the Sp1 sites in the AR mediated modulation of the cyclin D1 promoter, the ability of AR to associate to the Sp1 sites containing sequence of the cyclin D1 promoter (59) was first examined by DAPA. The association between AR and the cyclin D1-Sp1 sequence was observed only upon DHT treatment, while AR binding could not be detected in nuclear cell lysates from untreated cells (Figure 6A). AR recruitment on the Sp1-containing region of the endogenous cyclin D1 promoter was also investigated by ChIP experiments using anti-AR or anti-RNA Pol II antibodies. AR was recruited to the Sp1 sites containing promoter region in a ligand dependent manner. Conversely,

upon DHT administration, RNA Pol II was released from the investigated promoter region (Figure 6B) supporting the hypothesis that this Sp1-containing DNA region might cooperate with the CCND1-ARE site in theAR-dependent regulation of CCND1.

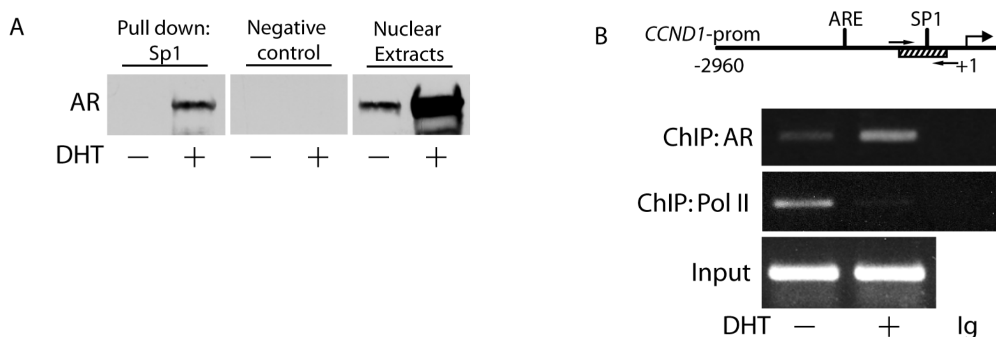


**Figure 6. DHT induces recruitment of AR to the Sp1-site containingv region of the cyclin D1 promoter.** (A) Nuclear extracts from MCF-7 cells treated with $10^{-7}$ M DHT or left untreated in PRF–CT for 2 h, were incubated with a biotinylated oligonucleotide containing the CCND1-Sp1 site and subjected to DNA affinity precipitation assay as described in the 'Materials and Methods' section. Specifically bound proteins were subjected to WB analysis using an antibody specific to AR. The specificity of the binding was tested by loading the unbound fraction (Negative control). MCF-7 cell nuclear extracts were used as positive control. (B) Chromatin IPs were carried out on serum starved MCF-7 cells as described in the 'Materials and Methods' section. Cells were treated with $10^{-7}$ M DHT or left untreated in PRF–CT for 2 h, lysed and DNA-associated proteins were precipitated using either anti-AR Ab or anti-RNA Pol II Ab (2 μg/sample each). In control samples (Ig), normal mouse or rabbit IgG was used, respectively, instead of the primary Abs as control of Ab specificity. The 5'-flanking sequence of the CCND1 containing the Sp1 site was detected by PCR with specific primers listed in the 'Materials and Methods' section. Inputs DNA were amplified as loading controls. Results are representative of three independent experiments.

## The AR corepressor DAX1 is recruited at the CCND1- ARE containing sequence of the cyclin D1 promoter

To assess whether the decrease of cyclin D1 promoter transcriptional activity might be caused by the cooperative interaction between AR and negative transcriptional regulators, we investigated the involvement of the orphan nuclear receptor DAX1, which has been shown to interact with and function as a negative coregulator of AR (60–62). DAX1 abundance was analysed in cytoplasmic and nuclear protein fractions obtained from MCF-7 cells stimulated or not with $10^{-7}$ M DHT. DAX1 was mainly present in the nuclear compartment and its abundance increased upon DHT treatment (Figure 7A). To investigate whether the DHT induced enhancement of DAX1 expression is paralleled by an AR/DAX1 physical interaction, co-IP assay was performed on nuclear and cytoplasmic protein fractions from MCF-7 cells. The formation of a AR/DAX1 complex was clearly evidenced into the nucleus upon DHT administration (Figure 7B), arguing for a DAX1 regulatory function on AR transcriptional activity.

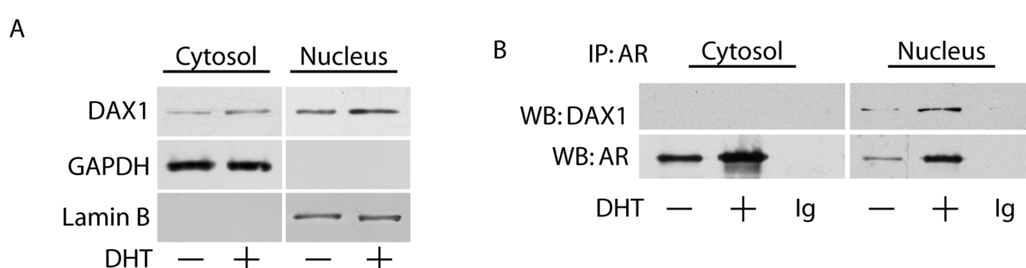


**Figure 7. DHT induces the formation of a AR/DAX1 complex into the nucleus.** (A) Serum starved MCF-7 cells were treated with $10^{-7}$ M DHT or left untreated in PRF–CT for 24 h. The expression of DAX1 and AR was determined by WB using 50 µg of either cytoplasmic or nuclear protein lysates. The expression of GAPDH and Lamin B was

assessed as control of protein loading and purity of lysate fractions. (B) Five hundred micrograms of either nuclear or cytoplasmic lysates from MCF-7 cells, treated with $10^{-7}$ M DHT or left untreated in PRF–CT for 24 h, were immunoprecipitated (IP) with anti-AR mAb and subjected to WB to detect DAX1 and AR protein levels. Results, obtained after repetitive stripping and reprobing of the same filters, are representative of three independent experiments.

To test whether DAX1 could assemble on the androgen responsive region of the cyclin D1 promoter, DAPA assay was performed. The binding affinity of DAX1 to the DNA probe containing the putative CCND1-ARE site (Figure 8A) or the Sp1 site (Figure 8B) was significantly changed in response to DHT. Furthermore, no binding of another AR corepressor, such as SMRT, to the CCND1- ARE containing DNA probe could be detected (data not shown). To verify that DAX1 is recruited within the androgen responsive region of human cyclin D1 promoter in vivo, ChIP assay was performed using anti-DAX1 antibody. DAX1 occupancy of the CCND1-ARE containing region of the cyclin D1 promoter significantly increased following DHT treatment. No recruitment of the steroid receptor coactivator AIB1, was detected under the same experimental condition (Figure 8C and D), showing the selectivity of corepressor recruitment. A similar pattern of DAX1 recruitment to the Sp1-containing region of the cyclin D1 promoter was observed (Figure 8E). Since histone deacetylation represents at least one of the mechanisms by which repressor proteins mediate transcriptional repression (63–64), we investigated HDAC1 and/or HDAC3 association on the AR responsive sequences within the cyclin D1 promoter by DAPA and ChIP assays. In both assays, DHT stimulation induces the recruitment of HDAC1 but not HDAC3 to either the CCND1-

ARE consensus sequence (Figure 8A, C and D), or the Sp1 site (Figure 8B and E).

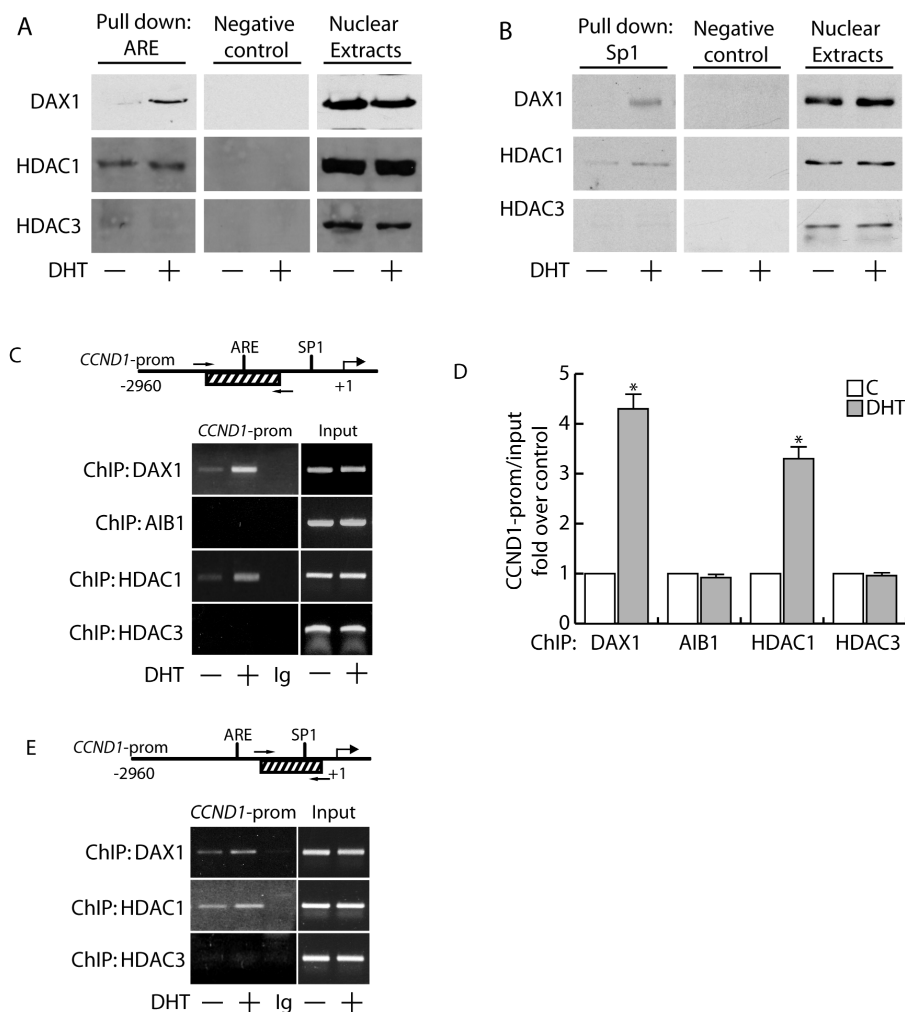


**Figure 8. DHT induces the recruitment of DAX1 and histone deacetylase 1 to the CCND1-ARE and Sp1 sites containing regions of the cyclin D1 promoter.** (A) and (B) Nuclear extracts from MCF-7 cells treated with $10^{-7}$ M DHT or left untreated in PRF–CT for 2 h were incubated with either a biotinylated oligonucleotide containing the CCND1-ARE site (A) or the Sp1 site (B) and subjected to DNA affinity precipitation assay as described in the 'Materials and Methods' section. Specifically bound proteins were subjected to WB analysis using antibodies specific to DAX1, HDAC1 or HDAC3. The specificity of the binding was tested by loading the unbound

fraction (Negative control). MCF-7 cell nuclear extracts were used as positive control. (C) Chromatin IPs were carried out on serum starved MCF-7 cells as described in the 'Materials and Methods' section. Cells were treated with $10^{-7}$M DHT or left untreated in PRF–CT for 2 h, lysed and DNA-associated proteins were precipitated using antibodies (Ab) specific to AR, AIB1, HDAC1, HDAC3 (2 μg/sample each). In control samples (Ig), normal mouse or rabbit IgG was used instead of the primary Abs as control of Ab specificity. The 5'-flanking sequence of the CCND1 containing the CCND1-ARE site was detected by PCR using specific primers listed in the 'Materials and Methods' section. Inputs DNA were amplified as loading controls. (D) Five microlitres volume of each sample and input from ChIP were used for real-time PCR as described in the 'Materials and Methods' section. Data were statistically analysed by Student's t-test, $*P<0.05$ versus untreated. (E) ChIP assays were carried out as above described. The 5'-flanking of the CCND1 containing the Sp1 sites was detected by PCR using specific primers reported in the 'Materials and Methods' section. Input DNAs were amplified as loading controls. Results are representative of three independent experiments.

To confirm the role of DAX1 in the AR-dependent modulation of the CCND1, RNA silencing was used to knockdown the expression of endogenous DAX1 in MCF-7 cells. A 50% decrease of DAX1 protein levels was achieved using anti-DAX1 siRNA in MCF-7 cells after 48 h (Figure 9A). ChIP analysis was then performed on MCF-7 cells transfected with DAX1 siRNA or scrambled control siRNA. Upon DHT stimulation, DAX1 knockdown caused the recovery of RNA Pol II recruitment within the CCND1-ARE (Figure 9B and C) and Sp1 (Figure 9D) containing region of the cyclin D1 promoter, whereas the scrambled siRNA showed no effect on RNA Pol II dismissal. AR recruitment on the responsive regions of the cyclin D1 promoter was not affected by DAX1 silencing (Figure 9B, C and D). Moreover, DAX1 knockdown resulted in the loss of the DHT inhibitory effect on cyclin D1 levels (Figure 9A), strengthening the role of

DAX1 as a component of the repressor complex participating in the AR-dependent inhibition of the CCND1.

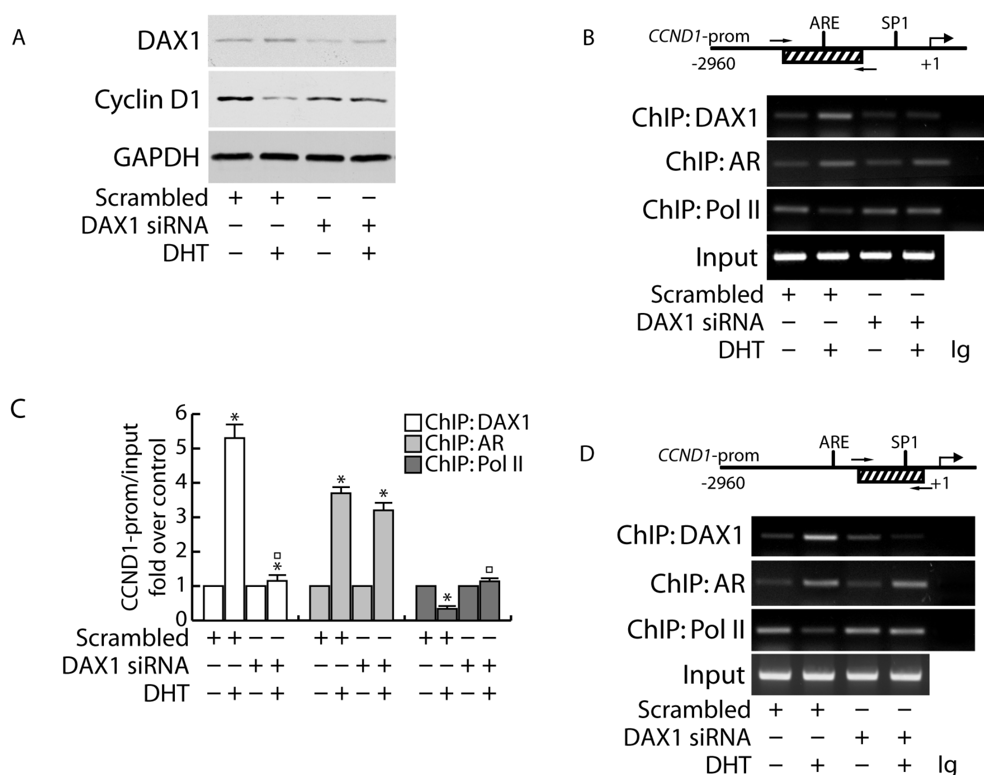


**Figure 9. Effect of DAX1 knockdown on the modulation of CCND1 expression by AR.** (A) Total cellular proteins were isolated from MCF-7 cells transfected with 5nM DAX1 siRNA or 5nM scrambled control siRNA (scrambled) and treated for 72 h with $10^{-7}$ M DHT or left untreated in PRF–CT. Fifty micrograms of protein lysates were analysed by WB to evaluate the expression of DAX1 and cyclin D1. The expression of GAPDH was assessed as control of protein loading. Results were obtained after repetitive stripping and reprobing of the same filters. (B) ChIP assays were carried out on serum starved MCF-7 cells transfected with 100nM DAX1 siRNA or 100nM scrambled control siRNA (scrambled). Cells were then treated with $10^{-7}$ M DHT for 2 h and DNA-associated proteins were precipitated using either anti-DAX1, AR or Pol II antibodies (2 μg/sample each). In control samples (Ig), normal mouse or rabbit IgG were used instead of the primary Abs, as control of Ab specificity. The region of the

cyclin D1 promoter containing the CCND1-ARE site, was detected by PCR using specific primers listed in the 'Materials and Methods' section. Inputs DNA were amplified as loading controls. (C) Five-microliters volume of each sample and input from ChIP were used for real-time PCR as reported in the 'Materials and Methods' section. Data were statistically analysed by Student's t-test, * $P<0.05$ and ▫$P<0.05$ versus scrambled DHT treated. (D) ChIP assays were carried out as above described. The 5'-flanking sequence of the CCND1 containing the Sp1 sites was detected by PCR using specific primers listed in the 'Materials and Methods' section. Input DNAs were amplified as loading controls. Results are representative of three independent experiments

# DISCUSSION

The AR represents a common characteristic in breast tumors, since it is expressed in a significant subset (60–90%) of both ERα-positive and -negative breast cancers (33–35). AR expression in breast cancer tissue samples has been shown to be associated with a better prognosis (35,38–41,65–66). Conversely, lack of AR expression correlates with transformation from in situ to invasive basal subtype of high-grade ductal breast carcinoma (48) and with a poor prognosis in lymph node-positive ER/PR/Her2-negative breast cancers (41); even though a recent report suggests that the AR may participate to the development of invasive ductal carcinoma by repressing E-cadherin expression (67). Despite the growing body of evidence indicating a protective role of AR in breast tumourigenesis, the mechanisms underlying the inhibition of the in vivo and/or in vitro breast cancer cells growth by activated AR, remain poorly defined. In this report we provide evidence that DHT-activated AR is a transcriptional repressor of the CCND1 in MCF-7 breast cancer cells. Analysis of the molecular events associated with this hormone-induced negative regulation of cyclin D1 expression allows, for the first time, the identification of a specific ARE within the CCND1 proximal promoter, and recognizes the recruitment of DAX1 and HDAC1 as components for AR-mediated transcriptional repression. Earlier studies have shown that DHT inhibits MCF-7 cells proliferation by targeting the G1/S transition of the cell cycle (28,30). Here, we demonstrated that, in MCF-7 cells, cyclin D1 expression is negatively modulated by DHT administration as evidenced by reduction of cyclin D1 mRNA and protein levels, and decrease of its promoter activity. The involvement of activated AR in this process is

substantiated by the observation that in AR-negative HEK-293 cells, DHT administration is able to interfere with cyclin D1 promoter activity only in the presence of exogenous AR expression. Moreover, the DHT inhibitory effect strictly depends on the integrity of the AR-DBD, suggesting the existence, in the cyclin D1 promoter, of a putative androgen responsive region which mediates androgen response. A number of studies examined the domains important for the functions of the cyclin D1 promoter demonstrating that it is a complex transcriptional unit composed of several distinct elements (59,68–70). Nevertheless, to date, no androgen response elements (AREs) have been identified within the cyclin D1 promoter. The present study provides evidence that AR-mediated inhibition of the CCND1 is consequent to direct binding of the AR to a specific androgen responsive site, located at position -570 bp as indicated by deletion and sequence analyses of the cyclin D1 proximal promoter. The identified CCND1-ARE motif 5'-TGCTAAattAGTTCT-3' resembles the so-called selective AREs that exhibit characteristics of partial direct repeats, instead of the conventional inverted repeats of the 5'-TGTTCT-3' monomer-binding element, and are recognized exclusively by the AR (58,71,72). This CCND1-ARE is functional, as demonstrated by transactivation studies, and capable to interact with the AR, in a ligand-dependent manner as evidenced by DAPA or EMSA. The physiological relevance of CCND1-ARE within the cyclin D1 promoter in vivo is pointed out by ChIP analysis showing that AR occupancy of the ARE containing promoter region is concomitant with a decrease in RNA Pol II recruitment, consistent with the reduced cyclin D1 transcriptional activity. Our study also indicates that AR binding to the Sp1 sites within the proximal cyclin D1 promoter is required for a full DHT-dependent inhibition of cyclin D1 promoter activity. This

observation is consistent with previous studies on the organization of transcription factor-binding sites within the regulatory regions of a selection of androgen-responsive genes, showing that a key feature of these genes is the presence of one or more AR-binding site (ARE, HRE) together with binding sequences for housekeeping (Sp1, NF-1), inducible (NF-kB, AP1) and tissue specific transcription factors (73). Therefore, androgen-regulated gene expression appears to be dependent on the coordinated interactions of the receptor protein and other transcription factors. Intriguingly, our data indicate that on CCND1 the promoter-bound DHT–AR complex functions as an inverse agonist, causing active repression of basal gene activity, eventually creating a repressive chromatin conformation associated with reduced RNA Polymerase II recruitment, in MCF-7 breast cancer cells. Our proposed model for AR-mediated repression of the CCND1 involves the recruitment of the atypical orphan nuclear receptor DAX1 that is expressed in tissues directly involved in steroid hormone production and reproductive function (74–75). DAX1 inhibits ligand-dependent transactivation by agonist-bound nuclear receptors like AR, ER and progesterone receptor (60–61,76). Moreover, DAX1 has been proposed to be involved in the development of cancers of a variety of tissues and has been found to positively correlate with AR and ER expression in breast cancer specimens (60–61,74,77). Our results suggest that, in MCF-7 cells, upon DHT stimulation, DAX1 is recruited on the androgen responsive region of the cyclin D1 promoter concomitantly to AR. The repressor complex formation also embraces the participation of HDAC1, further contributing to explain the AR-mediated inhibition of CCND1 transcription (Figure 1). Given the importance of cyclin D1 in breast cancer cell proliferation, androgen-mediated regulation of cyclin D1

levels appears to play a crucial role in cell-cycle control. The importance of our results is highlighted by recent studies demonstrating that biologically active DHT is locally produced in breast carcinoma tissues. Interestingly, intratumoural DHT levels are positively associated with AR and 5α reductase 1 expression but inversely correlated with tumour size, Ki-67 and aromatase expression (78), suggesting that in AR-positive breast carcinomas the use of aromatase inhibitors may be more effective by accumulation of the local DHT concentration. The biological significance of DHT-induced inhibition of cyclin D1 expression is also pointed out by clinical studies using tamoxifen as an adjuvant therapy in ER-positive breast cancers, showing a higher response and better survival rate in cancers with cyclin D1 low/moderate expression (7–8). These observations, supported by the widespread expression of AR in primary and metastatic breast tumours, suggest the possibility that targeting the AR-signalling pathway could be helpful in improving new molecular and pharmacological approaches for breast cancer treatment and to potentiate the effectiveness of anti-oestrogen adjuvant therapies.

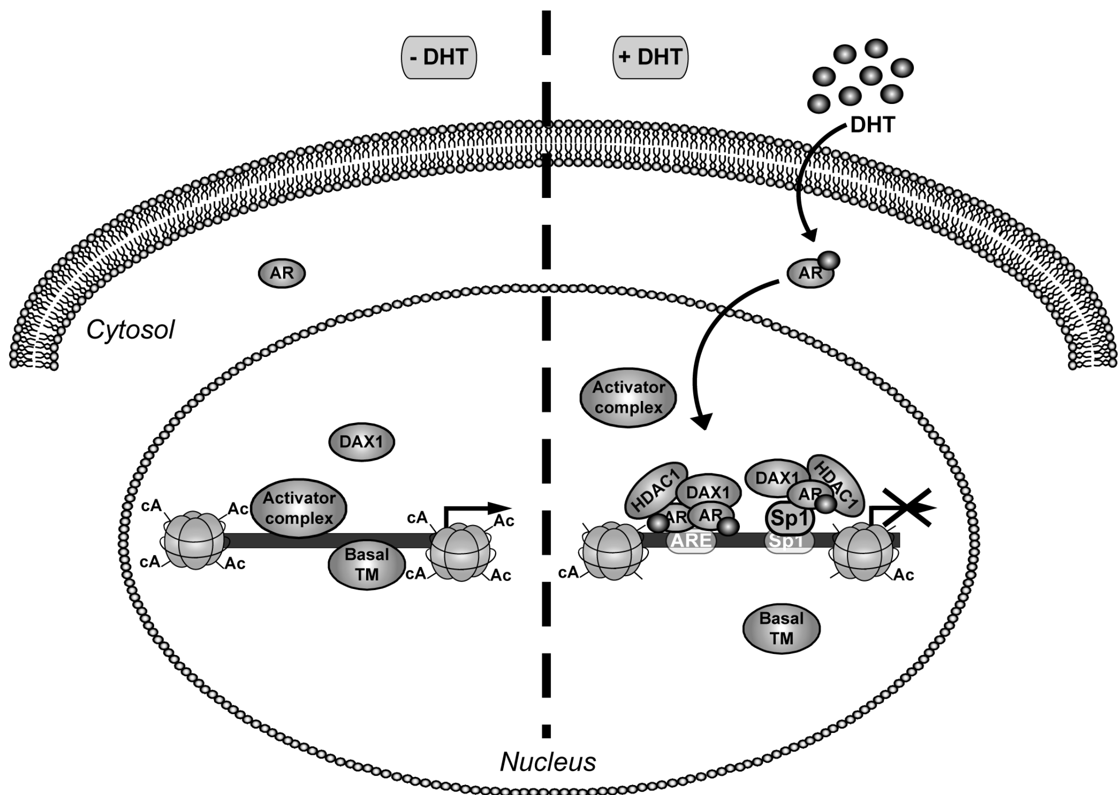


**Figure 1. Proposed model for AR-mediated repression of the CCND1.** In the absence of DHT, CCND1 basal activity is regulated by several serum factors acting through multiple regulatory region of the cyclin D1 promoter and enabling a permissive chromatin conformation and gene transcription (13,15,16). Upon DHT treatment, AR is recruited on the ARE- and Sp1-containing region of the proximal cyclin D1 promoter, causing displacement of RNA Polymerase II and recruitment of a corepressor complex containing DAX1 and HDAC1, with consequent repression of cyclin D1 expression.

## REFERENCES:

1. **Arnold,A. and Papanikolaou,A.** (2005) Cyclin D1 in breast cancer pathogenesis. J. Clin. Oncol., 23, 4215–4224.

2. **Ormandy,C.J., Musgrove,E.A., Hui,R., Daly,R.J. And Sutherland,R.L.** (2003) Cyclin D1, EMS1 and 11q13 amplification in breast cancer. Breast Cancer Res. Treat., 78,323–335.

3. **Gillett,C., Fantl,V., Smith,R., Fisher,C., Bartek,J., Dickson,C., Barnes,D. and Peters,G**. (1994) Amplification and overexpression of cyclin D1 in breast cancer detected by immunohistochemical staining. Cancer Res., 54, 1812–1817.

4. **Buckley,M.F., Sweeney,K.J., Hamilton,J.A., Sini,R.L., Manning,D.L., Nicholson,R.I.,deFazio,A., Watts,C.K.,Musgrove,E.A. and Sutherland,R.L.** (1993) Expression and amplification of cyclin genes in human breast cancer. Oncogene, 8, 2127–2133.

5. **Hodges,L.C., Cook,J.D., Lobenhofer,E.K., Li,L., Bennett,L., Bushel,P.R., Aldaz,C.M., Afshari,C.A. and Walker,C.L**. (2003) Tamoxifen functions as a molecular agonist inducing cell cycle-associated genes in breast cancer cells. Mol. Cancer Res., 1, 300–311.

6. **Hui,R., Finney,G.L., Carroll,J.S., Lee,C.S., Musgrove,E.A. And Sutherland,R.L.** (2002) Constitutive overexpression of cyclin D1 but not cyclin E confers acute resistance to antiestrogens in T-47D breast cancer cells. Cancer Res., 62, 6916–6923.

7. **Jirstrom,K., Stendahl,M., Ryden,L., Kronblad,A., Bendahl,P.O., Stal,O. and Landberg,G.** (2005) Adverse effect of adjuvant tamoxifen in premenopausal breast cancer with cyclin D1 gene amplification. Cancer Res., 65, 8009–8016.

8. **Stendahl,M., Kronblad,A., Ryden,L., Emdin,S., Bengtsson,N.O. and**

**Landberg,G.** (2004) Cyclin D1 overexpression is a negative predictive factor for tamoxifen response in postmenopausal breast cancer patients. Br. J. Cancer, 90, 1942–1948.

9. **Wang,T.C., Cardiff,R.D., Zukerberg,L., Lees,E., Arnold,A. And Schmidt,E.V.** (1994) Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice. Nature, 369, 669–671.

10. **Lee,R.J., Albanese,C., Fu,M., D'Amico,M., Lin,B., Watanabe,G., Haines,G.K. 3rd, Siegel,P.M., Hung,M.C., Yarden,Y. et al**. (2000) Cyclin D1 is required for transformation by activated Neu and is induced through an E2F-dependent signaling pathway. Mol. Cell. Biol., 20, 672–683.

11. **Yu,Q., Geng,Y. and Sicinski,P.** (2001) Specific protection against breast cancers by cyclin D1 ablation. Nature, 411, 1017–1021.

12. **Grillo,M., Bott,M.J., Khandke,N., McGinnis,J.P., Miranda,M., Meyyappan,M., Rosfjord,E.C. and Rabindran,S.K.** (2006) Validation of cyclin D1/CDK4 as an anticancer drug target in MCF-7 breast cancer cells: effect of regulated overexpression of cyclin D1 and siRNA-mediated inhibition of endogenous cyclin D1 and CDK4 expression. Breast Cancer Res. Treat., 95, 185–194.

13. **Dupont,J., Karas,M. and LeRoith,D.** (2000) The potentiation of estrogen on insulin-like growth factor I action in MCF-7 human breast cancer cells includes cell cycle components. J. Biol. Chem., 275, 35893–35901.

14. **Liu,M.M., Albanese,C., Anderson,C.M., Hilty,K., Webb,P., Uht,R.M., Price,R.H. Jr, Pestell,R.G. and Kushner,P.J.** (2002) Opposing action of estrogen receptors alpha and beta on cyclin D1 gene expression. J. Biol. Chem., 277, 24353–24360.

15. **Musgrove,E.A., Hamilton,J.A., Lee,C.S., Sweeney,K.J., Watts,C.K. and Sutherland,R.L.** (1993) Growth factor, steroid, and steroid antagonist regulation of cyclin gene expression with changes in T-47D human breast cancer cell cycle progression. Mol. Cell. Biol., 13, 3577–3587.

16. **Eeckhoute,J., Carroll,J.S., Geistlinger,T.R., Torres-Arzayus,M.I. and Brown,M**. (2006) A cell-type-specific transcriptional network required for estrogen regulation of cyclin D1 and cell cycle progression in breast cancer. Genes Dev., 20, 2513–2526.

17 **Powell S.M., Brooke G.N.,. Whitaker H.C, Reebye V., Gamble D., Chotai S.C., Dart D.A., Belandia B., Bevan C.L.** (2006) Mechanisms of androgen receptor repression in prostate cancer. Biochemical Society Transactions 34, part 6

18.**Brinkmann A.O., Klaasen P., Kuiper G:G:J:M:, Korput J:A:G:M:, Bolt J., Boer W., Smit A., Faber PW., Rooij J., Geurts van Kessel A., et al.** (1988) Structure and function of the androgen receptor. Urol. Res., 17,87-93

19. **Birrell,S.N., Hall,R.E. and Tilley,W.D**. (1998) Role of the androgen receptor in human breast cancer. J Mammary Gland Biol. Neoplasia, 3, 95–103.

20. **Labrie,F., Luu-The,V., Labrie,C., Belanger,A., Simard,J., Lin,S.X. and Pelletier,G.** (2003) Endocrine and intracrine sources of androgens in women: inhibition of breast cancer and other roles of androgens and their precursor dehydroepiandrosterone. Endocr. Rev., 24, 152–182.

21. **Yeh,S., Hu,Y.C., Wang,P.H., Xie,C., Xu,Q., Tsai,M.Y., Dong,Z., Wang,R.S., Lee,T.H. and Chang,C**. (2003) Abnormal mammary gland development and growth retardation in female mice and MCF7 breast cancer cells lacking androgen receptor. J. Exp. Med., 198, 1899–1908.

22. **Tworoger,S.S., Missmer,S.A., Barbieri,R.L., Willett,W.C., Colditz,G.A. and Hankinson,S.E.** (2005) Plasma sex hormone concentrations and subsequent risk of breast cancer among women using postmenopausal hormones. J. Natl Cancer Inst., 97, 595–602.

23. **Missmer,S.A., Eliassen,A.H., Barbieri,R.L. and Hankinson,S.E.** (2004) Endogenous estrogen, androgen, and progesterone concentrations and breast cancer risk among postmenopausal women. J. Natl Cancer Inst., 96, 1856–1865.

24. **Dimitrakakis,C., Zhou,J., Wang,J., Belanger,A., LaBrie,F., Cheng,C., Powell,D. and Bondy,C.** (2003) A physiologic role for Nucleic Acids Research, 2010, Vol. 38, No. 16 5363 testosterone in limiting estrogenic stimulation of the breast. Menopause, 10, 292–298.

25. **Murphy,L.C. and Watson,P.** (2002) Steroid receptors in human breast tumorigenesis and breast cancer progression. Biomed. Pharmacother., 56, 65–77.

26. **Zhou,J., Ng,S., Adesanya-Famuiya,O., Anderson,K. And Bondy,C.A.** (2000) Testosterone inhibits estrogen-induced mammary epithelial proliferation and suppresses estrogen receptor expression. FASEB J., 14, 1725–1730.

27. **Lanzino,M., De Amicis,F., McPhaul,M.J., Marsico,S., Panno,M.L. and Ando,S**. (2005) Endogenous coactivator ARA70 interacts with estrogen receptor alpha (ERalpha) and modulates the functional ERalpha/androgen receptor interplay in MCF-7 cells. J. Biol. Chem., 280, 20421–20430.

28. **Ando,S., De Amicis,F., Rago,V., Carpino,A., Maggiolini,M., Panno,M.L. and Lanzino,M.** (2002) Breast cancer: from estrogen to androgen receptor. Mol. Cell. Endocrinol., 193, 121–128.

29. **de Launoit,Y., Dauvois,S., Dufour,M., Simard,J. and Labrie,F.** (1991) Inhibition of cell cycle kinetics and proliferation by the androgen 5 alpha-dihydrotestosterone and antiestrogen N,n-butyl-N-methyl-11-[16 0 alpha-chloro-30,17 beta-dihydroxy-estra-1 0,30,50-(100)triene-70 alpha-yl] undecanamide in human breast cancer ZR-75-1 cells. Cancer Res., 51, 2797–2802.

30. **Greeve,M.A., Allan,R.K., Harvey,J.M. and Bentel,J.M**. (2004) Inhibition of MCF-7 breast cancer cell proliferation by 5alpha-dihydrotestosterone; a role for p21(Cip1/Waf1). J. Mol. Endocrinol., 32, 793–810.

31. **Lanzino,M., Garofalo,C., Morelli,C., Le Pera,M., Casaburi,I., McPhaul,M.J., Surmacz,E., Ando,S. and Sisci,D.** (2009) Insulin receptor substrate 1 modulates the transcriptional activity and the stability of androgen receptor in breast cancer cells. Breast Cancer Res. Treat., 115, 297–306.

32. **Lea,O.A., Kvinnsland,S. and Thorsen,T.** (1989) Improved measurement of androgen receptors in human breast cancer. Cancer Res., 49, 7162–7167.

33. **Moinfar,F., Okcu,M., Tsybrovskyy,O., Regitnig,P., Lax,S.F., Weybora,W., Ratschek,M., Tavassoli,F.A. and Denk,H.** (2003) Androgen receptors frequently are expressed in breast carcinomas: potential relevance to new therapeutic strategies. Cancer, 98, 703–711.

34. **Riva,C., Dainese,E., Caprara,G., Rocca,P.C., Massarelli,G., Tot,T., Capella,C. and Eusebi,V.** (2005) Immunohistochemical study of androgen receptors in breast carcinoma. Evidence of their frequent expression in lobular carcinoma. Virchows Arch., 447, 695–700.

35. **Agoff,S.N., Swanson,P.E., Linden,H., Hawes,S.E. And Lawton,T.J**.

(2003) Androgen receptor expression in estrogen receptor-negative breast cancer. Immunohistochemical, clinical, and prognostic associations. Am. J. Clin. Pathol., 120, 725–731.

36. **Soreide,J.A., Lea,O.A., Varhaug,J.E., Skarstein,A. And Kvinnsland,S**. (1992) Androgen receptors in operable breast cancer: relation to other steroid hormone receptors, correlations to prognostic factors and predictive value for effect of adjuvant tamoxifen treatment. Eur. J. Surg. Oncol., 18, 112–118.

37. **Kimura,N., Mizokami,A., Oonuma,T., Sasano,H. and Nagura,H.** (1993) Immunocytochemical localization of androgen receptor with polyclonal antibody in paraffin-embedded human tissues. J. Histochem. Cytochem., 41, 671–678.

38. **Sauter,E.R., Lininger,J., Magklara,A., Hewett,J.E. And Diamandis,E.P**. (2004) Association of kallikrein expression in nipple aspirate fluid with breast cancer risk. Int. J. Cancer, 108, 588–591.

39. **Sauter,E.R., Tichansky,D.S., Chervoneva,I. and Diamandis,E.P.** (2002) Circulating testosterone and prostate-specific antigen in nipple aspirate fluid and tissue are associated with breast cancer. Environ. Health Perspect., 110, 241–246.

40. **Schippinger,W., Regitnig,P., Dandachi,N., Wernecke,K.D., Bauernhofer,T., Samonigg,H. and Moinfar,F.** (2006) Evaluation of the prognostic significance of androgen receptor expression in metastatic breast cancer. Virchows Arch., 449, 24–30.

41. **Rakha,E.A., El-Sayed,M.E., Green,A.R., Lee,A.H., Robertson,J.F. and Ellis,I.O.** (2007) Prognostic markers in triple-negative breast cancer. Cancer, 109, 25–32.

42. **Lobaccaro,J.M., Lumbroso,S., Belon,C., Galtier-Dereure,F.,**

**Bringer,J., Lesimple,T., Heron,J.F., Pujol,H. and Sultan,C.** (1993) Male breast cancer and the androgen receptor gene. Nat. Genet., 5, 109–110.

43. **Kollara,A., Kahn,H.J., Marks,A. and Brown,T.J.** (2001) Loss of androgen receptor associated protein 70 (ARA70) expression in a subset of HER2-positive breast cancers. Breast Cancer Res. Treat., 67, 245–253.

44. **Tormey,D.C., Lippman,M.E., Edwards,B.K. and Cassidy,J.G.** (1983) Evaluation of tamoxifen doses with and without fluoxymesterone in advanced breast cancer. Ann. Intern. Med., 98, 139–144.

45. **Ingle,J.N., Twito,D.I., Schaid,D.J., Cullinan,S.A., Krook,J.E., Mailliard,J.A., Tschetter,L.K., Long,H.J., Gerstner,J.G., Windschitl,H.E. et al.** (1991) Combination hormonal therapy with tamoxifen plus fluoxymesterone versus tamoxifen alone in postmenopausal women with metastatic breast cancer. An updated analysis. Cancer, 67, 886–891.

46. **Macedo,L.F., Guo,Z., Tilghman,S.L., Sabnis,G.J., Qiu,Y. And Brodie,A.** (2006) Role of androgens on MCF-7 breast cancer cell growth and on the inhibitory effect of letrozole. Cancer Res., 66, 7775–7782.

47. **Sasano,H., Suzuki,T., Miki,Y. and Moriya,T.** (2008) Intracrinology of estrogens and androgens in breast carcinoma. J. Steroid Biochem. Mol. Biol., 108, 181–185.

48. **Hanley,K., Wang,J., Bourne,P., Yang,Q., Gao,A.C., Lyman,G. and Tang,P.** (2008) Lack of expression of androgen receptor may play a critical role in transformation from in situ to invasive basal subtype of high-grade ductal carcinoma of the breast. Hum. Pathol., 39, 386–392.

49. **Gonzalez-Angulo,A.M., Stemke-Hale,K., Palla,S.L., Carey,M., Agarwal,R., Meric-Berstam,F., Traina,T.A., Hudis,C., Hortobagyi,G.N., Gerald,W.L. et al.** (2009) Androgen receptor levels and

association with PIK3CA mutations and prognosis in breast cancer. Clin. Cancer Res., 15, 2472–2478.

50. **Holter E, Kotoja N, Makela S, Strauss L, Kietz S, Janne OA, Gustafsson JA, Palvimo JJ, Treuter E.** (2002) Inhibition of Androgen Receptor (AR) Function by the Reproductive Orphan Nuclear Receptor DAX-1. Mol. Endocrinol.16(3), 515-518.

51. **Zoppi,S., Marcelli,M., Deslypere,J.P., Griffin,J.E., Wilson,J.D. and McPhaul,M.J.** (1992) Amino acid substitutions in the DNA-binding domain of the human androgen receptor are a frequent cause of receptor-binding positive androgen resistance. Mol. Endocrinol., 6, 409–415.

52. **Recchia,A.G., Musti,A.M., Lanzino,M., Panno,M.L., Turano,E., Zumpano,R., Belfiore,A., Ando,S. and Maggiolini,M**. (2009) A cross-talk between the androgen receptor and the epidermal growth factor receptor leads to p38MAPK-dependent activation of mTOR and cyclinD1 expression in prostate and lung cancer cells. Int. J. Biochem. Cell Biol., 41, 603–614.

53. **Morelli,C., Garofalo,C., Sisci,D., del Rincon,S., Cascio,S., Tu,X., Vecchione,A., Sauter,E.R., Miller,W.H. Jr and Surmacz,E.** (2004) Nuclear insulin receptor substrate 1 interacts with estrogen receptor alpha at ERE promoters. Oncogene, 23, 7517–7526.

54. **Catalano,S., Giordano,C., Rizza,P., Gu,G., Barone,I., Bonofiglio,D., Giordano,F., Malivindi,R., Gaccione,D., Lanzino,M. et al.** (2009) Evidence that leptin through STAT and CREB signaling enhances cyclin D1 expression and promotes human endometrial cancer proliferation. J. Cell. Physiol., 218, 490–500.

55. **Cascio,S., Bartella,V., Garofalo,C., Russo,A., Giordano,A. And Surmacz,E.** (2007) Insulin-like growth factor 1 differentially regulates

estrogen receptor-dependent transcription at estrogen response element and AP-1 sites in breast cancer cells. J. Biol. Chem., 282, 3498–3506.

56. **Lanzino,M., Garofalo,C., Morelli,C., Le Pera,M., Casaburi,I., McPhaul,M.J., Surmacz,E., Ando,S. and Sisci,D.** (2009) Insulin receptor substrate 1 modulates the transcriptional activity and the stability of androgen receptor in breast cancer cells. Breast Cancer Res. Treat, 115, 297–306.

57. **Yeap,B.B., Krueger,R.G. and Leedman,P.J.** (1999) Differential posttranscriptional regulation of androgen receptor gene expression by androgen in prostate and breast cancer cells. Endocrinology, 140, 3282–3291.

58. **Verrijdt,G., Haelens,A. and Claessens,F**. (2003) Selective DNA recognition by the androgen receptor as a mechanism for hormone-specific regulation of gene expression. Mol. Genet. Metab., 78, 175–185.

59. **Castro-Rivera,E., Samudio,I. and Safe,S.** (2001) Estrogen regulation of cyclin D1 gene expression in ZR-75 breast cancer cells involves multiple enhancer elements. J. Biol. Chem., 276, 30853–30861.

60. **Holter,E., Kotaja,N., Makela,S., Strauss,L., Kietz,S., Janne,O.A., Gustafsson,J.A., Palvimo,J.J. and Treuter,E**. (2002) Inhibition of androgen receptor (AR) function by the reproductive orphan nuclear receptor DAX-1. Mol. Endocrinol., 16, 515–528.

61. **Agoulnik,I.U., Krause,W.C., Bingman,W.E. 3rd, Rahman,H.T., Amrikachi,M., Ayala,G.E. And Weigel,N.L.** (2003) Repressors of androgen and progesterone receptor action. J. Biol. Chem., 278, 31136–31148.

62. **Yuan,X., Lu,M.L., Li,T. and Balk,S.P**. (2001) SRY interacts with and negatively regulates androgen receptor transcriptional activity. J. Biol.

Chem., 276, 46647–46654.

63. **Haberland,M., Montgomery,R.L. and Olson,E.N**. (2009) The many roles of histone deacetylases in development and physiology: implications for disease and therapy. Nat. Rev. Genet., 10, 32–42.

64. **White,J.H., Fernandes,I., Mader,S. and Yang,X.J.** (2004) Corepressor recruitment by agonist bound nuclear receptors. Vitam. Horm., 68, 123–143.

65. **Peters,A.A., Buchanan,G., Ricciardelli,C., Bianco-Miotto,T., Centenera,M.M., Harris,J.M., Jindal,S., Segara,D., Jia,L., Moore,N.L. et al.** (2009) Androgen receptor inhibits estrogen receptor alpha activity and is prognostic in breast cancer. Cancer Res., 69, 6131–6140.

66. **Ogawa,Y., Hai,E., Matsumoto,K., Ikeda,K., Tokunaga,S., Nagahara,H., Sakurai,K., Inoue,T. and Nishiguchi,Y.** (2008) Androgen receptor expression in breast cancer: relationship with clinicopathological factors and biomarkers. Int. J. Clin. Oncol., 13, 431–435.

67. **Liu,Y.N., Liu,Y., Lee,H.J., Hsu,Y.H. and Chen,J.H**. (2008) Activated androgen receptor downregulates E-cadherin gene expression and promotes tumor metastasis. Mol. Cell. Biol., 28, 7096–7108.

68. **Cicatiello,L., Addeo,R., Sasso,A., Altucci,L., Petrizzi,V.B., Borgo,R., Cancemi,M., Caporali,S., Caristi,S., Scafoglio,C. et al**. (2004) Estrogens and progesterone promote persistent CCND1 gengene activation during G1 by inducing transcriptional derepression via c-Jun/c-Fos/estrogen receptor (progesterone receptor) complex assembly to a distal regulatory element and recruitment of cyclin D1 to its own gene promoter. Mol. Cell. Biol., 24, 7260–7274.

69. **Sabbah,M., Courilleau,D., Mester,J. and Redeuilh,G.** (1999) Estrogen induction of the cyclin D1 promoter: involvement of a cAMP

response-like element. Proc. Natl Acad. Sci. USA, 96, 11217–11222.

70. **Herber,B., Truss,M., Beato,M. and Muller,R.** (1994) Inducible regulatory elements in the human cyclin D1 promoter. Oncogene, 9, 2105–2107.

71. **Moehren,U., Denayer,S., Podvinec,M., Verrijdt,G. And Claessens,F.** (2008) Identification of androgen-selective androgen-response elements in the human aquaporin-5 and Rad9 genes. Biochem. J., 411, 679–686.

72. **Haelens,A., Verrijdt,G., Callewaert,L., Christiaens,V., Schauwaers,K., Peeters,B., Rombauts,W. and Claessens,F.** (2003) DNA recognition by the androgen receptor: evidence for an alternative DNA-dependent dimerization, and an active role of sequences flanking the response element on transactivation. Biochem. J., 369, 141–151.

73. **McEwan,I.J**. (2004) Molecular mechanisms of androgen receptor-mediated gene regulation: structure-function analysis of the AF-1 domain. Endocr. Relat. Cancer, 11, 281–293.

74. **Giguere,V.** (1999) Orphan nuclear receptors: from gene to function. Endocr. Rev., 20, 689–725.

75. **Lalli,E. and Sassone-Corsi,P**. (2003) DAX-1, an unusual orphan receptor at the crossroads of steroidogenic function and sexual differentiation. Mol. Endocrinol., 17, 1445–1453.

76. **Zhang,H., Thomsen,J.S., Johansson,L., Gustafsson,J.A. And Treuter,E**. (2000) DAX-1 functions as an LXXLL-containing corepressor for activated estrogen receptors. J. Biol. Chem., 275, 39855–39859.

77. **Conde,I., Alfaro,J.M., Fraile,B., Ruiz,A., Paniagua,R. And Arenas,M.I**. (2004) DAX-1 expression in human breast cancer: comparison with estrogen receptors ER-alpha, ER-beta and androgen receptor status. Breast Cancer Res., 6, R140–R148.

78. **Suzuki,T., Miki,Y., Moriya,T., Akahira,J., Ishida,T., Hirakawa,H., Yamaguchi,Y., Hayashi,S. and Sasano,H.** (2007) 5Alpha-reductase type 1 and aromatase in breast carcinoma as regulators of in situ androgen production. Int. J. Cancer, 120, 285–291. Nucleic Acids Research, 2010, Vol. 38, No. 16 5365

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# Inhibition of cyclin D1 expression by androgen receptor in breast cancer cells—identification of a novel androgen response element

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## ABSTRACT

**Cyclin D1 gene (*CCND1*) is a critical mitogen-regulated cell-cycle control element whose transcriptional modulation plays a crucial role in breast cancer growth and progression. Here we demonstrate that the non-aromatizable androgen 5-α-dihydrotestosterone (DHT) inhibits endogenous cyclin D1 expression, as evidenced by reduction of cyclin D1 mRNA and protein levels, and decrease of *CCND1*-promoter activity, in MCF-7 cells. The DHT-dependent inhibition of *CCND1* gene activity requires the involvement and the integrity of the androgen receptor (AR) DNA-binding domain. Site directed mutagenesis, DNA affinity precipitation assay, electrophoretic mobility shift assay and chromatin immunoprecipitation analyses indicate that this inhibitory effect is ligand dependent and it is mediated by direct binding of AR to an androgen response element (*CCND1*-ARE) located at −570 to −556-bp upstream of the transcription start site, in the cyclin D1 proximal promoter. Moreover, AR-mediated repression of the *CCND1* involves the recruitment of the atypical orphan nuclear receptor DAX1 as a component of a multiprotein repressor complex also embracing the participation of Histone Deacetylase 1. In conclusion, identification of the *CCND1*-ARE allows defining cyclin D1 as a specific androgen target gene in breast and might contribute to explain the molecular basis of the inhibitory role of androgens on breast cancer cells proliferation.**

## INTRODUCTION

Over-expression of cyclin D1 has been linked to breast cancer growth and progression (1–4), as well as development of resistance to hormone therapy (5–8). The biologically relevant role of cyclin D1 in breast tumourigenesis has been evidenced by several findings: mammary gland-targeted cyclin D1 over-expression resulted in mammary hyperplasia and adenocarcinoma in transgenic mice (9); cyclin D1 antisense blocked ErbB2-induced mammary tumour growth *in vivo* (10), and cyclin D1-deficient mice were resistant to ErbB2- or Ras-induced mammary tumourigenesis (11). In addition, the correlation between cyclin D1 expression levels and cellular proliferation in breast cancer cells has been further confirmed by silencing experiments (1,12).

Several hormones are involved in breast cancer cells proliferation, so that cyclin D1 represents an important target of their intracellular-signalling pathways (13–17).

Emerging evidences indicate that the androgen-signalling pathway mainly exerts inhibitory effects on the growth of normal mammary epithelial cells and plays a protective role in the pathogenesis of breast cancer (18–21). Nonetheless, there are also some epidemiologic reports supporting the concept that androgens, in certain settings, can contribute to breast cancer growth (22–23). Androgens excess (e.g. in congenital adrenal hyperplasia) suppresses breast development (20), while mice lacking a functional androgen receptor (AR) display defective mammary gland development and morphogenesis (21). Furthermore, *in vivo* studies evidenced that blocking the action of endogenous androgens results in a significant increase in mammary epithelial cell proliferation (24–25).

*In vitro*, androgen signalling may counteract the proliferative effect of estrogens in AR-positive breast cancer

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

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cells, (26), while over-expression of the AR in MCF-7 cells markedly decreases oestrogen receptor α (ERα) transcriptional activity (27–28). Furthermore, the non-aromatizable androgen 5-α-dihydrotestosterone (DHT) is able to inhibit serum as well as estradiol-induced proliferation in ERα positive breast cancer cell lines (18,28–31), through a mechanism involving an increase in AR protein cell content (28) concomitantly with the down-regulation of the G1/S transition of the cell cycle (28,30).

The AR is present in both primary breast tumours (70–90%) and metastases (75%) (32–34) and shows significant association with important clinical and pathologic prognostic factors (35), since AR expression and functional activity correlate with a low tumour grade, smaller tumour size, improved response to hormone therapy and longer patient survival (33,35–41). Conversely, reduced or impaired AR signalling has been demonstrated in hereditary male breast cancer (42) as well as in HER2-positive breast cancers, generally associated with a worse outcome (43). Androgens have been previously used in the adjuvant therapy of breast cancer in both pre-menopausal and post-menopausal women, with an efficacy comparable to that of current hormonal treatment (44–45), and combined hormonal therapy using tamoxifen plus the androgen fluoxymesterone offered some therapeutic advantage over tamoxifen alone in metastatic breast carcinoma (45). Furthermore, it has been suggested that in AR-positive breast cancer cells and breast carcinomas, the anti-proliferative effect of aromatase inhibitors seems to be due not only to the reduction in estrogens biosynthesis but also to the unmasking of the inhibitory effect of androgens acting via the AR (46–47).

Thus, AR is not only frequently expressed in breast tumours and related to prognosis (48), but it also may serve as a predictive marker for adjuvant hormonal therapy (49). However, events following AR activation and leading to inhibition of cell growth are not clearly identified in breast cancer cells.

Here we investigated whether DHT-dependent inhibition of breast cancer cell proliferation might be due to the modulation of cyclin D1, whose induction represents a key rate-limiting event in mitogenic signalling leading to S-phase entry. We report the identification of a novel androgen-mediated mechanism that controls the expression of cyclin D1 in MCF-7 breast cancer cells by negatively regulating cyclin D1 transcript and protein levels. Indeed, we identified, in the human *cyclin D1* promoter a functional androgen responsive element (ARE), which binds the AR in response to DHT stimulation. Transcriptional repression of *CCND1* by AR appears to be consequent to the recruitment of a multiprotein repressor complex involving the participation of the AR corepressor DAX1 and containing histone deacetylase activity.

## MATERIALS AND METHODS

### Cell culture and treatments

Breast cancer epithelial cell line MCF-7 and human embryonic kidney cell line HEK-293 were grown in DMEM/F12 (Gibco, USA) supplemented with 5% calf serum (CS; Gibco) and in DMEM plus 10% foetal calf serum, respectively. 5α-DHT) (Sigma, USA) and hydroxyflutamide (OH-Fl; Sigma) were used at a concentration of $10^{-7}$ M and $10^{-6}$ M, respectively. Before each experiment, cells were grown in phenol red-free (PRF) DMEM, containing 5% charcoal-treated foetal calf serum (PRF–CT) for 3 days and then serum starved in PRF for 24 h to synchronize the cells. All the experiments were performed in 2.5% PRF–CT.

### Cell proliferation assays

MCF-7 cells were seeded on six-well plates ($10^5$ cells/well) in 2.5% PRF–CT. After 24 h, cells were exposed for 3 days to $10^{-7}$ M DHT and/or $10^{-6}$ M OHFl or left untreated. Media were renewed daily. The effects of the various drugs on cell proliferation were measured 0, 24, 48 and 72 h following initial exposure to treatments by counting MCF-7 cells using a Burker's chamber, with cell viability determined by trypan blue dye exclusion.

In the same experimental conditions, cell viability was also examined using the method of transcriptional and translational (MTT) colorimetric assay (50). At the above indicated time points, 100 μl of MTT (5 mg/ml) were added to each well, and the plates were incubated for 4 h at 37°C. Then, 1 ml 0.04N HCl in isopropanol was added to solubilise the cells. The absorbance was measured with the Ultrospec 2100 Prospectrophotometer (Amersham-Biosciences, Italy) at a test wavelength of 570 nm.

### Cell-cycle analysis

MCF-7 cells were seeded on six-well plates ($10^5$ cells/well) in 2.5% PRF–CT. After 24 h, cells were exposed to $10^{-7}$ M DHT or left untreated. Cell-cycle analysis was performed 72 h following initial exposure to treatment as previously described (28).

### Plasmids, transfections and luciferase reporter assays

The following plasmids were used: pcDNA3-AR (AR) encoding full-length AR [27]; CMV-P881 ($AR_{(Cys574 \rightarrow Arg)}$) encoding the full-length AR carrying a mutation in the DNA-binding domain (DBD; Cys-574→Arg) (51); D1Δ-2960, D1Δ-944, D1Δ-848, D1Δ-254, D1Δ-136 and D1Δ-96, carrying fragments from the human *CCND1* promoter and inserted into the luciferase vector pXP2 (a gift from Dr A. Weitz, University of Naples, Italy); the vector-based pSiAR plasmid, coding for small interfering RNA targeting the 5′-untranslated region of AR mRNA, and the scrambled control construct pSiCon (52); The Renilla reniformis luciferase expression vector used was pRL-Tk (Promega, USA). MCF-7 cells were transfected using Fugene 6 (Roche, CH, USA) according to the manufacturer's instructions. pRL-Tk was used to assess transfection efficiency. Luciferase activity was measured using dual luciferase assay System (Promega), normalized to renilla luciferase activity and expressed as relative luciferase units.

For western blotting (WB) assays, MCF-7 cells were plated on 60-mm dishes and transfected with an

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appropriate amount of various plasmids, as indicated in figure legends.

### Immunoprecipitation and WB

Total cell proteins and the cytoplasmic and nuclear fractions were obtained from 70% confluent cell cultures. Immunoprecipitation (IP) and WB were performed as previously described (53). The following monoclonal (m) and polyclonal (p) antibodies (Ab) were used: anti-AR mAb (441), anti-DAX1 pAb (K-17), anti-Lamin B pAb (C-20), anti-GAPDH pAb (FL-335) and normal mouse immunoglobulin G (Ig) (Santa Cruz Biotechnology, USA).

### Real-time reverse transcription–PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions and treated with DNase I (Ambion, Austin, TX, USA). Two micrograms of total RNA were reverse transcribed with the ImProm-II Reverse transcription system kit (Promega); cDNA was diluted 1 : 3 in nuclease-free water and 5 µl were analysed in triplicates by real-time PCR in an iCycler iQ Detection System (Bio-Rad, USA) using SYBR Green Universal PCR Master Mix (Bio-Rad) with 0.1 µmol/l of each primer in a total volume of 30 µl reaction mixture. Primers used for the amplification were 5′-CGTGGCCTCTAAGATGAAGGA-3′ (forward) and 5′-CGGTGTAGATGCACAGCTTCTC-3′ (reverse). Negative controls contained water instead of first strand cDNA. Each sample was normalized on its 18S rRNA content. The 18S quantification was done using a TaqMan rRNA Reagent kit (Applied Biosystems, USA) following the manufacturer instructions. The relative gene expression levels were normalized to a calibrator that was chosen to be the basal, untreated sample. Final results were expressed as n-fold differences in gene expression relative to 18S rRNA and calibrator, calculated using the $\Delta\Delta C_t$ method as follows: $n\text{-fold} = 2^{-(\Delta C\text{tsample}-\Delta C\text{tcalibrator})}$, where $\Delta C_t$ values of the sample and calibrator were determined by subtracting the average $C_t$ value of the 18S rRNA reference gene from the average $C_t$ value of the different genes analysed.

### Site-directed mutagenesis

The cyclin D1 promoter plasmids bearing the AR responsive (*CCND1*-ARE) mutated sites (D1Δ-944mARE) were created by site-directed mutagenesis using Quick Change kit (Stratagene, La Jolla, CA, USA), as previously described (54), using the following mutagenic primers (mutations are shown as lowercase letters): *CCND1*-ARE (forward) 5′-TTGTGTGCCCGGTCCTCCCCGT CCTTGCATaaaAAATTAGggtTTGCAATTTACACGT GTTAATGAAA-3′ and *CCND1*-ARE (reverse) 5′-TTTC ATTAACACGTGTAAATTGCA AaccCTAATTTtttAT GCAAGGACGGGGAGGACCGGGCACACAA-3′; Sp1 (forward) 5′-GCCCCCTCCCCCTGCaaCCaaCC CCaaCaCCCTCCCGCTCCCAT-3′ and Sp1 (reverse) 5′-ATGGGAGCGGGAGGGtGttGGGGttGGttGCAG GGGGAGGGGGC-3′. The mutated expression vectors were confirmed by DNA sequencing.

### DNA affinity precipitation assay

DNA affinity precipitation assay was performed as previously described (55). The DNA motif probes were prepared by annealing a biotinylated sense oligonucleotide (for *CCND1*-ARE, 5′-[Bio]-GCTAAATTAGTTCTTGC AATTTAC-3′; for *CCND1*-mutatedARE, 5′-[Bio]-CAT AAAA-AATTAGGGTTTGCAAT-3′; for Sp1, 5′-[Bio]-TGCCCGCGCCCCCTCCCCCTGCGCCCG-CCCCCG CCCCCCT-3′) with the respective unbiotinylated complementary oligonucleotide (for *CCND1*-ARE, 5′-GTAAAT TGCAAGAACTAATTTAGC-3′; for *CCND1*-mutatedARE, 5′-ATTGCAAACCCTAATTTTTTATG -3′; for Sp1, 5′-AGGGGGGCGGGGGCGGGCGCAG GGG-GAGGGGGCGCGGGCA-3′).

### Electrophoretic mobility shift assay

Nuclear protein extracts were prepared as previously described (54). The double-stranded oligonucleotides used as probes were end labelled with $^{[\gamma-32P]}$ATP and T4 polynucleotide kinase and purified using Sephadex G50 spin columns. The oligonucleotides used as probes and as cold competitors (Sigma Genosys, UK) were: (nucleotide motifs of interest are underlined): Probe: 5′-TGCA<u>TG CTAA</u>AAT<u>AGTTCT</u>TGCAA-3′; Mutated probe; 5′-TGCA<u>TaaaAA</u>AAT<u>AGggtT</u>TG-CAA-3′. Nuclear extracts (20 µg) were incubated with 50 000 c.p.m. of labelled probe, under conditions previously reported (54). The mixture was incubated at room temperature for 20 min in the presence or absence of the unlabelled competitor oligonucleotide. Mouse anti-AR monoclonal antibody (441), or rabbit anti-AR polyclonal antibody (C-19) or normal rabbit IgG (Santa Cruz Biotechnology), were included in some of the reaction mixtures with an additional 12-h incubation at 4°C before addition of labelled probe. The entire reaction mixture was electrophoresed through a 6% polyacrylamide gel for 3 h at 150 V.

### Chromatin IP

Chromatin IP (ChIP) assay was performed as previously described (56). The immuno-cleared chromatin was precipitated with anti-AR mAb, anti-DAX1 pAb, anti-Polymerase II pAb, anti-HDAC1 mAb, anti-HDAC3 pAb and anti-AIB1 mAb (Santa Cruz, USA). A 4-µl volume of each sample was used as template for PCR with specific primers. The following specific primer pairs were used to amplify 168 bp of the ARE-containing cyclin D1 promoter fragment: 5′-TACCCCTTGGGCATTTGC AACGA-3′ (forward); 5′-ACAGACGGCCAAAGAATC TCA-3′ (reverse), and 228 bp of the Sp1-sites containing cyclin D1 promoter fragment 5′-GGCGATTTGCATTTC TATGA-3′ (forward) and 5′-CAAAACTCCCCTGTAGT CCGT-3′ (reverse).

Immunoprecipitated DNA was also analysed in triplicates by real-time PCR by using 5 µl of the diluted (1 : 3) template DNA as described earlier. The following primer pairs were used: 5′-GCGCCGGAATGAAACTTG-3′ (forward); 5′-CTGCATCTTCTTTCATTTTCATTAAC AC-3′ (reverse) to amplify the ARE-containing cylin D1

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promoter fragment. Real-time PCR data were normalized with respect to unprocessed lysates (input DNA). Inputs DNA quantification was performed by using 5 µl of the diluted (1/50) template DNA. The relative antibody-bound fractions were normalized to a calibrator that was chosen to be the basal, untreated sample. Final results were expressed as fold differences with respect to the relative inputs.

### RNA silencing

For AR gene silencing experiments, MCF-7 cells were transfected using the vector-based pSiAR plasmid or the scrambled control construct pSiCon (52), as described in the 'Plasmids, transfections and luciferase reporter assays' paragraph.

For DAX1 gene silencing experiments, custom synthesized siRNA (Ambion) annealed duplexes were used for effective depletion of DAX1 mRNA. A scrambled siRNA that does not match with any human mRNA was used as a control for non-sequence-specific effects (Ambion). Growing cells were switched to PRF for 24 h and then switched to PRF–CT medium for 48 h. After that, cells were trypsinized and transfected in suspension with 5 nM siRNA (siDAX1 or scrambled siRNA) in 35-mm dishes, using Lipofectamine 2000 (Invitrogen), following the manufactuer's instructions. Cells were incubated with the siRNA-Lipofectamine 2000 complex at 37°C for 4 h and then switched to fresh PRF and treated or not with DHT ($10^{-7}$ M) for 72 h before analysis. For ChIP assay 100 nM siDAX1 was used to silence 60% confluent cells plated in 150-mm dishes.

### Statistical analysis

All data were expressed as the mean ± SD of at least three independent experiments. Statistical significances were tested using Student's *t*-test.

## RESULTS

### DHT administration inhibits serum-induced MCF-7 cells proliferation

We previously demonstrated that MCF-7 cells are androgen responsive and that DHT treatment induces a transient increase in AR protein levels (27), similar to those seen in other cell types (57).

To investigate the role of activated AR on breast cancer cell proliferation, the response of MCF-7 cells to the non-aromatizable androgen DHT and/or the AR antagonist OHFl was measured after 24, 48 and 72 h of treatment in PRF DMEM implemented with 2.5% of steroid-depleted serum (PRF–CT). DHT concentration was chosen based on previous studies demonstrating dose-dependent inhibitory effects of DHT on MCF-7 cells proliferation (28,30).

As expected, cells grown in presence of PRF–CT proliferated; DHT treatment instead inhibited serum-induced MCF-7 cells proliferation and, by the end of the treatment, the mean number of DHT-treated cells was ~30% of respective controls. Addition of the AR antagonist OHFl (or bicalutamide, data not shown) effectively reversed the inhibitory effect of DHT on MCF-7 cells proliferation, suggesting that it was mediated by the AR (Figure 1A). A similar pattern of DHT-dependent effect on MCF-7 cells proliferation was obtained by simultaneously performed MTT colorimetric assay (Figure 1B). These data well correlated with cell-cycle analysis showing an increase of the percentage of cells in G0/G1 phase and a concurrent decrease in the S phase, following 72 h of DHT treatment. At this time point the presence of sub-G1 apoptotic cells was undetectable (Figure 1C).

### Activated AR decreases cyclin D1 expression and promoter activity'

Since DHT administration reduces the G1/S phase transition in MCF-7 cells (28,30), we inquired whether DHT-induced decrease of MCF-7 cells proliferation might be consequent to the modulation of cyclin D1 expression, whose induction represents a key rate-limiting event in mitogenic signalling leading to S-phase entry. To this aim, serum starved MCF-7 cells were left untreated or treated with $10^{-7}$ M DHT for 24 h, 48 h or 72 h and cyclin D1 expression was assessed by real-time RT–PCR and WB analysis. As shown in Figure 2A, MCF-7 cells exhibited a decrease in the serum-dependent levels of cyclin D1 mRNA (Figure 2A) and protein (Figure 2B) following 48 and 72 h of DHT treatment. The involvement of the AR in the negative regulation of cyclin D1 expression was ascertained by silencing AR expression in MCF-7 cells (Figure 2B).

To test whether activated AR might negatively modulate cyclin D1 promoter activity, MCF-7 cells were transiently transfected with a cyclin D1 promoter reporter plasmid (D1Δ-2996) and left untreated or treated with $10^{-7}$ M DHT.

The cyclin D1 promoter was induced by serum, while DHT treatment inhibited basal cyclin D1 promoter activity, decreasing the serum-induced signal by ~50%. OHFl addition reversed this effect, suggesting that it was due to AR activation (Figure 2C).

To substantiate the AR involvement in the modulation of *CCND1* expression, we evaluated the effects of ectopic AR expression on the transcriptional activity of the cyclin D1 promoter. HEK-293 cells, which do not express AR, were co-transfected with the D1Δ-2996 plasmid and increasing amounts of a full length AR-encoding plasmid. In the absence of exogenous AR expression, DHT treatment cannot influence cyclin D1 promoter activity. On the contrary, in the presence of ectopic AR, a dose-dependent decrease in the serum-induced cyclin D1 promoter activity was observed upon DHT administration (Figure 2D).

To investigate whether the effect of AR on cyclin D1 promoter activity is dependent on its transactivation properties, luciferase assay was performed in AR-negative HEK-293 cells transfected with an expression plasmid encoding an AR carrying a mutation ($AR_{Cys574\rightarrow Arg}$) in the DBD, which disrupts its ability to bind target DNA sequences (27,51). In these

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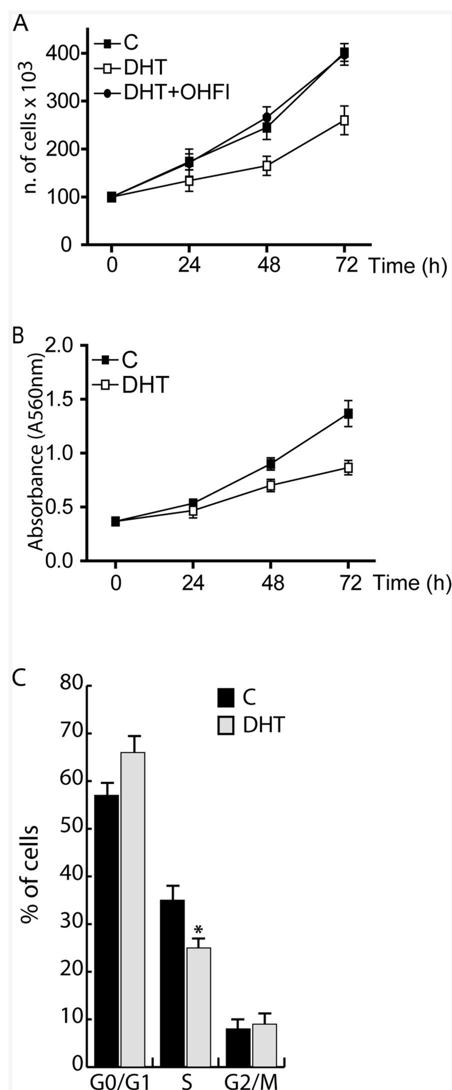


**Figure 1.** Proliferative response of MCF-7 cells to DHT. Serum starved MCF-7 cells were grown in PRF–CT in absence or presence of $10^{-7}$ M DHT and/or $10^{-6}$ M OH-Fl. The effects of the various drugs on cell proliferation were measured 0, 24, 48 and 72 h following initial exposure to treatments by: (**A**) cell counting using a Burker's Chamber, with cell viability determined by trypan blue dye exclusion or (**B**) the MTT assay as described in 'Materials and Methods' section. (**B**) Serum starved MCF-7 cells were grown in PRF–CT in absence or presence of $10^{-7}$ M DHT for 72 h and then subjected to cell-cycle analysis as described in 'Materials and Methods' section. Data, representing a mean ± SD of three independent experiments, each in triplicate, were statistically analysed by Student's *t*-test, $*P < 0.05$ versus untreated.

circumstances, no decrease in cyclin D1 promoter activity could be detected (Figure 2D), suggesting the existence, in the cyclin D1 promoter, of putative androgen responsive region(s).

## Characterization of functional androgen responsive region(s) in the cyclin D1 promoter

To define the AR responsive region(s) of the cyclin D1 promoter, a series of 5′-promoter-deleted mutants were used and tested for both androgen sensitivity and promoter activity, in MCF-7 cells. The constructs D1Δ-2960, D1Δ-944 and D1Δ-848, which include 2.960 kb, 0.944 kb and 0.848 kb of the cyclin D1 promoter fragment, respectively, showed a decreased transcriptional activity upon DHT stimulation with respect to untreated controls. A weaker inhibition of the cyclin D1 promoter signal was evidenced using the D1Δ-254 and D1Δ-136 constructs, while the D1Δ-96 plasmid, failed to respond to DHT (Figure 3).

Sequence analysis of the −848 bp to −254 bp cyclin D1 promoter DNA fragment revealed a likely ARE sequence (TGCTAAattAGTTCT) located at the −570 bp position of the promoter. This putative ARE (*CCND1*-ARE) is homologous to the AREs found in the promoters of the *sc* (*sc*-ARE1.2) and *slp* (*slp*-HRE2) genes (58) (Figure 4A). To assess the relative importance of this sequence we used site-directed mutagenesis to alter it. Nucleotide substitutions were introduced into the −570-bp to −556-bp fragment of D1Δ-944 and the mutant promoter D1Δ-944-mARE was assayed in parallel with the unmutated D1Δ-944 promoter. Disruption of the putative ARE consensus site resulted in a significant loss of the inhibitory effect of DHT on cyclin D1 promoter activity (Figure 4B). These results address the *CCND1*-ARE as a crucial sequence in mediating cyclin D1 promoter inhibition upon DHT exposure.

Nevertheless, mutations at the putative ARE did not completely abolish cyclin D1 promoter responsiveness to DHT, as indicated by the persistence of a weak DHT-induced decrease in the luciferase activity of the D1Δ-136 construct, containing two Sp1 sites. To investigate whether this additional *cis*-element might be involved in the androgen response, as previously demonstrated for other AR target genes (57,58), site-directed mutagenesis was used to introduce nucleotide substitutions into the −117-bp to −101-bp fragment of D1Δ-944 (D1Δ-944-mSp1) or D1Δ-944-mARE (D1Δ-944-mARE/Sp1) and the mutant promoters were assayed in functional analysis. As depicted in Figure 4B, DHT treatment is able to induce only an about 30% inhibition of D1Δ-944-mSp1 promoter activity, indicating that Sp1 sites are important for an AR full inhibitory effect on the cyclin D1 promoter. Consistent with these observations, mutation of both ARE and Sp1 motifs completely abolishes DHT responsiveness of the cyclin D1 promoter further suggesting that its regulation occurs through a functional interaction of the AR with the *CCND1*-ARE and Sp1 responsive sequences.

## AR interacts with the ARE containing region of the cyclin D1 promoter

To support the functional importance of the identified ARE sequence, a double-stranded oligonucleotide containing the putative ARE sequence was used in a DNA affinity precipitation assay (DAPA) to determine whether AR can bind to the *CCND1*-ARE consensus sequence. Endogenous AR was found associated with the putative consensus oligonucleotide following DHT administration only, while AR binding was undetectable in nuclear cell lysates from untreated cells (Figure 5A). Moreover, AR

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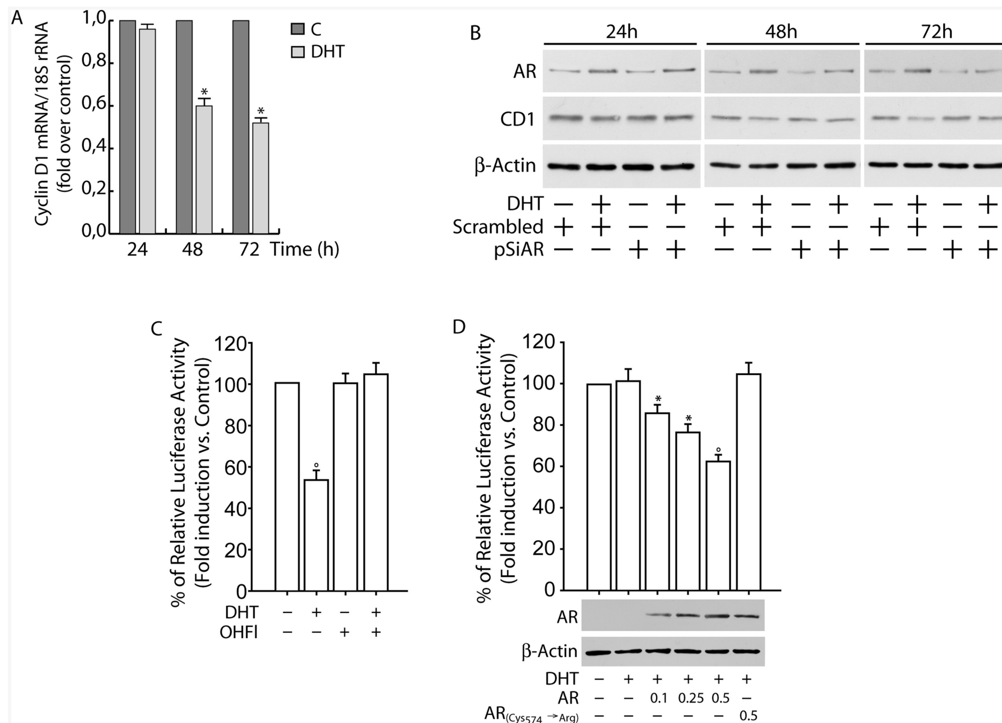


**Figure 2.** *CCND1* expression and promoter activity are inhibited by DHT-activated AR. **(A)** Serum-starved MCF-7 cells were treated or left untreated with $10^{-7}$ M DHT for 24, 48 or 72 h in PRF–CT and total RNA was extracted. The abundance of cyclin D1 mRNA was detected by real-time reverse transcription-PCR, as described in 'Materials and Methods' section. **(B)** Total proteins were isolated from MCF-7 cells transfected with 0.5 µg pSiAR or 0.5 µg pSiCon scrambled control and treated for 24, 48 and 72 h with $10^{-7}$ M DHT or left untreated in PRF–CT. A 50 µg of protein lysates were analysed by WB to evaluate the expression of cyclin D1 and AR. The expression of β-actin was assessed as control of protein loading. Results are representative of three independent experiments. **(C)** Serum-starved MCF-7 cells were transiently transfected with D1Δ-2960 reporter plasmid (0.25 µg/well). Upon transfection, cells were treated with $10^{-7}$ M DHT and/or $10^{-6}$ M OH-Fl, or left untreated in PRF–CT for 24 h. Firefly luciferase activity was detected and expressed as percentage of Relative Luciferase Activity. Activation of the reporter gene D1Δ-2960 in the presence of PRF–CT is arbitrarily set at 100%. Results represent the means ± SD of five separate experiments each in triplicate. Data were statistically analysed by student's *t*-test, $^{*}P < 0.01$ versus untreated. **(D)** AR negative HEK 293 cells were co-transfected with D1Δ-2960 (0.25 µg/well) plus increasing quantities (µg) of pcDNA3-AR (AR) or CMVP881 ($AR_{Cys\text{-}574 \rightarrow Arg}$) (0.5 µg/well) as indicated. Upon transfection, cells were treated with $10^{-7}$ M DHT, or left untreated in PRF–CT for 24 h. Firefly luciferase activity was detected and expressed as percentage of relative luciferase activity. Activation of the reporter gene D1Δ-2960 in the presence of PRF–CT is arbitrarily set at 100%. Linear relation between transfected AR plasmid and expressed AR protein quantity was evaluated by WB. Results represent the mean ± SD from five independent experiments. Data were statistically analysed by Student's *t*-test, $^{*}P < 0.05$ and $^{\circ}P < 0.01$ versus untreated.

from DHT treated MCF-7 lysates was unable to bind to a cyclin D1 promoter oligo in which the putative ARE consensus site was mutated.

To further determine the specificity of the putative ARE site we performed electrophoretic mobility shift assay (EMSA). As shown in Figure 5B, factors present in MCF-7 cells nuclear extracts retarded the mobility of the putative ARE domain in a specific manner, since the formation of a protein–DNA complex was found (Figure 5B, lane 1). Of note, DHT treatment induced a strong increase in the protein–DNA association (Figure 5B, lane 2) whose appearance was competed effectively by a 100-fold molar excess of unlabelled probe (Figure 5B, lane 3), demonstrating the specificity of the DNA-binding complex. This inhibition was no longer observed using a mutated putative ARE oligonucleotide as competitor (Figure 5B, lane 4). Contemporary administration of the anti-androgen bicalutamide caused a dramatic decrease in the DNA-binding complex induced by DHT (Figure 5B, lane 6) suggesting that the AR is involved in the binding to the *CCND1*-ARE. Finally the specificity of these bands was proved by the drastic attenuation of the complex in the presence of a mouse anti-AR monoclonal antibody, indicating that this antibody recognizes AR epitopes that interact or interfere with AR association to ARE (Figure 5B, lane 7). Moreover, the addition of a rabbit anti-AR polyclonal antibody to the EMSA generated a molecular weight super shift (Figure 5B, lane 8), further indicating the involvement of the AR. Normal rabbit IgG addition did not affect protein–DNA complex formation (Figure 5B, lane 9).

To better determine the physiological relevance of the *CCND1*-ARE, we investigated whether AR interacts with this region of the *CCND1* as it exist in native chromatin, performing ChIP in AR-positive MCF-7 andT47D breast cancer cells, using anti-AR or anti-RNA Polymerase (Pol) II antibodies. AR occupancy of the *CCND1*-ARE containing region of cyclin D1 promoter was induced in a ligand-dependent manner, since AR recruitment was enhanced by DHT (Figure 5C). The inhibitory role of

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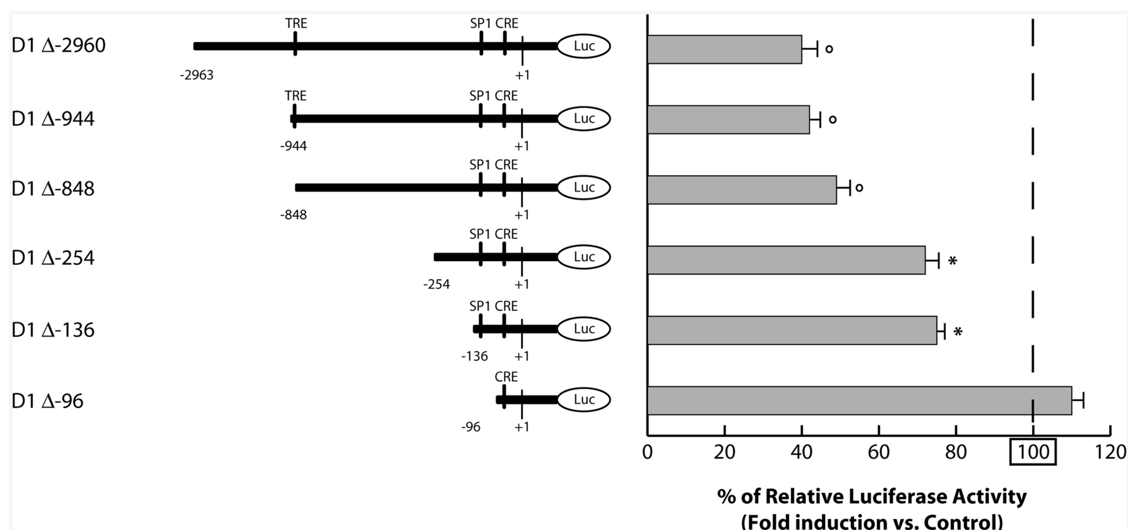


**Figure 3.** Functional analysis of the promoter regions involved in AR-mediated modulation of the cyclin D1 promoter. (**Left panel**) Schematic representation of human cyclinD1 promoter fragments used in this study. All of the promoter constructs contain the same 3′-boundary (+142). The 5′-boundaries of the promoter fragments varied from −2960 to −96. (**Right panel**) Transcriptional activity of cyclin D1 promoter constructs following DHT stimulation is shown. Serum-starved MCF-7 cells were transiently transfected with the indicated mutated plasmid (0.25 µg/well), as reported in 'Materials and Methods' section. Upon transfection, cells were treated with $10^{-7}$ M DHT or left untreated in PRF–CT for 24 h. Firefly luciferase activity was detected and expressed as percentage of Relative Luciferase Activity. Activation of reporter plasmids in the presence of PRF–CT only is arbitrarily set at 100. Results represent the means ± SD of three separate experiments each in triplicate. Data were statistically analysed by Student's *t*-test, $^{*}P < 0.05$ and $^{\circ}P < 0.01$ versus untreated.

AR on cyclin D1 promoter, is further evidenced by the dynamic of RNA Pol II recruitment onto the cyclin D1 promoter, that appears to be drastically reduced upon DHT treatment (Figure 5C).

### AR associates with the SP1 sites present in the cyclin D1 proximal promoter

To further investigate the functional importance of the Sp1 sites in the AR-mediated modulation of the cyclin D1 promoter, the ability of AR to associate to the Sp1 sites containing sequence of the cyclin D1 promoter (59) was first examined by DAPA. The association between AR and the cyclin D1-Sp1 sequence was observed only upon DHT treatment, while AR binding could not be detected in nuclear cell lysates from untreated cells (Figure 6A).

AR recruitment on the Sp1-containing region of the endogenous cyclin D1 promoter was also investigated by ChIP experiments using anti-AR or anti-RNA Pol II antibodies. AR was recruited to the Sp1 sites containing promoter region in a ligand-dependent manner. Conversely, upon DHT administration, RNA Pol II was released from the investigated promoter region (Figure 6B) supporting the hypothesis that this Sp1-containing DNA region might cooperate with the *CCND1*-ARE site in the AR-dependent regulation of *CCND1*.

### The AR corepressor DAX1 is recruited at the *CCND1*-ARE containing sequence of the cyclin D1 promoter

To assess whether the decrease of cyclin D1 promoter transcriptional activity might be caused by the cooperative interaction between AR and negative transcriptional regulators, we investigated the involvement of the orphan nuclear receptor DAX1, which has been shown to interact with and function as a negative coregulator of AR (60–62).

DAX1 abundance was analysed in cytoplasmic and nuclear protein fractions obtained from MCF-7 cells stimulated or not with $10^{-7}$ M DHT. DAX1 was mainly present in the nuclear compartment and its abundance increased upon DHT treatment (Figure 7A). To investigate whether the DHT-induced enhancement of DAX1 expression is paralleled by an AR/DAX1 physical interaction, co-IP assay was performed on nuclear and cytoplasmic protein fractions from MCF-7 cells. The formation of a AR/DAX1 complex was clearly evidenced into the nucleus upon DHT administration (Figure 7B), arguing for a DAX1 regulatory function on AR transcriptional activity.

To test whether DAX1 could assemble on the androgen responsive region of the cyclin D1 promoter, DAPA assay was performed. The binding affinity of DAX1 to the DNA probe containing the putative *CCND1*-ARE site (Figure 8A) or the Sp1 site (Figure 8B) was significantly changed in response to DHT. Furthermore, no binding of another AR corepressor, such as SMRT, to the *CCND1*-ARE containing DNA probe could be detected (data not shown).

To verify that DAX1 is recruited within the androgen responsive region of human cyclin D1 promoter *in vivo*, ChIP assay was performed using anti-DAX1 antibody. DAX1 occupancy of the *CCND1*-ARE containing region of the cyclin D1 promoter significantly increased following DHT treatment. No recruitment of the steroid receptor coactivator AIB1, was detected under the same

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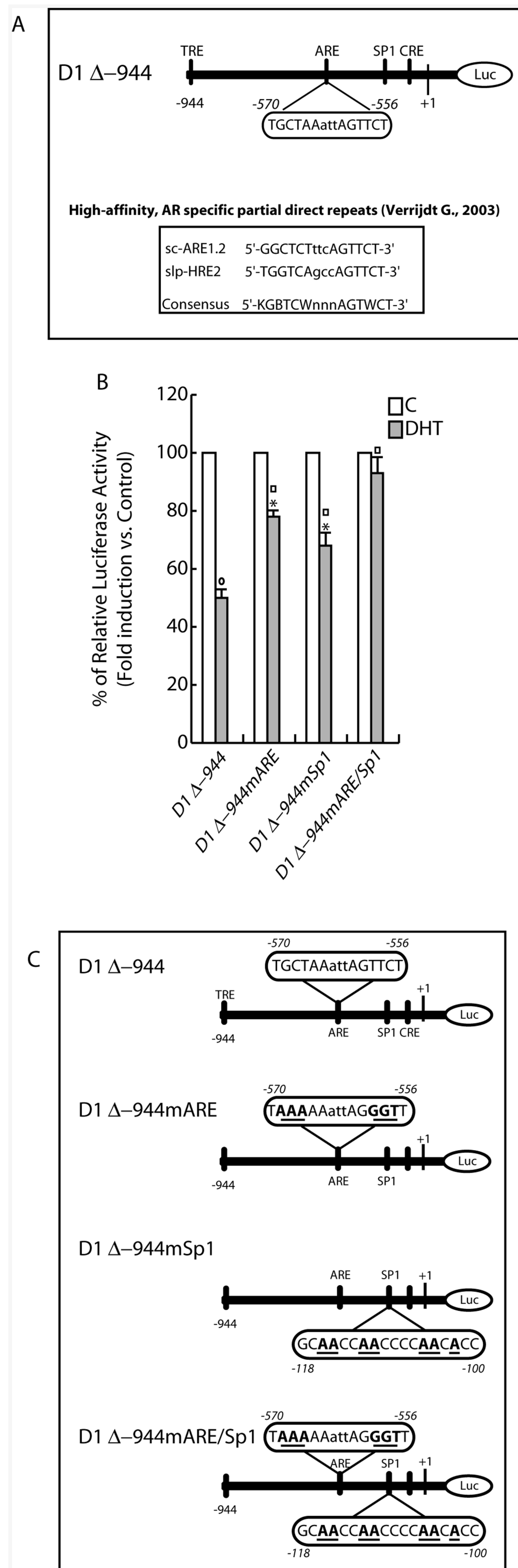


**Figure 4.** Mutations of the putative ARE site or the Sp1 sites reduce the ability of AR to down-regulate cyclin D1 transcriptional activity. **(A)** Representation of the putative ARE site location within the cyclin D1 promoter. A list of known high affinity AR-specific ARE sequences is listed in the lower panel. **(B)** Site-directed mutagenesis was performed as described in 'Materials and Methods' section. MCF-7 cells were co-transfected, as reported in 'Materials and Methods' section, by using the mutated promoter constructs, schematically represented in **(C)**. Upon transfection, cells were treated with $10^{-7}$M DHT or left untreated in PRF–CT for 24 h. Firefly luciferase activity was detected and expressed as percentage of Relative Luciferase Activity. Activation of reporter gene in the presence of PRF–CT is arbitrarily set at 100. Results represent the means ± SD of three separate experiments each in triplicate. Data were statistically analysed by Student's *t*-test, $*P < 0.05$ and $^{\circ}P < 0.01$ versus control (C); $^{\square}P < 0.01$ versus DHT treated D1Δ-944.

experimental condition (Figure 8C and D), showing the selectivity of corepressor recruitment. A similar pattern of DAX1 recruitment to the Sp1-containing region of the cyclin D1 promoter was observed (Figure 8E). Since histone deacetylation represents at least one of the mechanisms by which repressor proteins mediate transcriptional repression (63–64), we investigated HDAC1 and/or HDAC3 association on the AR responsive sequences within the cyclin D1 promoter by DAPA and ChIP assays. In both assays, DHT stimulation induces the recruitment of HDAC1 but not HDAC3 to either the *CCND1*-ARE consensus sequence (Figure 8A, C and D), or the Sp1 site (Figure 8B and E).

To confirm the role of DAX1 in the AR-dependent modulation of the *CCND1*, RNA silencing was used to knockdown the expression of endogenous DAX1 in MCF-7 cells. A 50% decrease of DAX1 protein levels was achieved using anti-DAX1 siRNA in MCF-7 cells after 48 h (Figure 9A). ChIP analysis was then performed on MCF-7 cells transfected with DAX1 siRNA or scrambled control siRNA. Upon DHT stimulation, DAX1 knockdown caused the recovery of RNA Pol II recruitment within the *CCND1*-ARE (Figure 9B and C) and Sp1 (Figure 9D) containing region of the cyclin D1 promoter, whereas the scrambled siRNA showed no effect on RNA Pol II dismissal. AR recruitment on the responsive regions of the cyclin D1 promoter was not affected by DAX1 silencing (Figure 9B, C and D). Moreover, DAX1 knockdown resulted in the loss of the DHT inhibitory effect on cyclin D1 levels (Figure 9A), strengthening the role of DAX1 as a component of the repressor complex participating in the AR-dependent inhibition of the *CCND1*.

## DISCUSSION

The AR represents a common characteristic in breast tumors, since it is expressed in a significant subset (60–90%) of both ERα-positive and -negative breast cancers (33–35). AR expression in breast cancer tissue samples has been shown to be associated with a better prognosis (35,38–41,65–66). Conversely, lack of AR expression correlates with transformation from in situ to invasive basal subtype of high-grade ductal breast carcinoma (48) and with a poor prognosis in lymph node-positive ER/PR/Her2-negative breast cancers (41); even though a recent report suggests that the AR may participate to the development of invasive ductal carcinoma by repressing E-cadherin expression (67).

Despite the growing body of evidence indicating a protective role of AR in breast tumourigenesis, the mechanisms underlying the inhibition of the *in vivo* and/or *in vitro*

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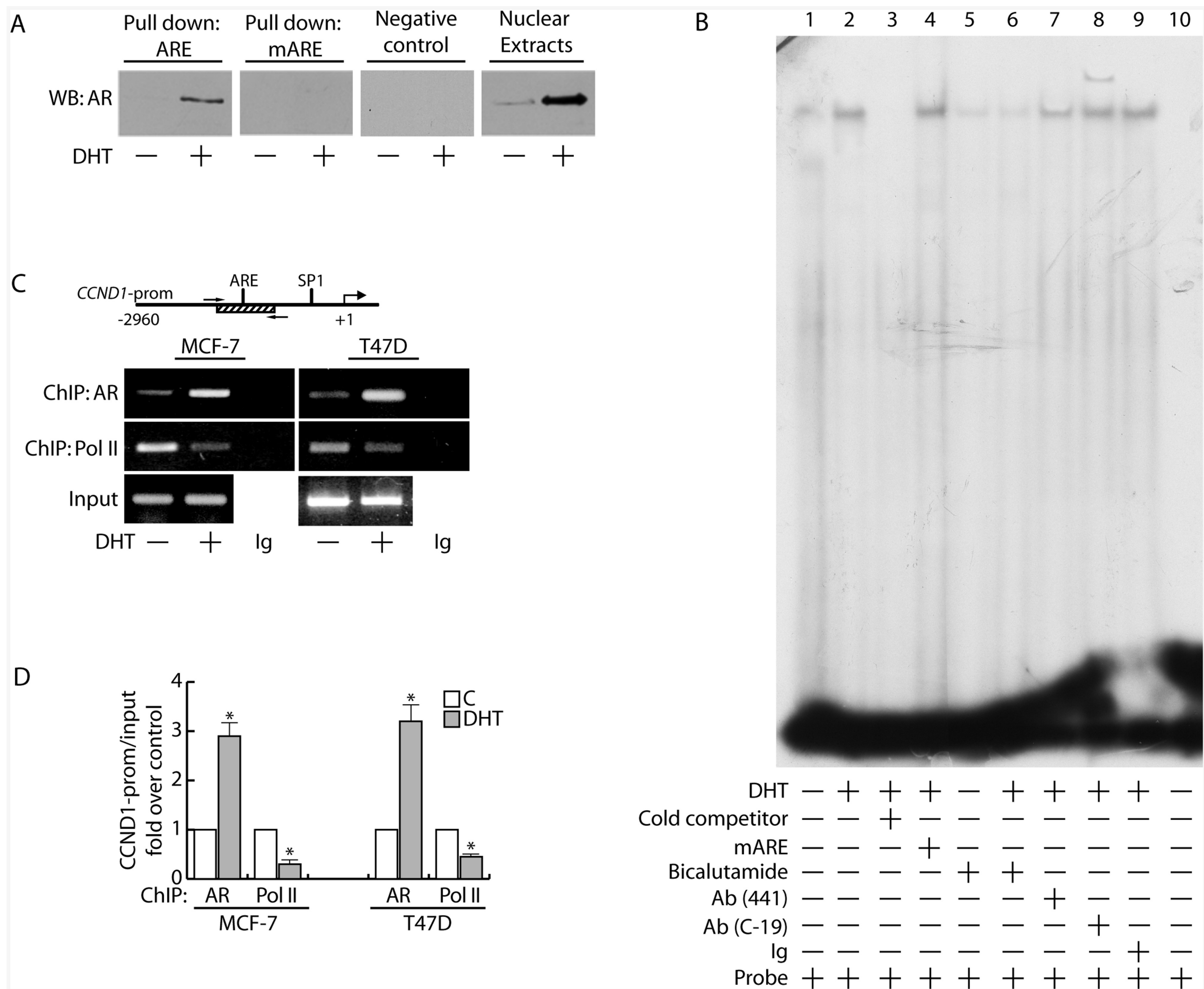


**Figure 5.** DHT induces activation of AR-DNA-binding activity in MCF-7 cells and AR recruitment to the ARE containing region of the cyclin D1 promoter. **(A)** Nuclear extracts from MCF-7 cells treated with $10^{-7}$ M DHT or left untreated in PRF–CT for 2 h, were incubated with either a biotinylated oligonucleotide containing the *CCND1*-ARE site or a biotinylated oligonucleotide mutated in the *CCND1*-ARE (mARE) consensus site and subjected to DNA affinity precipitation assay as described in 'Materials and Methods' section. Specifically bound proteins were subjected to WB analysis using an antibody specific to AR. The specificity of the binding was tested by loading the unbound fraction (Negative control). MCF-7 cell nuclear extracts were used as positive control. **(B)** Nuclear extracts from MCF-7 cells left untreated (lane 1) or treated with $10^{-7}$ M DHT for 2 h (lanes 2–4 and 6–9) were incubated with a double-stranded *CCND1*-ARE-specific consensus sequence probe labelled with [$\lambda^{32}$P]ATP and subjected to electrophoresis in a 6% polyacrylamide gel. Competition experiments were done by adding as competitor a 100-fold molar excess of unlabelled probe (lane 3) or a 100-fold molar excess of unlabelled oligonucleotide containing a mutated *CCND1*-ARE (lane 4). The formation of the DNA–AR complex was blocked by the addition of $10^{-5}$ M Bicalutamide (lanes 5 and 6). The specificity of the binding was tested by adding an anti-AR antibodies to the reaction mixture (lane 7-8) or IgG (lane 9). Lane 10 contains probe alone. **(C)** Chromatin IPs were carried out on serum starved MCF-7 and T47D cells as described in 'Materials and Methods' section. Cells were treated with $10^{-7}$ M DHT or left untreated in PRF–CT for 2 h, lysed and DNA-associated proteins were precipitated using either anti-AR Ab or anti-RNA Pol II Ab (2 µg/sample each). In control samples (Ig), normal mouse or rabbit IgG, respectively, was used instead of the primary Abs as control of Ab specificity. The 5′-flanking sequence of the *CCND1* containing the ARE site was detected by PCR with specific primers listed in 'Materials and Methods' section. Inputs DNA were amplified as loading controls. **(D)** Five-microlitres volume of each sample and input from ChIP were used for real-time PCR as described in the 'Materials and Methods' section. Data were statistically analysed by Student's *t*-test, $*P < 0.05$ versus untreated.

breast cancer cells growth by activated AR, remain poorly defined.

In this report we provide evidence that DHT-activated AR is a transcriptional repressor of the *CCND1* in MCF-7 breast cancer cells. Analysis of the molecular events associated with this hormone-induced negative regulation of cyclin D1 expression allows, for the first time, the identification of a specific ARE within the *CCND1* proximal promoter, and recognizes the recruitment of DAX1 and HDAC1 as components for AR-mediated transcriptional repression.

Earlier studies have shown that DHT inhibits MCF-7 cells proliferation by targeting the G1/S transition of the cell cycle (28,30). Here, we demonstrated that, in MCF-7 cells, cyclin D1 expression is negatively modulated by DHT administration as evidenced by reduction of

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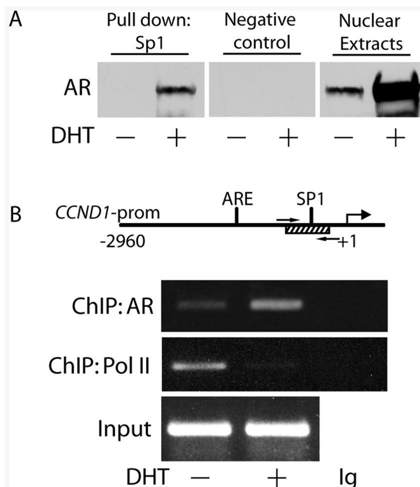


**Figure 6.** DHT induces recruitment of AR to the Sp1-site containing region of the cyclin D1 promoter. **(A)** Nuclear extracts from MCF-7 cells treated with $10^{-7}$ M DHT or left untreated in PRF–CT for 2 h, were incubated with a biotinylated oligonucleotide containing the *CCND1*-Sp1 site and subjected to DNA affinity precipitation assay as described in the 'Materials and Methods' section. Specifically bound proteins were subjected to WB analysis using an antibody specific to AR. The specificity of the binding was tested by loading the unbound fraction (Negative control). MCF-7 cell nuclear extracts were used as positive control. **(B)** Chromatin IPs were carried out on serum starved MCF-7 cells as described in the 'Materials and Methods' section. Cells were treated with $10^{-7}$ M DHT or left untreated in PRF–CT for 2 h, lysed and DNA-associated proteins were precipitated using either anti-AR Ab or anti-RNA Pol II Ab (2 μg/sample each). In control samples (Ig), normal mouse or rabbit IgG was used, respectively, instead of the primary Abs as control of Ab specificity. The 5′-flanking sequence of the *CCND1* containing the Sp1 site was detected by PCR with specific primers listed in the 'Materials and Methods' section. Inputs DNA were amplified as loading controls. Results are representative of three independent experiments.

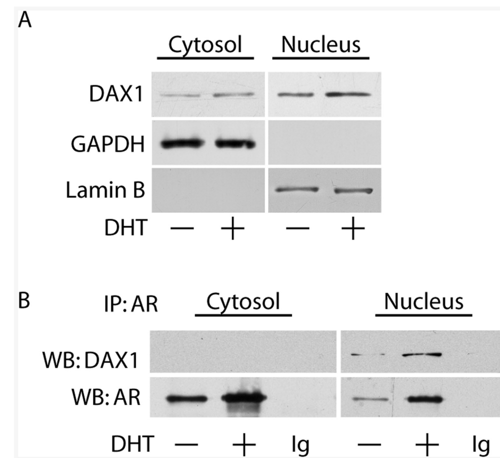


**Figure 7.** DHT induces the formation of a AR/DAX1 complex into the nucleus. **(A)** Serum starved MCF-7 cells were treated with $10^{-7}$ M DHT or left untreated in PRF–CT for 24 h. The expression of DAX1 and AR was determined by WB using 50 μg of either cytoplasmic or nuclear protein lysates. The expression of GAPDH and Lamin B was assessed as control of protein loading and purity of lysate fractions. **(B)** Five hundred micrograms of either nuclear or cytoplasmic lysates from MCF-7 cells, treated with $10^{-7}$ M DHT or left untreated in PRF–CT for 24 h, were immunoprecipitated (IP) with anti-AR mAb and subjected to WB to detect DAX1 and AR protein levels. Results, obtained after repetitive stripping and reprobing of the same filters, are representative of three independent experiments.

cyclin D1 mRNA and protein levels, and decrease of its promoter activity. The involvement of activated AR in this process is substantiated by the observation that in AR-negative HEK-293 cells, DHT administration is able to interfere with cyclin D1 promoter activity only in the presence of exogenous AR expression. Moreover, the DHT inhibitory effect strictly depends on the integrity of the AR-DBD, suggesting the existence, in the cyclin D1 promoter, of a putative androgen responsive region which mediates androgen response.

A number of studies examined the domains important for the functions of the cyclin D1 promoter demonstrating that it is a complex transcriptional unit composed of several distinct elements (17,59,68–70). Nevertheless, to date, no androgen response elements (AREs) have been identified within the cyclin D1 promoter. The present study provides evidence that AR-mediated inhibition of the *CCND1* is consequent to direct binding of the AR to a specific androgen responsive site, located at position −570 bp as indicated by deletion and sequence analyses of the cyclin D1 proximal promoter. The identified *CCND1*-ARE motif 5′-TGCTAAattAGTTCT-3′ resembles the so-called selective AREs that exhibit characteristics of partial direct repeats, instead of the conventional inverted repeats of the 5′-TGTTCT-3′ monomer-binding element, and are recognized exclusively by the AR (58,71,72).

This *CCND1*-ARE is functional, as demonstrated by transactivation studies, and capable to interact with the AR, in a ligand-dependent manner as evidenced by DAPA or EMSA. The physiological relevance of *CCND1*-ARE within the cyclin D1 promoter *in vivo* is pointed out by ChIP analysis showing that AR occupancy of the ARE containing promoter region is concomitant with a decrease in RNA Pol II recruitment, consistent with the reduced cyclin D1 transcriptional activity.

Our study also indicates that AR binding to the Sp1 sites within the proximal cyclin D1 promoter is required for a full DHT-dependent inhibition of cyclin D1 promoter activity. This observation is consistent with previous studies on the organization of transcription factor-binding sites within the regulatory regions of a selection of androgen-responsive genes, showing that a key feature of these genes is the presence of one or more AR-binding site (ARE, HRE) together with binding sequences for housekeeping (Sp1, NF-1), inducible (NF-kB, AP1) and tissue specific transcription factors (73). Therefore, androgen-regulated gene expression appears to be dependent on the coordinated interactions of the receptor protein and other transcription factors.

Intriguingly, our data indicate that on *CCND1* the promoter-bound DHT–AR complex functions as an inverse agonist, causing active repression of basal gene activity, eventually creating a repressive chromatin conformation associated with reduced RNA Polymerase II recruitment, in MCF-7 breast cancer cells.

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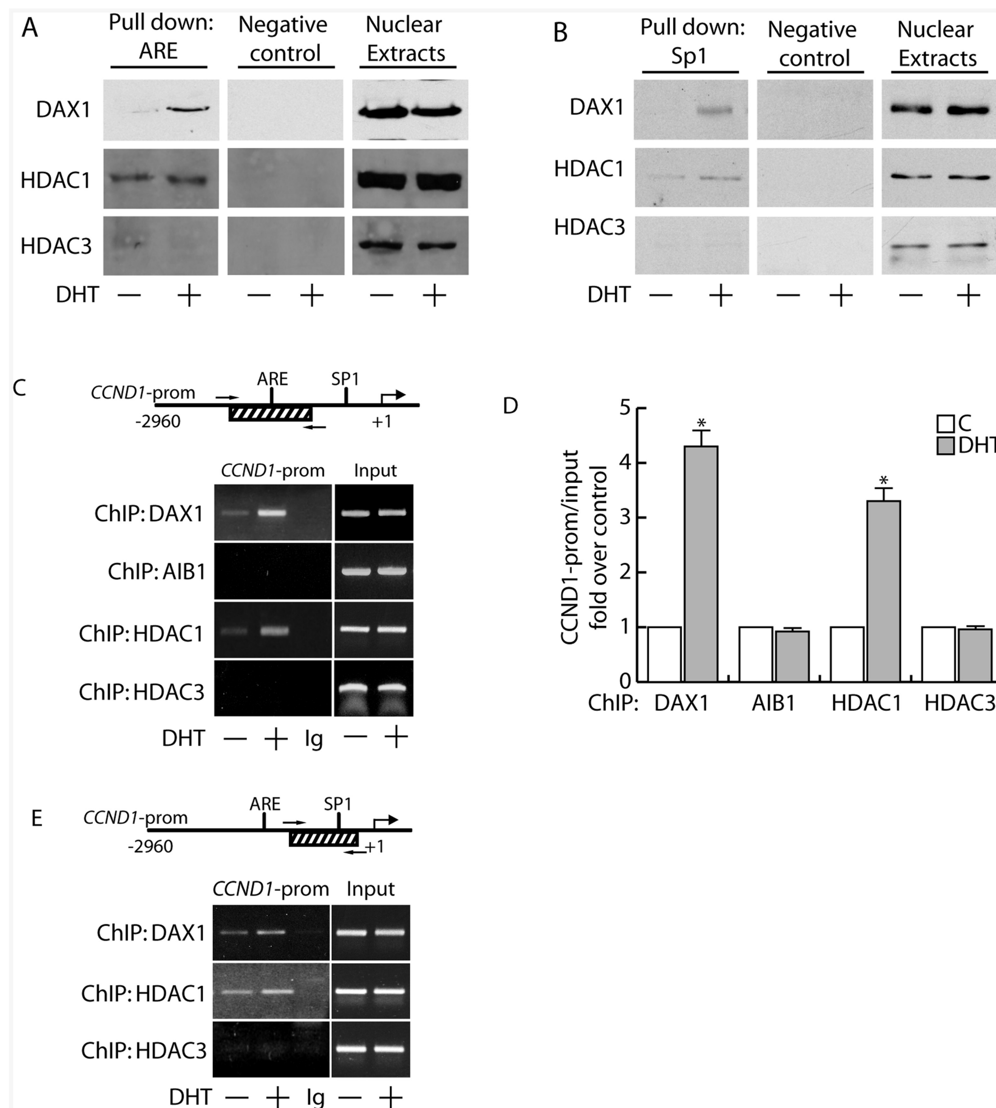


**Figure 8.** DHT induces the recruitment of DAX1 and histone deacetylase 1 to the CCND1-ARE and Sp1 sites containing regions of the cyclin D1 promoter. (**A**) and (**B**) Nuclear extracts from MCF-7 cells treated with $10^{-7}$ M DHT or left untreated in PRF–CT for 2 h were incubated with either a biotinylated oligonucleotide containing the *CCND1*-ARE site (A) or the Sp1 site (B) and subjected to DNA affinity precipitation assay as described in the 'Materials and Methods' section. Specifically bound proteins were subjected to WB analysis using antibodies specific to DAX1, HDAC1 or HDAC3. The specificity of the binding was tested by loading the unbound fraction (Negative control). MCF-7 cell nuclear extracts were used as positive control. **(C)** Chromatin IPs were carried out on serum starved MCF-7 cells as described in the 'Materials and Methods' section. Cells were treated with $10^{-7}$ M DHT or left untreated in PRF–CT for 2 h, lysed and DNA-associated proteins were precipitated using antibodies (Ab) specific to AR, AIB1, HDAC1, HDAC3 (2 µg/sample each). In control samples (Ig), normal mouse or rabbit IgG was used instead of the primary Abs as control of Ab specificity. The 5′-flanking sequence of the *CCND1* containing the CCND1-ARE site was detected by PCR using specific primers listed in the 'Materials and Methods section. Inputs DNA were amplified as loading controls. **(D)** Five-microlitres volume of each sample and input from ChIP were used for real-time PCR as described in the 'Materials and Methods' section. Data were statistically analysed by Student's *t*-test, $*P < 0.05$ versus untreated. **(E)** ChIP assays were carried out as above described. The 5′-flanking sequence of the *CCND1* containing the Sp1 sites was detected by PCR using specific primers reported in the 'Materials and Methods' section. Input DNAs were amplified as loading controls. Results are representative of three independent experiments.

Our proposed model for AR-mediated repression of the *CCND1* involves the recruitment of the atypical orphan nuclear receptor DAX1 that is expressed in tissues directly involved in steroid hormone production and reproductive function (74–75). DAX1 inhibits ligand-dependent trans-activation by agonist-bound nuclear receptors like AR, ER and progesterone receptor (60–61,76). Moreover, DAX1 has been proposed to be involved in the development of cancers of a variety of tissues and has been found to positively correlate with AR and ER expression in breast cancer specimens (60–61,74,77). Our results suggest that, in MCF-7 cells, upon DHT stimulation, DAX1 is recruited on the androgen responsive region of the cyclin D1 promoter concomitantly to AR. The repressor complex formation also embraces the participation of HDAC1, further contributing to explain the AR-mediated inhibition of *CCND1* transcription (Figure 10).

Given the importance of cyclin D1 in breast cancer cell proliferation, androgen-mediated regulation of cyclin D1 levels appears to play a crucial role in cell-cycle control.

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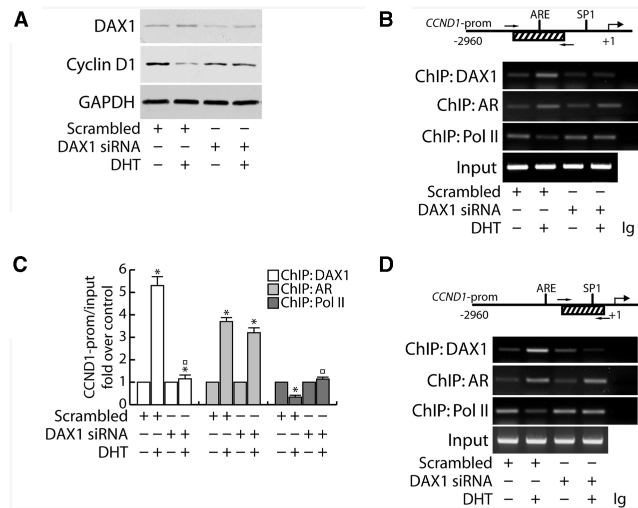


**Figure 9.** Effect of DAX1 knockdown on the modulation of *CCND1* expression by AR.**(A)** Total cellular proteins were isolated from MCF-7 cells transfected with 5 nM DAX1 siRNA or 5 nM scrambled control siRNA (scrambled) and treated for 72 h with $10^{-7}$ M DHT or left untreated in PRF–CT. Fifty micrograms of protein lysates were analysed by WB to evaluate the expression of DAX1 and cyclin D1. The expression of GAPDH was assessed as control of protein loading. Results were obtained after repetitive stripping and reprobing of the same filters. **(B)** ChIP assays were carried out on serum starved MCF-7 cells transfected with 100 nM DAX1 siRNA or 100 nM scrambled control siRNA (scrambled). Cells were then treated with $10^{-7}$ M DHT for 2 h and DNA-associated proteins were precipitated using either anti-DAX1, AR or Pol II antibodies (2 µg/sample each). In control samples (Ig), normal mouse or rabbit IgG were used instead of the primary Abs, as control of Ab specificity. The region of the cyclin D1 promoter containing the *CCND1*-ARE site, was detected by PCR using specific primers listed in the 'Materials and Methods' section. Inputs DNA were amplified as loading controls. **(C)** Five-microliters volume of each sample and input from ChIP were used for real-time PCR as reported in the 'Materials and Methods' section. Data were statistically analysed by Student's t-test, $*P < 0.05$ and $^{\square}P < 0.05$ versus scrambled DHT treated. **(D)** ChIP assays were carried out as above described. The 5′-flanking sequence of the *CCND1* containing the Sp1 sites was detected by PCR using specific primers listed in the 'Materials and Methods' section. Input DNAs were amplified as loading controls. Results are representative of three independent experiments.

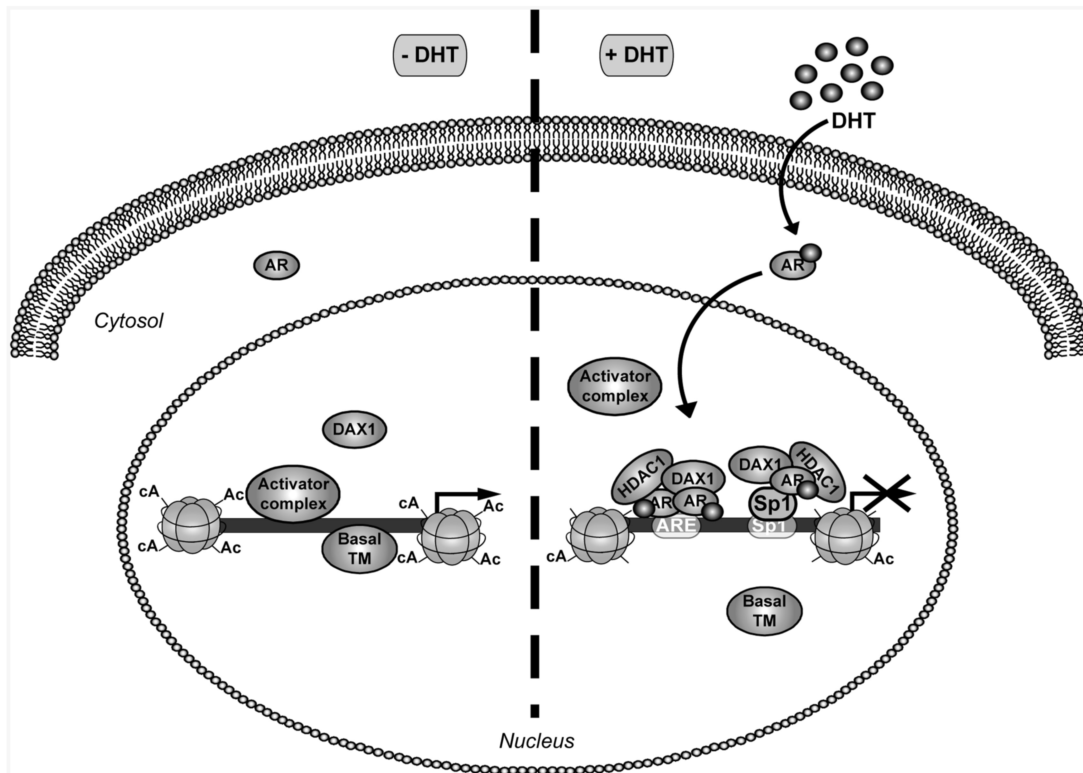


**Figure 10.** Proposed model for AR-mediated repression of the *CCND1*. In the absence of DHT, *CCND1* basal activity is regulated by several serum factors acting through multiple regulatory region of the cyclin D1 promoter and enabling a permissive chromatin conformation and gene transcription (13,15,17). Upon DHT treatment, AR is recruited on the ARE- and Sp1-containing region of the proximal cyclin D1 promoter, causing displacement of RNA Polymerase II and recruitment of a corepressor complex containing DAX1 and HDAC1, with consequent repression of cyclin D1 expression.

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The importance of our results is highlighted by recent studies demonstrating that biologically active DHT is locally produced in breast carcinoma tissues. Interestingly, intratumoural DHT levels are positively associated with AR and 5α-reductase 1 expression but inversely correlated with tumour size, Ki-67 and aromatase expression (78), suggesting that in AR-positive breast carcinomas the use of aromatase inhibitors may be more effective by accumulation of the local DHT concentration. The biological significance of DHT-induced inhibition of cyclin D1 expression is also pointed out by clinical studies using tamoxifen as an adjuvant therapy in ER-positive breast cancers, showing a higher response and better survival rate in cancers with cyclin D1 low/moderate expression (7–8). These observations, supported by the widespread expression of AR in primary and metastatic breast tumours, suggest the possibility that targeting the AR-signalling pathway could be helpful in improving new molecular and pharmacological approaches for breast cancer treatment and to potentiate the effectiveness of anti-oestrogen adjuvant therapies.

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## REFERENCES

1. Arnold,A. and Papanikolaou,A. (2005) Cyclin D1 in breast cancer pathogenesis. *J. Clin. Oncol.*, **23**, 4215–4224.
2. Ormandy,C.J., Musgrove,E.A., Hui,R., Daly,R.J. and Sutherland,R.L. (2003) Cyclin D1, EMS1 and 11q13 amplification in breast cancer. *Breast Cancer Res. Treat.*, **78**, 323–335.
3. Gillett,C., Fantl,V., Smith,R., Fisher,C., Bartek,J., Dickson,C., Barnes,D. and Peters,G. (1994) Amplification and overexpression of cyclin D1 in breast cancer detected by immunohistochemical staining. *Cancer Res.*, **54**, 1812–1817.
4. Buckley,M.F., Sweeney,K.J., Hamilton,J.A., Sini,R.L., Manning,D.L., Nicholson,R.I., deFazio,A., Watts,C.K., Musgrove,E.A. and Sutherland,R.L. (1993) Expression and amplification of cyclin genes in human breast cancer. *Oncogene*, **8**, 2127–2133.
5. Hodges,L.C., Cook,J.D., Lobenhofer,E.K., Li,L., Bennett,L., Bushel,P.R., Aldaz,C.M., Afshari,C.A. and Walker,C.L. (2003) Tamoxifen functions as a molecular agonist inducing cell cycle-associated genes in breast cancer cells. *Mol. Cancer Res.*, **1**, 300–311.
6. Hui,R., Finney,G.L., Carroll,J.S., Lee,C.S., Musgrove,E.A. and Sutherland,R.L. (2002) Constitutive overexpression of cyclin D1 but not cyclin E confers acute resistance to antiestrogens in T-47D breast cancer cells. *Cancer Res.*, **62**, 6916–6923.
7. Jirstrom,K., Stendahl,M., Ryden,L., Kronblad,A., Bendahl,P.O., Stal,O. and Landberg,G. (2005) Adverse effect of adjuvant tamoxifen in premenopausal breast cancer with cyclin D1 gene amplification. *Cancer Res.*, **65**, 8009–8016.
8. Stendahl,M., Kronblad,A., Ryden,L., Emdin,S., Bengtsson,N.O. and Landberg,G. (2004) Cyclin D1 overexpression is a negative predictive factor for tamoxifen response in postmenopausal breast cancer patients. *Br. J. Cancer*, **90**, 1942–1948.
9. Wang,T.C., Cardiff,R.D., Zukerberg,L., Lees,E., Arnold,A. and Schmidt,E.V. (1994) Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice. *Nature*, **369**, 669–671.
10. Lee,R.J., Albanese,C., Fu,M., D'Amico,M., Lin,B., Watanabe,G., Haines,G.K. 3rd, Siegel,P.M., Hung,M.C., Yarden,Y. *et al.* (2000) Cyclin D1 is required for transformation by activated Neu and is induced through an E2F-dependent signaling pathway. *Mol. Cell. Biol.*, **20**, 672–683.
11. Yu,Q., Geng,Y. and Sicinski,P. (2001) Specific protection against breast cancers by cyclin D1 ablation. *Nature*, **411**, 1017–1021.
12. Grillo,M., Bott,M.J., Khandke,N., McGinnis,J.P., Miranda,M., Meyyappan,M., Rosfjord,E.C. and Rabindran,S.K. (2006) Validation of cyclin D1/CDK4 as an anticancer drug target in MCF-7 breast cancer cells: effect of regulated overexpression of cyclin D1 and siRNA-mediated inhibition of endogenous cyclin D1 and CDK4 expression. *Breast Cancer Res. Treat.*, **95**, 185–194.
13. Dupont,J., Karas,M. and LeRoith,D. (2000) The potentiation of estrogen on insulin-like growth factor I action in MCF-7 human breast cancer cells includes cell cycle components. *J. Biol. Chem.*, **275**, 35893–35901.
14. Liu,M.M., Albanese,C., Anderson,C.M., Hilty,K., Webb,P., Uht,R.M., Price,R.H. Jr, Pestell,R.G. and Kushner,P.J. (2002) Opposing action of estrogen receptors alpha and beta on cyclin D1 gene expression. *J. Biol. Chem.*, **277**, 24353–24360.
15. Musgrove,E.A., Hamilton,J.A., Lee,C.S., Sweeney,K.J., Watts,C.K. and Sutherland,R.L. (1993) Growth factor, steroid, and steroid antagonist regulation of cyclin gene expression associated with changes in T-47D human breast cancer cell cycle progression. *Mol. Cell. Biol.*, **13**, 3577–3587.
16. Altucci,L., Addeo,R., Cicatiello,L., Dauvois,S., Parker,M.G., Truss,M., Beato,M., Sica,V., Bresciani,F. and Weisz,A. (1996) 17beta-Estradiol induces cyclin D1 gene transcription, p36D1-p34cdk4 complex activation and p105Rb phosphorylation during mitogenic stimulation of G(1)-arrested human breast cancer cells. *Oncogene*, **12**, 2315–2324.
17. Eeckhoute,J., Carroll,J.S., Geistlinger,T.R., Torres-Arzayus,M.I. and Brown,M. (2006) A cell-type-specific transcriptional network required for estrogen regulation of cyclin D1 and cell cycle progression in breast cancer. *Genes Dev.*, **20**, 2513–2526.
18. Birrell,S.N., Hall,R.E. and Tilley,W.D. (1998) Role of the androgen receptor in human breast cancer. *J Mammary Gland Biol. Neoplasia*, **3**, 95–103.
19. Somboonporn,W. and Davis,S.R. (2004) Testosterone effects on the breast: implications for testosterone therapy for women. *Endocr. Rev.*, **25**, 374–388.
20. Labrie,F., Luu-The,V., Labrie,C., Belanger,A., Simard,J., Lin,S.X. and Pelletier,G. (2003) Endocrine and intracrine sources of androgens in women: inhibition of breast cancer and other roles of androgens and their precursor dehydroepiandrosterone. *Endocr. Rev.*, **24**, 152–182.
21. Yeh,S., Hu,Y.C., Wang,P.H., Xie,C., Xu,Q., Tsai,M.Y., Dong,Z., Wang,R.S., Lee,T.H. and Chang,C. (2003) Abnormal mammary gland development and growth retardation in female mice and MCF7 breast cancer cells lacking androgen receptor. *J. Exp. Med.*, **198**, 1899–1908.
22. Tworoger,S.S., Missmer,S.A., Barbieri,R.L., Willett,W.C., Colditz,G.A. and Hankinson,S.E. (2005) Plasma sex hormone concentrations and subsequent risk of breast cancer among women using postmenopausal hormones. *J. Natl Cancer Inst.*, **97**, 595–602.
23. Missmer,S.A., Eliassen,A.H., Barbieri,R.L. and Hankinson,S.E. (2004) Endogenous estrogen, androgen, and progesterone concentrations and breast cancer risk among postmenopausal women. *J. Natl Cancer Inst.*, **96**, 1856–1865.
24. Dimitrakakis,C., Zhou,J., Wang,J., Belanger,A., LaBrie,F., Cheng,C., Powell,D. and Bondy,C. (2003) A physiologic role for

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testosterone in limiting estrogenic stimulation of the breast. *Menopause*, **10**, 292–298.
25. Murphy,L.C. and Watson,P. (2002) Steroid receptors in human breast tumorigenesis and breast cancer progression. *Biomed. Pharmacother.*, **56**, 65–77.
26. Zhou,J., Ng,S., Adesanya-Famuiya,O., Anderson,K. and Bondy,C.A. (2000) Testosterone inhibits estrogen-induced mammary epithelial proliferation and suppresses estrogen receptor expression. *FASEB J.*, **14**, 1725–1730.
27. Lanzino,M., De Amicis,F., McPhaul,M.J., Marsico,S., Panno,M.L. and Ando,S. (2005) Endogenous coactivator ARA70 interacts with estrogen receptor alpha (ERalpha) and modulates the functional ERalpha/androgen receptor interplay in MCF-7 cells. *J. Biol. Chem.*, **280**, 20421–20430.
28. Ando,S., De Amicis,F., Rago,V., Carpino,A., Maggiolini,M., Panno,M.L. and Lanzino,M. (2002) Breast cancer: from estrogen to androgen receptor. *Mol. Cell. Endocrinol.*, **193**, 121–128.
29. de Launoit,Y., Dauvois,S., Dufour,M., Simard,J. and Labrie,F. (1991) Inhibition of cell cycle kinetics and proliferation by the androgen 5 alpha-dihydrotestosterone and antiestrogen N,n-butyl-N-methyl-11-[16′ alpha-chloro-3′,17 beta-dihydroxy-estra-1′,3′,5′-(10′)triene-7′ alpha-yl] undecanamide in human breast cancer ZR-75-1 cells. *Cancer Res.*, **51**, 2797–2802.
30. Greeve,M.A., Allan,R.K., Harvey,J.M. and Bentel,J.M. (2004) Inhibition of MCF-7 breast cancer cell proliferation by 5alpha-dihydrotestosterone; a role for p21(Cip1/Waf1). *J. Mol. Endocrinol.*, **32**, 793–810.
31. Lanzino,M., Garofalo,C., Morelli,C., Le Pera,M., Casaburi,I., McPhaul,M.J., Surmacz,E., Ando,S. and Sisci,D. (2009) Insulin receptor substrate 1 modulates the transcriptional activity and the stability of androgen receptor in breast cancer cells. *Breast Cancer Res. Treat.*, **115**, 297–306.
32. Lea,O.A., Kvinnsland,S. and Thorsen,T. (1989) Improved measurement of androgen receptors in human breast cancer. *Cancer Res.*, **49**, 7162–7167.
33. Moinfar,F., Okcu,M., Tsybrovskyy,O., Regitnig,P., Lax,S.F., Weybora,W., Ratschek,M., Tavassoli,F.A. and Denk,H. (2003) Androgen receptors frequently are expressed in breast carcinomas: potential relevance to new therapeutic strategies. *Cancer*, **98**, 703–711.
34. Riva,C., Dainese,E., Caprara,G., Rocca,P.C., Massarelli,G., Tot,T., Capella,C. and Eusebi,V. (2005) Immunohistochemical study of androgen receptors in breast carcinoma. Evidence of their frequent expression in lobular carcinoma. *Virchows Arch.*, **447**, 695–700.
35. Agoff,S.N., Swanson,P.E., Linden,H., Hawes,S.E. and Lawton,T.J. (2003) Androgen receptor expression in estrogen receptor-negative breast cancer. Immunohistochemical, clinical, and prognostic associations. *Am. J. Clin. Pathol.*, **120**, 725–731.
36. Soreide,J.A., Lea,O.A., Varhaug,J.E., Skarstein,A. and Kvinnsland,S. (1992) Androgen receptors in operable breast cancer: relation to other steroid hormone receptors, correlations to prognostic factors and predictive value for effect of adjuvant tamoxifen treatment. *Eur. J. Surg. Oncol.*, **18**, 112–118.
37. Kimura,N., Mizokami,A., Oonuma,T., Sasano,H. and Nagura,H. (1993) Immunocytochemical localization of androgen receptor with polyclonal antibody in paraffin-embedded human tissues. *J. Histochem. Cytochem.*, **41**, 671–678.
38. Sauter,E.R., Lininger,J., Magklara,A., Hewett,J.E. and Diamandis,E.P. (2004) Association of kallikrein expression in nipple aspirate fluid with breast cancer risk. *Int. J. Cancer*, **108**, 588–591.
39. Sauter,E.R., Tichansky,D.S., Chervoneva,I. and Diamandis,E.P. (2002) Circulating testosterone and prostate-specific antigen in nipple aspirate fluid and tissue are associated with breast cancer. *Environ. Health Perspect.*, **110**, 241–246.
40. Schippinger,W., Regitnig,P., Dandachi,N., Wernecke,K.D., Bauernhofer,T., Samonigg,H. and Moinfar,F. (2006) Evaluation of the prognostic significance of androgen receptor expression in metastatic breast cancer. *Virchows Arch.*, **449**, 24–30.
41. Rakha,E.A., El-Sayed,M.E., Green,A.R., Lee,A.H., Robertson,J.F. and Ellis,I.O. (2007) Prognostic markers in triple-negative breast cancer. *Cancer*, **109**, 25–32.
42. Lobaccaro,J.M., Lumbroso,S., Belon,C., Galtier-Dereure,F., Bringer,J., Lesimple,T., Heron,J.F., Pujol,H. and Sultan,C. (1993) Male breast cancer and the androgen receptor gene. *Nat. Genet.*, **5**, 109–110.
43. Kollara,A., Kahn,H.J., Marks,A. and Brown,T.J. (2001) Loss of androgen receptor associated protein 70 (ARA70) expression in a subset of HER2-positive breast cancers. *Breast Cancer Res. Treat.*, **67**, 245–253.
44. Tormey,D.C., Lippman,M.E., Edwards,B.K. and Cassidy,J.G. (1983) Evaluation of tamoxifen doses with and without fluoxymesterone in advanced breast cancer. *Ann. Intern. Med.*, **98**, 139–144.
45. Ingle,J.N., Twito,D.I., Schaid,D.J., Cullinan,S.A., Krook,J.E., Mailliard,J.A., Tschetter,L.K., Long,H.J., Gerstner,J.G., Windschitl,H.E. *et al.* (1991) Combination hormonal therapy with tamoxifen plus fluoxymesterone versus tamoxifen alone in postmenopausal women with metastatic breast cancer. An updated analysis. *Cancer*, **67**, 886–891.
46. Macedo,L.F., Guo,Z., Tilghman,S.L., Sabnis,G.J., Qiu,Y. and Brodie,A. (2006) Role of androgens on MCF-7 breast cancer cell growth and on the inhibitory effect of letrozole. *Cancer Res.*, **66**, 7775–7782.
47. Sasano,H., Suzuki,T., Miki,Y. and Moriya,T. (2008) Intracrinology of estrogens and androgens in breast carcinoma. *J. Steroid Biochem. Mol. Biol.*, **108**, 181–185.
48. Hanley,K., Wang,J., Bourne,P., Yang,Q., Gao,A.C., Lyman,G. and Tang,P. (2008) Lack of expression of androgen receptor may play a critical role in transformation from in situ to invasive basal subtype of high-grade ductal carcinoma of the breast. *Hum. Pathol.*, **39**, 386–392.
49. Gonzalez-Angulo,A.M., Stemke-Hale,K., Palla,S.L., Carey,M., Agarwal,R., Meric-Berstam,F., Traina,T.A., Hudis,C., Hortobagyi,G.N., Gerald,W.L. *et al.* (2009) Androgen receptor levels and association with PIK3CA mutations and prognosis in breast cancer. *Clin. Cancer Res.*, **15**, 2472–2478.
50. Mosmann,T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods*, **65**, 55–63.
51. Zoppi,S., Marcelli,M., Deslypere,J.P., Griffin,J.E., Wilson,J.D. and McPhaul,M.J. (1992) Amino acid substitutions in the DNA-binding domain of the human androgen receptor are a frequent cause of receptor-binding positive androgen resistance. *Mol. Endocrinol.*, **6**, 409–415.
52. Recchia,A.G., Musti,A.M., Lanzino,M., Panno,M.L., Turano,E., Zumpano,R., Belfiore,A., Ando,S. and Maggiolini,M. (2009) A cross-talk between the androgen receptor and the epidermal growth factor receptor leads to p38MAPK-dependent activation of mTOR and cyclinD1 expression in prostate and lung cancer cells. *Int. J. Biochem. Cell Biol.*, **41**, 603–614.
53. Morelli,C., Garofalo,C., Sisci,D., del Rincon,S., Cascio,S., Tu,X., Vecchione,A., Sauter,E.R., Miller,W.H. Jr and Surmacz,E. (2004) Nuclear insulin receptor substrate 1 interacts with estrogen receptor alpha at ERE promoters. *Oncogene*, **23**, 7517–7526.
54. Catalano,S., Giordano,C., Rizza,P., Gu,G., Barone,I., Bonofiglio,D., Giordano,F., Malivindi,R., Gaccione,D., Lanzino,M. *et al.* (2009) Evidence that leptin through STAT and CREB signaling enhances cyclin D1 expression and promotes human endometrial cancer proliferation. *J. Cell. Physiol.*, **218**, 490–500.
55. Cascio,S., Bartella,V., Garofalo,C., Russo,A., Giordano,A. and Surmacz,E. (2007) Insulin-like growth factor 1 differentially regulates estrogen receptor-dependent transcription at estrogen response element and AP-1 sites in breast cancer cells. *J. Biol. Chem.*, **282**, 3498–3506.
56. Lanzino,M., Garofalo,C., Morelli,C., Le Pera,M., Casaburi,I., McPhaul,M.J., Surmacz,E., Ando,S. and Sisci,D. (2009) Insulin receptor substrate 1 modulates the transcriptional activity and the stability of androgen receptor in breast cancer cells. *Breast Cancer Res. Treat*, **115**, 297–306.
57. Yeap,B.B., Krueger,R.G. and Leedman,P.J. (1999) Differential posttranscriptional regulation of androgen receptor gene expression by androgen in prostate and breast cancer cells. *Endocrinology*, **140**, 3282–3291.

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58. Verrijdt,G., Haelens,A. and Claessens,F. (2003) Selective DNA recognition by the androgen receptor as a mechanism for hormone-specific regulation of gene expression. *Mol. Genet. Metab.*, **78**, 175–185.
59. Castro-Rivera,E., Samudio,I. and Safe,S. (2001) Estrogen regulation of cyclin D1 gene expression in ZR-75 breast cancer cells involves multiple enhancer elements. *J. Biol. Chem.*, **276**, 30853–30861.
60. Holter,E., Kotaja,N., Makela,S., Strauss,L., Kietz,S., Janne,O.A., Gustafsson,J.A., Palvimo,J.J. and Treuter,E. (2002) Inhibition of androgen receptor (AR) function by the reproductive orphan nuclear receptor DAX-1. *Mol. Endocrinol.*, **16**, 515–528.
61. Agoulnik,I.U., Krause,W.C., Bingman,W.E. 3rd, Rahman,H.T., Amrikachi,M., Ayala,G.E. and Weigel,N.L. (2003) Repressors of androgen and progesterone receptor action. *J. Biol. Chem.*, **278**, 31136–31148.
62. Yuan,X., Lu,M.L., Li,T. and Balk,S.P. (2001) SRY interacts with and negatively regulates androgen receptor transcriptional activity. *J. Biol. Chem.*, **276**, 46647–46654.
63. Haberland,M., Montgomery,R.L. and Olson,E.N. (2009) The many roles of histone deacetylases in development and physiology: implications for disease and therapy. *Nat. Rev. Genet.*, **10**, 32–42.
64. White,J.H., Fernandes,I., Mader,S. and Yang,X.J. (2004) Corepressor recruitment by agonist-bound nuclear receptors. *Vitam. Horm.*, **68**, 123–143.
65. Peters,A.A., Buchanan,G., Ricciardelli,C., Bianco-Miotto,T., Centenera,M.M., Harris,J.M., Jindal,S., Segara,D., Jia,L., Moore,N.L. *et al.* (2009) Androgen receptor inhibits estrogen receptor-alpha activity and is prognostic in breast cancer. *Cancer Res.*, **69**, 6131–6140.
66. Ogawa,Y., Hai,E., Matsumoto,K., Ikeda,K., Tokunaga,S., Nagahara,H., Sakurai,K., Inoue,T. and Nishiguchi,Y. (2008) Androgen receptor expression in breast cancer: relationship with clinicopathological factors and biomarkers. *Int. J. Clin. Oncol.*, **13**, 431–435.
67. Liu,Y.N., Liu,Y., Lee,H.J., Hsu,Y.H. and Chen,J.H. (2008) Activated androgen receptor downregulates E-cadherin gene expression and promotes tumor metastasis. *Mol. Cell. Biol.*, **28**, 7096–7108.
68. Cicatiello,L., Addeo,R., Sasso,A., Altucci,L., Petrizzi,V.B., Borgo,R., Cancemi,M., Caporali,S., Caristi,S., Scafoglio,C. *et al.* (2004) Estrogens and progesterone promote persistent CCND1 gene activation during G1 by inducing transcriptional derepression via c-Jun/c-Fos/estrogen receptor (progesterone receptor) complex assembly to a distal regulatory element and recruitment of cyclin D1 to its own gene promoter. *Mol. Cell. Biol.*, **24**, 7260–7274.
69. Sabbah,M., Courilleau,D., Mester,J. and Redeuilh,G. (1999) Estrogen induction of the cyclin D1 promoter: involvement of a cAMP response-like element. *Proc. Natl Acad. Sci. USA*, **96**, 11217–11222.
70. Herber,B., Truss,M., Beato,M. and Muller,R. (1994) Inducible regulatory elements in the human cyclin D1 promoter. *Oncogene*, **9**, 2105–2107.
71. Moehren,U., Denayer,S., Podvinec,M., Verrijdt,G. and Claessens,F. (2008) Identification of androgen-selective androgen-response elements in the human aquaporin-5 and Rad9 genes. *Biochem. J.*, **411**, 679–686.
72. Haelens,A., Verrijdt,G., Callewaert,L., Christiaens,V., Schauwaers,K., Peeters,B., Rombauts,W. and Claessens,F. (2003) DNA recognition by the androgen receptor: evidence for an alternative DNA-dependent dimerization, and an active role of sequences flanking the response element on transactivation. *Biochem. J.*, **369**, 141–151.
73. McEwan,I.J. (2004) Molecular mechanisms of androgen receptor-mediated gene regulation: structure-function analysis of the AF-1 domain. *Endocr. Relat. Cancer*, **11**, 281–293.
74. Giguere,V. (1999) Orphan nuclear receptors: from gene to function. *Endocr. Rev.*, **20**, 689–725.
75. Lalli,E. and Sassone-Corsi,P. (2003) DAX-1, an unusual orphan receptor at the crossroads of steroidogenic function and sexual differentiation. *Mol. Endocrinol.*, **17**, 1445–1453.
76. Zhang,H., Thomsen,J.S., Johansson,L., Gustafsson,J.A. and Treuter,E. (2000) DAX-1 functions as an LXXLL-containing corepressor for activated estrogen receptors. *J. Biol. Chem.*, **275**, 39855–39859.
77. Conde,I., Alfaro,J.M., Fraile,B., Ruiz,A., Paniagua,R. and Arenas,M.I. (2004) DAX-1 expression in human breast cancer: comparison with estrogen receptors ER-alpha, ER-beta and androgen receptor status. *Breast Cancer Res.*, **6**, R140–R148.
78. Suzuki,T., Miki,Y., Moriya,T., Akahira,J., Ishida,T., Hirakawa,H., Yamaguchi,Y., Hayashi,S. and Sasano,H. (2007) 5Alpha-reductase type 1 and aromatase in breast carcinoma as regulators of in situ androgen production. *Int. J. Cancer*, **120**, 285–291.


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