ERK and AKT Signaling Drive MED1 Overexpression in Prostate Cancer in Association with Elevated Proliferation and Tumorigenicity

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Abstract
MED1 is a key coactivator of the androgen receptor (AR) and other signal-activated transcription factors. Whereas MED1 is overexpressed in prostate cancer cell lines and is thought to coactivate distinct target genes involved in cell-cycle progression and castration-resistant growth, the underlying mechanisms by which MED1 becomes overexpressed and its oncogenic role in clinical prostate cancer have remained unclear. Here, we report that MED1 is overexpressed in the epithelium of clinically localized human prostate cancer patients, which correlated with elevated cellular proliferation. In a Nkx3.1:Pt en mutant mouse model of prostate cancer that recapitulates the human disease, MED1 protein levels were markedly elevated in the epithelium of both invasive and castration-resistant adenocarcinoma prostate tissues. Mechanistic evidence showed that hyperactivated ERK and/or AKT signaling pathways promoted MED1 overexpression in prostate cancer cells. Notably, ectopic MED1 overexpression in prostate cancer xenografts significantly promoted tumor growth in nude mice. Furthermore, MED1 expression in prostate cancer cells promoted the expression of a number of novel genes involved in inflammation, cell proliferation, and survival. Together, these findings suggest that elevated MED1 is a critical molecular event associated with prostate oncogenesis.

Visual Overview: http://mcr.aacrjournals.org/content/11/7/736/F1.large.jpg.

Introduction
Nearly all prostate cancers are initially dependent on androgens for growth and survival, and consequently, current treatments for prostate cancer include androgen ablation approaches that initially result in tumor regression (1). The physiologic actions of androgens in the prostate are mediated primarily through the androgen receptor (AR), a member of the nuclear hormone receptor (NR) family of ligand-activated transcription factors (2). AR is abundantly expressed in the prostate epithelium and its functional activity is critically dependent on interactions with multiple coregulatory factors and enzymes (3).

Given the importance of these cofactors in regulating transcriptional activity, it has been proposed that overexpression or hyperactivation of distinct coactivators or corepressors may influence prostate tumorigenesis and/or promote progression of the disease into a lethal castration-resistant malignancy (4–6).

Mediator is an evolutionary conserved coactivator complex best known for its ability to functionally bridge gene-specific activators with the RNA polymerase II basal transcription machinery, and more recently, implicated in facilitating chromosomal looping events, coordinating chromatin modification, and promoting transcriptional elongation (7). The complex was originally isolated from human cells as a coactivator bound to NRs and was consequently shown to be essential for NR-dependent transcription (8). Most NRs, including AR, recruit Mediator via direct, ligand-dependent interactions with mediator subunit 1 (MED1; refs. 9–11). MED1 has emerged as a pivotal player in Mediator function, not only as a primary binding target for NRs, but also as a key coactivator for other types of gene-specific activators involved in growth and development including the GATA family of proteins, Pit-1, BRCA-1, p53, C/EBPβ, and GABPα (7, 8).

MED1 plays an important role in coactivating AR-dependent transcription in prostate cancer cells (9, 12, 13) and in vitro from chromatinized templates (14). Indeed, MED1 was shown to be indispensable for robust androgen-
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dependent transcription of the well-characterized AR targets prostate-specific antigen (PSA; ref. 13) and human glandular kallikrein (KLK2; ref. 15). MED1 is overexpressed in several prostate cancer cell lines and its knockdown results in cell-cycle arrest, decreased proliferation, and increased cell death (15). Notably, MED1 overexpression in castration-resistant prostate cancer cells results in its selective recruitment to enhancers of distinct AR target genes specifically involved in cell-cycle progression including UBE2C, an oncogene that inactivates the M-phase checkpoint (16, 17). Furthermore, MED1 coactivates AR-dependent expression of Cdk6, a DNA replication licensing factor that promotes G1-S-phase transition (12). Consistent with these findings, conditional Med1 ablation in murine hepatocytes impairs cell-cycle progression and mitogen-induced proliferative responses (18).

Despite its functional importance as a coactivator for signal-activated transcription factors, MED1 is only variably associated with the core Mediator complex (19, 20) and exists in a subpopulation (<10%) of the total nuclear complex (21). MED1 is a phosphorylation target of the mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (ERK; ref. 22, 23) which significantly increases MED1 nuclear half-life (23) and promotes its association with the core Mediator complex thereby enhancing its coactivator activity (24). MED1 is also a phosphorylation target for activated phosphoinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathways (16), and both MAPK-ERK and activated PI3K/AKT signaling lead to MED1 phosphorylation at a common site (16, 23). Given that ERK and AKT are both commonly hyperactivated in prostate cancer (1, 22, 23) which significantly increases MED1 nuclear half-life (23) and promotes its association with the core Mediator complex thereby enhancing its coactivator activity (24), MED1 is also a phosphorylation target for activated phosphoinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathways (16), and both MAPK-ERK and activated PI3K/AKT signaling lead to MED1 phosphorylation at a common site (16, 23). Given that ERK and AKT are both commonly hyperactivated in prostate cancer (1, 22, 23), we have proposed that aberrant ERK and/or AKT phosphorylation events might promote MED1 overexpression in malignant prostate epithelium (15). Supporting this notion, MED1 levels were recently found to be elevated in tamoxifen-resistant breast cancer cells in which both ERK and AKT signaling pathways are amplified (27, 28). Increased MED1 expression in prostate cancer cells has also been shown to involve epigenetic repression of microRNA-205 (miR-205) that normally targets MED1 for transcriptional silencing (29), whereas MED1 overexpression in a cohort of primary breast cancers was shown to involve amplification of the MED1 gene locus (30).

In this report, we show that MED1 is overexpressed in the epithelium of clinically localized human prostate cancer specimens, and in a Nkx3.1:Ptén–mutant mouse model of prostate cancer progression and castration resistance, and that MED1 overexpression correlates with cellular proliferation. We also show that ERK and AKT signaling pathways are involved in the underlying molecular mechanism of MED1 overexpression. Significantly, ectopic MED1 overexpression in prostate cancer xenografts promotes tumorigenicity in nude mice and upregulates the expression of a number of promitotic, antiapoptotic, and proinflammatory genes. Taken together, these findings suggest that elevated MED1 expression is an important oncogenic event associated with prostate cancer cell growth and proliferation and as such, may represent a novel target for therapeutic intervention.

Materials and Methods

Prostate cell lines and culture

LNCaP, DU-145, and PC3 cell lines were all obtained from the American Type Culture Collection. The lines were authenticated within the past 3 months on the basis of androgen-dependent and androgen-independent cell growth and gene expression, morphology, and expression of prostate-specific cell markers. LNCaP cells were maintained in RPMI 1640 (Invitrogen) containing 10% fetal bovine serum (FBS; Gemini BioProducts). DU-145 and PC3 cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% FBS. For androgen starvation experiment, cells were grown in phenol red-free medium containing 10% charcoal/dextran-stripped FBS (CD5-FBS, Gemini Bioproducts). R1881 and DHT were from Sigma. LY294002 was from Cell Signaling Technology. U0126 was from ENZO Life Sciences. PD-0325901 was from Millipore-Calbiochem.

Plasmids

The pSG5-HA-MED1 and pMCL-HA-MKK1(NΔ4) expression vectors were described previously (23). The pCMV5-HA-AKT-myr vector expressing a constitutively active myristoylated form of AKT was from Dario Alessi (University of Dundee, UK). The pCIN4-HA-MED1 expression vector was generated by subcloning the full-length hemagglutinin (HA)-tagged human MED1 cDNA into pCIN4 containing a neomycin selectable marker.

Stable MED1-expressing LNCaP lines

LNCaP cells were transfected with either pCIN4-HA-MED1 or an empty pCIN4 vector using Lipofectamine and Plus Reagent (Invitrogen). Two days posttransfection, stable clones were selected with 500 μg/mL G418 (Invitrogen) and then expanded for over expression analyses by anti-HA immunoblot.

Tissue microarray, immunohistochemistry, and computerized image analyses

Tissue microarrays (TMA) were from the Cooperative Prostate Cancer Tissue Resource (National Cancer Institute). TMA-1 contained radical prostatectomy specimens from 299 patients with prostate cancer based on the longest follow-up data from the resource, as well as 28 nondiseased control prostate specimens. TMA-2 contained 250 prostate cancer patient specimens [58 of which are also represented by high-grade prostatic intraepithelial neoplasia (HGPIN) cores], as well as 14 nondiseased control prostate specimens. Only 218 cancer specimens on TMA-1, and 223 cancer specimens on TMA-2 (49 of which were HGPIN) were scored in this study due to the fact that some cores on the slides were unreadable. The TMAs were stained using antibodies against mouse MED1 (sc-8998; Santa Cruz Biotechnology) as described (15). Slides were deparaffinized and antigen retrieval was carried out using Cell Conditioning
Stained specimens were scored by a pathologist (M.M.) using a Scanscope digital slide scanner (Aperio). To establish a proliferation index, TMA-2 was stained with semiquantitative analysis by a pathologist (M. Chekmareva). The relative ESI indices were validated via blind staining intensity (ESI) index was generated for each specimen. The relative ESI indices were validated via blind semiquantitative analysis by a pathologist (M. Chekmareva).

**Immunohistochemical analyses of Nkx3.1;Pten mouse prostate specimens**

The Nkx3.1;Pten-mutant mice were described previously (32, 33). Procedures and criteria for generating and classifying HGPIN/adenocarcinoma as a consequence of aging, as well as castration-resistant adenocarcinoma have been described in detail (32, 33). Mouse prostate tissues were formalin fixed and paraffin embedded. Four-micrometer sections were mounted on slides and stained with antibodies specific for MED1, normal rabbit IgG (sc-2027; Santa Cruz Biotechnology), activated phosphorylated AKT (p-AKT, cat. #4370; Cell Signaling Technology), and activated phosphorylated ERK1/2 (p-ERK1/2, cat. #4370; Cell Signaling Technology). Staining conditions and immunochemistry were carried out as described above. The stained specimens were scored by a pathologist (M.M. Ittmann) for extent (0–3) and intensity (0–3). A staining index was determined by multiplying the extent score by the intensity score such that the final index ranged from 0 (no staining) to 9 (strong and extensive staining).

**Soft agar assays**

LNCaP cells were starved in 10% CDS-FBS for 48 hours and then plated at a density of 5 × 10^3 cells/well in 24 well-plates containing RPMI, 10% FBS, and 0.7% agarose with or without R1881 (10 nmol/L). Subsequent to plating, cells were treated with 200 μL of 10% CDS-FBS RPMI medium with or without R1881 (10 nmol/L). The media was changed every 3 days. After day 14, cells were stained with 1% crystal violet (Sigma) and colony numbers were quantitated using Quantity One software (Bio-Rad). Experiments were conducted in triplicate.

**Tumorigenesis assay**

Five- to 6-week-old male nude mice (Taconic Farms, Inc.) were inoculated with 1 × 10^6 LNCaP cells stably expressing pCIN4-HA-MED1 or control empty vector controls. Briefly, the cells were resuspended in 0.1 mL PBS and 0.1 mL Matrigel (BD Biosciences) and injected into the flanks of nude mice. Tumor size was monitored using a digital caliper. Tumor volume was calculated according to the formula: (Length × Width × Height) / 2. After 4 weeks, the nude mice were sacrificed and the tumors were removed for pathohistologic examination as described above in the mouse prostate immunohistochemistry analyses section. A Ki-67 proliferation index was determined as outlined above in the TMA section.

**Antiphospho-MED1 antibodies**

Polyclonal antiphosphorylated-MED1 (Thr 1032) antibodies were generated in rabbits and affinity purified by YenZym Antibodies. A purified human phospho-MED1 peptide (amino acids 1025–1037; CSSNRPF-pT-PPTST) was used for immunization. Immune rabbit serum was first affinity purified using the phospho-peptide followed by affinity absorption to the nonmodified peptide (CSSNRFPFTPPTST) to ensure depletion of antibodies that cross react with nonphosphorylated MED1.

**Immunofluorescence and immunoblotting**

LNCaP cells were treated with 50 μmol/L LY294002 or U0126, or vehicle alone for 8 hours. Cells were washed with PBS, and then fixed with 3.7% formaldehyde in PBS containing 0.1% Tween 20 for 20 minutes. After blocking with 5% goat serum in PBS plus 0.1% Tween 20, the cells were stained with rabbit anti-MED1 or antiphospho-MED1 antibodies followed by fluorescein isothiocyanate (FITC) conjugated donkey antirabbit IgG (Santa Cruz Biotechnology). As a control, cells were also stained in parallel with mouse anti-β-actin antibodies (sc-47778; Santa Cruz Biotechnology) followed by FITC-conjugated goat antimouse IgG (Sigma-Aldrich). All cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Wide-field fluorescence microscopy was carried out using a Nikon Eclipse E1000 motorized fluorescence microscope. For immunoblot analyses, LNCaP cells were treated with 50 μmol/L LY294002, 50 μmol/L U0126, or vehicle for 8 hours as described above, or transfected with HA-MKK1(NA4), HA-AKT-myr, or empty vector using Lipofectamine 2000 (Invitrogen) for 24 hours. Whole-cell extract was then prepared, fractionated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed by immunoblot using anti-MED1, antiphospho-MED1, anti-HA, and anti-β-actin antibodies. Immunoblot signals were digitalized and quantified using Image J software (NIH). All experiments were repeated at least 3 times to verify reproducibility.

**Cell-cycle analyses**

MED1-overexpressing or empty vector control LNCaP cells (1 × 10^6) were cultured in 10 nmol/L DHT and then washed in PBS and fixed with ice-cold 70% ethanol. The fixed cells were stained with 20 μg/mL propidium iodide, 100 μg/mL RNase and 0.1% Tween 20. The cells were analyzed using a Cytomics FC500 flow cytometer (Beckman Coulter, Inc.). Experiments were conducted in triplicate.

**Gene microarray**

LNCaP cells (5 × 10^6) were transfected with 4 μg of pSG5-HA-MED1 or empty pSG5 vector for 24 hours. Total RNA (10 μg) was extracted using the RNeasy Mini Kit.
(Qiagen) and then submitted for gene microarray analyses at the Functional Genomics Core Facility (Cancer Institute of New Jersey) using the Affymetrix HG-U133A arrays (Affymetrix). Two independent experiments were conducted. The data has been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE41150 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE41150). The heat map (Fig. 6A) was generated by GeneSpring GX 12.0 (Agilent Technologies).

RNA extraction, reverse transcription (RT), and real-time PCR

Total RNA was extracted from transiently transfected LNCaP cells (as per gene microarray section above) using an RNasy Mini Kit (Qiagen). One micrograms of total RNA was incubated with reverse transcriptase (Invitrogen) and 200 mmol/L deoxynucleoside triphosphates (dNTP) to generate first-strand cDNA. Real-time PCR was conducted using an Opticon Continuous Fluorescence Detection System (MJ Research) with a power SYBR Green PCR Mix (Applied Biosystems) and with primers specific for MED1 (Forward: 5’-GAG AAT CCT GTG AGC TGT CCG-3’; Reverse: 5’-GTT GCT TTC CAG TAC ATA ATT GC-3’), IFIT2 (Forward: 5’-GAG TGA GAA CAA TAA GAA TTC C-3’; Reverse: 5’-GAA TTC ACG ATT CTG AAA CTC AG-3’), IFL27 (Forward: 5’-GGC CAG GAT TGC TAC AGT TTG-3’; Reverse: 5’-CAG TGG CTC CCA GTG ACT G-3’), CCL5 (Forward: 5’-GCC TCC CCA TAT TCC TCG -3’; Reverse: 5’-GGG TGA CAA AGA CGA CTG C -3’), PSA (15), Cdc6 (12), or β-actin (23) for 25 cycles.

Results

MED1 is overexpressed in human prostate cancers

We previously showed that MED1 knockdown in prostate cancer cell lines decreased both androgen-dependent and androgen-independent proliferation, inhibited cell-cycle progression, and increased apoptosis (15), thus suggesting a potential role for MED1 in prostate tumorigenesis. To evaluate MED1 expression in clinically localized human prostate cancer, we carried out immunohistochemistry on prostate cancer TMAs using an antibody specific for MED1 (Supplementary Fig. S1). TMA-1 contained radical prosta
tectomy specimens from 218 patients with prostate cancer, whereas TMA-2 contained specimens from an additional 223 patients with cancer, 49 of which are also represented by HGPIN cores. To obtain an objective evaluation of MED1 expression, the stained slides were digitized and scored quantitatively via computerized image analysis thus generating an ESI index for each specimen (see Materials and Methods). The relative ESI indices were validated via blind semiquantitative analysis by a clinical pathologist. MED1 staining was clearly present in the nuclei of basal and luminal epithelial cells with some staining of stromal cell nuclei (Fig. 1).

In agreement with our earlier findings (15), both normal and prostate cancer specimens on both TMAs exhibited a broad range of nuclear MED1 staining indices. Nonetheless, greater than two-thirds (67%) of the prostate cancers on TMA-1 exhibited intermediate to strong nuclear MED1 staining (ESI >130), whereas only one-third of the normal specimens (33%) had comparable levels of staining. Similarly, nearly 70% of the prostate cancers on TMA-2 exhibited intermediate to strong nuclear MED1 staining (ESI >38), whereas less than 42% of the normal specimens had comparable levels of staining. For both TMA-1 and -2, there was a statistically significant increase in the MED1 staining index in the prostate cancer specimens (P < 0.005, t test; Fig. 2A and B), and for TMA-2, there was also a statistically significant increase in MED1 staining of the HGPIN specimens over the normal prostate (P < 0.05, t test; Fig. 2B). Furthermore, when TMA-2 was evaluated for expression of the proliferation marker Ki-67, prostate cancer specimens with strong MED1 expression (ESI > 50) exhibited a significantly higher proliferation index (P < 0.0005, t test; Fig. 2C). These data show for the first time that MED1 is overexpressed at the protein level in the malignant epithelium of statistically significant numbers of patients with clinically localized human prostate cancer and further show that MED1 overexpression correlates with prostate cancer cellular proliferation.

MED1 overexpression in a Nkx3.1;Pten-mutant mouse model of prostate cancer

To further assess the pathologic relevance of MED1 amplification in prostate cancer epithelial cells, we examined whether MED1 expression was likewise amplified in a genetically engineered animal model of prostate cancer that recapitulates the human disease. Toward this end, we utilized a well-characterized mouse model of prostate...
tumorigenesis based on the loss-of-function of Nkx3.1 and Pten (32, 33). In humans, NKKX3.1 encodes a homeobox gene located in a region on chromosome 8p21 that is frequently lost during the early stages of prostate cancer (reviewed in refs. 1, 34), whereas PTEN encodes a tumor suppressor commonly inactivated in prostate cancer via deletion, mutation, and/or downregulation of its protein expression (reviewed in ref. 25). Compound-mutant mice containing germ-line deletions of one allele of Nkx3.1 and one allele of Pten (Nkx3.1<sup>+/−</sup>;Pten<sup>+/−</sup>, hereafter denoted as Nkx3.1<sup>−/−</sup>;Pten<sup>−/−</sup>-mutant mice) develop PIN and subsequently invasive prostate HGPIN/adenocarcinoma as a consequence of aging, as well as castration-resistant adenocarcinoma following castration (32, 33).

To evaluate MED1 expression levels in Nkx3.1<sup>−/−</sup>;Pten<sup>−/−</sup>-mutant mouse prostates, HGPIN/adenocarcinoma and castration-resistant adenocarcinoma tissues were stained with an anti-MED1 antibody and subsequently compared with MED1-stained prostates from age-matched wild-type mice (Fig. 3). The stained specimens were then scored for both extent of staining (scale, 0–3) and intensity of staining (scale, 0–3). A staining index was determined by multiplying the extent score by the intensity score such that the final index ranged from 0 (no staining) to 9 (strong and extensive staining). As with the human specimens, MED1 staining was predominant in the nuclei of basal and luminal epithelial cells. Notably, we found that the average MED1 staining indices for HGPIN/adenocarcinoma and castration-resistant adenocarcinoma tissues were more than double than that for normal prostate specimens (Fig. 3E; P < 0.000001, t test). MED1 staining was also elevated in Nkx3.1<sup>−/−</sup>;Pten<sup>−/−</sup>-mutant mouse PIN (data not shown). Previous studies showed that advanced staged prostate cancers in Nkx3.1<sup>−/−</sup>;Pten<sup>−/−</sup>-mutant mice exhibit hyperactivated ERK and AKT signaling pathways (35, 36). Indeed, ERK is often concomitantly deregulated together with AKT in human prostate cancer cells and is thought to promote tumor progression into a castration-resistant malignancy (35–39). In agreement with the earlier studies, we observed elevated levels of activated ERK and AKT in both adenocarcinoma and castration-resistant adenocarcinoma tissues as compared with normal prostate (Fig. 3F, data not shown). Overall, these data show that MED1 protein expression levels are markedly higher in Nkx3.1<sup>−/−</sup>;Pten<sup>−/−</sup>-mutant prostate cancers and further suggest that hyperactivated ERK and AKT signaling pathways may play an underlying etiologic role in MED1 overexpression.

ERK and AKT signaling pathways modulate MED1 expression in prostate cancer cells

Phosphorylation via ERK and/or activated AKT signaling pathways has been implicated in significantly increasing the nuclear half-life of the MED1 polypeptide in vivo as well as stimulating its functional coactivator activity (23, 24). Given the elevated levels of activated ERK and AKT in Nkx3.1<sup>−/−</sup>;Pten<sup>−/−</sup>-mutant mouse prostate cancers and the associated elevated MED1 levels in the epithelium of these tissues (Fig. 3), we next asked whether activated ERK or AKT signaling pathways influence MED1 expression in prostate cancer cells. To more directly correlate MED1 expression with phosphorylation, we generated antibodies against...
phospho-MED1 specific for the phosphorylation site recognized and phosphorylated by both ERK and AKT (Thr 1032; refs. 16, 23; see Methods; Supplementary Fig. S2).

To determine whether ERK and AKT signaling pathways modulate MED1 expression in prostate cancer cells, we first cultured human prostate cancer LNCaP cells in the presence of MAPK- and PI3K/AKT-specific chemical inhibitors (U0126 and LY294002, respectively) and then measured MED1 and phosph-MED1 levels in situ by immunoﬂuorescence. As shown in Fig. 4A, treatment with either LY294002 or U0126 markedly abrogated MED1 phosphorylation and signiﬁcantly decreased MED1 expression. Similarly, when MED1 expression and phosphorylation in LNCaP cells was assayed by immunoblot (Fig. 4B), treatment with either LY294002 or U0126 signiﬁcantly decreased MED1 phosphorylation (70%–80% decrease) and expression (50%–70% decrease). Treatment of other prostate cancer cell lines including DU145 and PC3 with LY294002 or U0126 resulted in similar decreases in MED1 expression and MED1 phosphorylation (Supplementary Fig. S3A and S3B). Importantly, transient transfection of LNCaP cells with constitutively active AKT or a constitutively active form of MAPK-kinase 1 (MKK1, the upstream activator of ERK) enhanced both MED1 phosphorylation (∼4-fold) and expression (>2.5-fold; Fig. 4C), whereas treatment of LNCaP cells with the MKK1 chemical
inhibitor PD-0325901 decreased MED1 expression and phosphorylation to levels similar to that observed with the other inhibitors (Supplementary Figs. S3C). Together these findings are consistent with our earlier data showing that MED1 phosphorylation stabilizes its expression in vivo (23), and when considered along with Nkx3.1;Pten-mutant mouse data (Fig. 3F), support the idea that MED1 phosphorylation via activated ERK and/or AKT signaling pathways is directly involved in stabilizing MED1 expression in prostate cancer cells.

MED1 overexpression in prostate cancer cells increases tumorigenicity and cellular proliferation

To explore whether MED1 overexpression in prostate cancer cells promotes tumor growth, we stably overexpressed ectopic full-length MED1 in androgen-responsive LNCaP cells (Fig. 5A). In anchorage-independent soft agar assays, we found that MED1 overexpressing clonal lines formed more than twice as many colonies as control clones transfected with an empty vector (Fig. 5B). To further investigate whether MED1 overexpression can influence tumorigenicity in vivo, MED1 overexpressing lines were subcutaneously injected into the flanks of nude mice and tumor growth was monitored for a period of 4 weeks. As shown in Fig. 5C and D, LNCaP cell tumors ectopically overexpressing MED1 were significantly larger than their age-matched counterparts transfected with an empty vector. To confirm that the observed increase in tumor size was the result of increased cellular proliferation, representative tumors were dissected, sectioned, and stained with an antibody specific for Ki-67. In agreement with the human prostate cancer TMA data, we found that tumors overexpressing MED1 contained nearly 4-fold more Ki-67-positive cells compared with tumors transfected with an empty vector (Fig. 5F and G). Moreover, ectopic overexpression of MED1 in LNCaP cells resulted in a 2-fold increase in percentage of cells in the G2/M cell-cycle phase (Fig. 5H). Together, these data suggest that MED1 amplification in prostate cancer cells promotes tumor growth and accelerates cellular proliferation.

MED1 overexpression in prostate cancer cells upregulates genes involved in inflammation, cell-cycle progression, and survival

Given the functional role of MED1 as a transcriptional coactivator for gene-specific activators involved in growth and development (7, 8), we were interested in identifying MED1 target genes potentially involved in prostate tumorigenesis. Accordingly, total RNA was isolated from LNCaP cells transiently transfected with HA-MED1 or an empty vector. The expression levels of a total of 111 genes were measured by reverse transcription quantitative PCR. We observed a significant upregulation of 11 genes involved in inflammation, cell-cycle progression, and survival (Fig. 6A). The upregulated genes included IL-1β, IL-6, IL-8, TNF-α, and MMP-9, which are known to be involved in inflammation. Additionally, several cell-cycle genes, such as cyclin D1, cyclin E, and cyclin B1, were upregulated, indicating increased cell proliferation. These findings suggest that MED1 overexpression in prostate cancer cells upregulates genes involved in inflammation, cell-cycle progression, and survival, which may contribute to the tumorigenic potential of these cells.
vector control and then analyzed using cDNA microarrays representing over 22,000 human genes (HG-U133A, Affymetrix). As expected, the well-characterized AR-targets KLK2 and PSA, previously shown to be coactivated by the MED1–Mediator complex (13, 15, 40), were among genes upregulated in MED1-overexpressing LNCaP cells (Fig. 6A).
and B). We also identified Cdc6, an essential regulator of DNA replication that plays a key role in activating checkpoint mechanisms during cell-cycle progression (41). Notably, Cdc6 gene expression in prostate cancer cells is regulated by AR in a cell-cycle–dependent manner and involves recruitment of MED1-mediator to an androgen–response element in the Cdc6 promoter region (12).

Chronic or recurrent inflammation is thought to play an important role in the development of prostate cancer (42). Strikingly, MED1 overexpression in LNCaP cells upregulated the expression of proinflammatory chemokines (CCL5, CCL10), cell survival factors (G1P3), and interferon-induced biomarkers (IFIT2, IFIT4, IFITM1) previously associated with epithelial cell proliferation and cancer (43–46). We also observed elevated expression of several potent promitotic growth factors (PDGFD, IGFBP3, FGF13), antiapoptotic factors (PI4KD, BIRC3), ubiquitin ligase and conjugating enzymes (HERC5, UBE21), and an ubiquitin-like modifier (ISG15) previously shown to amplified in high-grade prostate cancer (47). In addition, ESE1, a member of the ETS family of transcription and characterized as having an epithelial-restricted expression pattern, was also upregulated (Fig. 6A). Interestingly, ESE1 is normally involved in epithelial cell differentiation and its altered expression has been implicated in the development of both breast and prostate cancer (48). Real-time RT-PCR confirmed that several genes including PSA, Cdc6, IFIT2, IFIT2, and CCL5 were upregulated by the overexpression of ectopic MED1 (Fig. 6B). Collectively, these data suggest that in prostate cancer cells, MED1 is involved in the coordinate regulation of proinflammatory, promitotic, and antiapoptotic gene expression.

**Discussion**

Emerging evidence suggests that alterations in the cellular concentration of distinct transcriptional coregulatory factors in steroid hormone-dependent cancers can adversely affect pathologic outcome by driving cellular proliferation and metastasis (49). In this study, we show for the first time that MED1 expression is elevated in the malignant epithelium of a statistically significant number of patients with clinical prostate cancer and that MED1 overexpression...
overexpression correlates with increased cellular proliferation (Fig. 2). Moreover, we found that MED1 levels are significantly elevated in a Nkx3.1:Pen-mutant mouse model of prostate cancer that recapitulates progression of the human disease (Fig. 3). Supporting the pathologic relevance of these findings, we show that ectopic MED1 overexpression in human prostate cancer xenografts significantly promotes tumorigenicity (Fig. 5) and upregulates the expression of genes involved in inflammation, cell-cycle progression, and survival (Fig. 6). Collectively, our findings suggest that MED1 overexpression may contribute to prostate oncogenesis by promoting prostate cancer cell growth and proliferation.

Inactivation of the tumor suppressor PTEN is among the most common genetic alterations associated with prostate cancer and ultimately leads to aberrant activation of AKT (1, 25). MAPK–ERK signaling is also commonly activated in prostate cancer (26), often concomitantly with AKT (35–39). Three lines of evidence presented here implicate ERK and AKT signaling in promoting MED1 overexpression in prostate cancer. First, MED1 expression levels were markedly elevated in Nkx3.1:Pen-mutant mouse prostate cancers in which ERK and AKT signaling pathways are genetically programmed to become hyperactivated in parallel with disease progression (35; Fig. 3). Second, activation or suppression of either AKT or ERK signaling pathways in cultured prostate cancer cells, respectively, increased or decreased MED1 protein expression (Fig. 4; Supplementary Fig. S3). Third, increases or decreases in MED1 expression were directly correlated with increases or decreases in MED1 phosphorylation at a site phosphorylated by both ERK and activated AKT signaling pathways (Fig. 4; Supplementary Fig. S3). These findings are in close agreement with earlier studies showing that phosphorylation of MED1 at this site is associated with a significant increase in its nuclear half-life (23). While our findings suggest that hyperactivated ERK and AKT promote MED1 overexpression in prostate cancer cells at the protein level, other mechanisms are likely involved in activating MED1 expression at the mRNA level including epigenetic repression of the miR-205 locus (29).

We recently observed that phosphorylation of MED1 can enhance its androgen-dependent binding to AR in vitro and promote its recruitment to AR target genes (10), and in this study we found that phosphorylated MED1 robustly binds AR in DHT-stimulated LNCaP cells (Supplementary Fig. S4). When taken into consideration with the other data presented here, we hypothesize that in prostate cancer cells, AKT and/or ERK signaling drives MED1 interaction with AR in 2 ways: first, by increasing MED1 protein concentration which in turn increases its availability to AR as a coactivator; and second, by enhancing the primary MED1–AR protein–protein interaction. Under such a scenario, MED1 phosphorylation could have clinical implications for prostate cancer progression to the terminal castration-resistant stage when endogenous androgen levels are therapeutically ablated. Moreover, MED1 phosphorylation by activated AKT signaling in cultured castration-resistant prostate cancer cells was recently shown to facilitate long-range chromatin looping events that specifically activate distinct genes associated with the castration-resistant phenotype (16).

While MED1 is best-characterized as a NR coactivator (8, 11), it also serves as a regulatory binding target for numerous other gene-specific transactivators, coregulatory factors, and chromatin-modifying enzymes (7). Thus, in addition to NR signaling, MED1 likely plays multiple coregulatory roles in cell growth, proliferation, and survival pathways. Indeed, MED1 acts as a tumor suppressor in melanoma and non–small-cell lung cancer cells and can regulate the expression of genes involved in malignant migration and invasion (50–52). We previously found that MED1 silencing in prostate cancer cell lines lacking AR still led to a decrease in cell-cycle progression (15), and in the study here, MED1 overexpression in AR-positive LNCaP cells clearly elevated the expression of several genes not previously identified as AR-targets including those for several proinflammatory proteins (Fig. 6). It is interesting to note in this regard that C/EBPβ, PGC-1α, the GATA family of transcription factors, and NRs for thyroid hormone, glucocorticoids, retinoids, estrogen, vitamin D3, and prostanoids have all been implicated in regulating inflammation (53–55) and all are direct binding targets for MED1 (7, 8, 56).

In summary, the results here show that MED1 protein levels are amplified in the epithelium of clinically localized human prostate cancers. A potential oncogenic role for MED1 is suggested by its ability to accelerate tumor growth in prostate cancer xenografts and by its ability to upregulate the expression of proinflammatory, promitotic, and anti-apoptotic gene expression in cultured prostate cancer cells. Importantly, our findings further reveal that one underlying mechanism of MED1 amplification in prostate cancer stems from hyperactivated ERK and/or AKT kinase activity. Given the well-documented role of AKT and ERK-signaling pathways in prostate cancer development, it is envisioned that MED1 could represent a novel biomarker in prostate cancer progression and a potential target for therapeutic intervention.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
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