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ELF3 is a Repressor of Androgen Receptor Action in Prostate Cancer Cells

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Abstract

The androgen receptor (AR) plays a critical role in the development and progression of prostate cancer (PC) and is a major therapeutic target in this disease. The transcriptional activity of AR is modulated by the co-regulators with which it interacts and consequently deregulation of cofactor expression and/or activity impacts the expression of genes whose products can play a role in PC pathogenesis. Here we report that ELF3, a member of the ETS family of transcription factors, is a repressor of AR transcriptional activity. Exogenous expression of ELF3 represses AR transcriptional activity when assessed using reporter-based transfection assays or when evaluated on endogenous AR target genes. Conversely, ELF3 knockdown increases the AR transcriptional activity. Biochemical dissection of this activity indicates that it results from the physical interaction between ELF3 and AR and that this interaction inhibits the recruitment of AR to specific androgen response elements within target gene promoters. Significantly, we observed that depletion of ELF3 expression in LNCaP cells promotes cell migration while increased ELF3 expression severely inhibits tumor growth *in vitro* and in a mouse xenograft model. Taken together, these results suggest that modulation of ELF3 expression and/or AR/ELF3 interaction may have utility in the treatment of PC.

Keywords

ETS factor; ChIP; nuclear receptor; LNCaP cells; xenograft

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

INTRODUCTION

The androgen receptor (AR) is a member of the nuclear hormone receptor superfamily of ligand-regulated transcription factors. The role of AR in benign hyperplasia and malignancy of the prostate is well established and has provided substantial validation for AR as a therapeutic^{1, 2}. Prostate cancer (PC) affects one in six men during their lifetime and is second only to lung cancer as a cause of cancer death in males³. Although surgical intervention is very effective in eradicating localized disease, chemical androgen ablation therapies such as administration of gonadotropin releasing hormone (GnRH) agonists and/or AR antagonists are still the most successful treatment options for patients with metastatic PC. Unfortunately, patients that have undergone androgen ablation therapy often develop resistance and progress to a hormone-refractory state, beyond which point drugs targeting the androgen axis have limited clinical utility⁴. Despite being refractory to androgen ablation therapy, studies in human biopsies as well as in xenograft models have suggested that AR remains transcriptionally active (or is re-activated) in late-stage disease. Several mechanisms have been proposed to explain the process by which cancers progress to a hormone-refractory state, including AR mutations, amplification and/or increases in local androgen production⁵⁻⁷. In addition, it has been shown that deregulation of cofactor expression or activity can, in certain circumstances, permit weak androgens and anti-androgens to activate those AR target genes responsible for cell growth, leading ultimately to the development of hormone-refractory PC⁸⁻¹⁴.

AR can either stimulate or repress gene transcription, an activity that is determined by promoter context, cofactor availability and the impact of different signaling pathways on the functionality of receptor-cofactor complexes. A large number of AR-interacting proteins have been identified to date, although the physiological and pathological significance of all but a few remains to be determined¹¹. Cofactors can modulate AR action by (a) regulating chromatin remodeling or histone modification, (b) serving as bridging elements between the receptor and DNA or with the basal transcriptional machinery or (c) by physically interfering with the interaction of the receptor with the transcription apparatus. Not surprisingly, it has been shown that the biological endpoints resulting from the activity of different AR-cofactor complexes are not equivalent, suggesting that pharmaceuticals that are able to differentially modulate specific receptor-cofactor interactions are likely to have distinct biological activities.

E74-like factor 3 (ELF3, also known as ESE-1) is a member of the ETS family of transcription factors^{15, 16}. Members of the ETS family play crucial roles in several biological processes, including the regulation of cell differentiation, hematopoiesis, angiogenesis as well as cartilage and skeletal development¹⁷. Like most transcription factors, ETS proteins can either activate or repress gene transcription depending on the cell- and promoter context in which they operate. Gene knockout studies have shown that ELF3 plays essential roles during development and, in the adult, it is expressed in organs that contain secretory epithelial cells such as the lung, kidney, liver, pancreas, gastrointestinal tract, prostate and the mammary gland^{15, 16, 18}. Genes shown to be regulated by ELF3 are involved in inflammation, differentiation, tumorigenesis and metastasis¹⁷. Cooperative interactions between ELF3 with other transcription factors such as NF- κ B, SP1 and AP-1

have been reported by other groups^{19, 20}. Whereas ELF3 has been implicated in cancer, most studies performed to date have focused on defining its role in breast cancer pathogenesis^{21–24}. While a specific role for ELF3 in PC has not been reported, chromosomal translocations that result in fusions between the ETS family members *ETV1*, *ERG*, *ETV4*, and *TMPRSS2* are found in a majority of advanced PCs^{25, 26}. It was of interest therefore that we recently identified ELF3 as a potential AR-interacting protein²⁷. Following up on this finding we determined that ELF3 is indeed a *bona fide* coregulator functioning as a negative modulator of AR transcriptional activity. Furthermore, manipulation of ELF3 inhibits androgen-dependent growth and migration of PC cells. These findings highlight the biological significance of the AR/ELF3 interaction in PC biology and suggest that this complex may be a target for novel therapeutic interventions.

RESULTS

Interaction between ELF3 and the androgen receptor

Previously, we reported the results of a high-throughput protein-protein interaction screen that employed T7 phage display technology to identify proteins that interacted either directly or indirectly with AR in the presence of different ligands. Over 300 interacting proteins were identified in this manner the functional significance of which remain under investigation²⁷. Among the proteins that were identified in this manner was ELF3, a member of the ETS family of transcription factors, a class of proteins that have been implicated in the pathology of solid tumors¹⁷. Furthermore, we utilized paired-sample data from GSE21034²⁸ to demonstrate in a case-by case manner that the expression of ELF3 is lost in primary prostate tumors when compared to expression in normal adjacent sites (Fig. 1A). Consequently, we proceeded to evaluate the possibility that ELF3 was a biologically relevant regulator of AR action in the prostate. As a first step in this process, we performed a co-immunoprecipitation assay to demonstrate an interaction between endogenous ELF3 and AR in LNCaP cells. As shown in Fig. 1B, ELF3 was detected in cell lysate immunoprecipitated with AR antibody. In addition, AR was also detected in cell lysate subjected to immunoprecipitation with ELF3 antibody. Interestingly, the interaction was significantly enhanced in the presence of R1881. Subsequently, *in vitro* GST-pull-down assays were used to show that recombinant AR and ELF3 interact directly and to define the domains within each protein that were required for the observed interaction. To this end, purified full length GST-ELF3 was incubated with either *in vitro* translated full length AR or fragments thereof encoding selected functional domains of the receptor as shown in Fig. 1C. The results of this analysis confirm that the two proteins can directly interact with each other, albeit weakly, and that both the amino- and carboxy-terminal domains of AR bind to ELF3. Furthermore, it was demonstrated that Flag-ELF3 co-localizes with AR in the nuclei of LNCaP cells following the addition of R1881 (Fig. 1D). In summary, the results of these protein-protein interaction assays performed *in vitro* and within intact cells imply the existence of a close physical association between ELF3 and AR, a finding that encouraged us to explore the functional consequences of this association.

ELF3 represses androgen receptor-dependent transcriptional activity

To address the functional role of ELF3 in AR signaling we first evaluated the impact of its expression on AR transcriptional activity in androgen responsive reporter gene assays in HEPG2 and LNCaP cells, respectively. In the AR-negative HEPG2 cells, transfection of an AR expression plasmid leads to robust R1881-dependent activation of the synthetic 2XARE-luciferase, PSA-luciferase and MMTV-luciferase reporter genes (Fig. 2A). Co-transfection of ELF3 dramatically inhibits the AR-dependent activation of both the 2XARE- and PSA-luciferase reporters. Minimal but significant inhibition of the MMTV-luciferase reporter was also observed in this assay. Similarly, ELF3 expression in LNCaP cells reduces the transcriptional activity of endogenous AR on the PSA, KLF5 and MMTV luciferase reporter genes (Fig. 2B). However, ectopic expression of ELF3 did not change AR protein levels in both HepG2 and LNCaP cells as shown by western blot (Fig 2A and B). Importantly, ELF3 inhibitory activity was noted at all concentrations of R1881 and its ability to repress the activity of the PSA promoter in HEPG2 cells expressing AR cannot be reversed by increasing doses of R1881 (Fig. 2C). We next examined the activity of ELF3 in the presence of the AR antagonist Casodex® (CSX) on AR transcriptional activity using the PSA reporter gene assay in HEPG2 cells (Fig. 2D). As expected, treatment with CSX blocked the R1881 induction of PSA promoter activity in control-transfected cells. Interestingly, a greater level of repression of the PSA promoter activity by CSX was observed in the presence of ELF3, a result that is consistent with ELF3 acting as an AR corepressor.

Identification of the domains within ELF3 required for AR repression

In an effort to define the mechanism(s) by which ELF3 impacts AR transcriptional activity we used a series of mutations to define the functions of this protein that are required for repression. ELF3 contains five distinct domains: 1) a pointed domain (PNT), which has been implicated in protein-protein interactions, 2) a trans-activation domain (TAD), 3) a serine- and aspartic acid-rich (SAR) domain which influences cellular transformation, 4) an AT-hook domain suggested to be involved in non-sequence-specific binding to AT-rich regions of DNA and in protein-protein interactions and, 5) an ETS domain which is required for DNA binding (Fig. 3). To examine the role of each of these domains in AR repression we used a series of expression constructs that encoded ELF3 proteins in which the integrity of these domains was disrupted and assayed their activity in HepG2 cells in which AR was co-expressed (Fig. 3). In fact, expression of the ETS domain alone (N270) acts as a dominant-negative inhibitor of wild type ELF3 activity and reproducibly increases AR activity by more than two-fold in this assay. In agreement with these results, disruption of the predicted α helices within the ETS domain, (A312P) and (I279P) point mutations also disrupted ELF3-mediated repression of PSA promoter activity. The role of the A/T hook domain in this process is more complex in that deletion of one hook motif (237–241) abolishes ELF3 repression activity while simultaneous deletion of both motifs (235–260 and 237–241+246–250) only partially impairs the repressor function of ELF3. Introduction of the point mutation, L143P, a manipulation shown previously to disrupt the TAD function of ELF3²⁹, reduces its repressor activity by 40%. It has been shown that the regions flanking the ETS domain within the ETS1 or ETS2 proteins regulate their ability to bind to DNA³⁰. Removal of the C-terminal tail of ELF3 (355–362) significantly reduces the ability of ELF3 to repress AR activity. This finding is of particular importance as the original T7 derived

fragment of ELF3 that interacted with AR contains this domain alone suggesting that the ability of ELF3 to interact with DNA may contribute to its AR-inhibitory activity.

AR target genes regulation following knockdown or over-expression of ELF3

To further investigate the functional role of ELF3 on AR action in an endogenous setting, the expression of a series of well-characterized AR-regulated genes were assessed in LNCaP cells following ELF3 knockdown. For these studies, two separate siRNAs (siELF3#1 and siELF3#2) that target the coding region of ELF3 were used to knockdown the expression of endogenous ELF3. A scrambled siRNA (siC) was used as a negative control in these knockdown experiments. In addition, for comparison, we also included in these experiments an siRNA directed against AR (siAR). Introduction of either siELF3#1 or siELF3#2 results in an efficient knockdown of ELF3 protein expression (Fig. 4A) and an increase in AR-target gene expression in LNCaP cells (Fig. 4B). This is particularly evident for genes whose induction was modestly but significantly induced upon activation of AR by R1881 i.e. *NDRG1*, *SLC16A6*, *NKX3.1* and *SLC45A3*. To examine the effect of ELF3 overexpression on AR function, lentiviruses were constructed that utilized the doxycycline inducible pLVX-TetOn system to conditionally express ELF3 at different levels in stably transfected LNCaP cells. Western blot analysis was used to confirm that ELF3 expression could be up-regulated in these cells following doxycycline addition (Fig. 4C). Importantly, we observed that the expression of all R1881-induced AR target genes examined was significantly reduced, albeit to a different degree, in cells expressing ELF3 (Fig. 4D).

Having established that ELF3 is a repressor of AR activity in the context of LNCaP cells we next focused our efforts on defining the mechanism(s) underlying this repressive activity. To this end, we used chromatin immunoprecipitation (ChIP) assays to assess the impact of ELF3 on AR recruitment to specific AREs located within the enhancers of three androgen-regulated genes. For this study, TetON-Flag-ELF3 LNCaP cells expressing ELF3 were treated with either vehicle or R1881 for 4 hrs. It was determined that R1881-induced recruitment of AR to functional AREs within the PSA, NKX3.1, TMPRSS2 genes regions was inhibited in cells expressing ELF3 (Fig. 4E). A ChIP assay using an antibody against the FLAG epitope failed to detect exogenous FLAG tagged ELF3 at these AR binding sites further suggesting that ELF3 inhibits AR function by interfering with its ability to interact with DNA (Fig. 4E). To confirm whether the repression function of AR by ELF3 through a direct protein-protein interaction and not by competing with AR recruitment to its DNA elements on its target genes, LNCaP cells were transiently transfected with either I279P or A312P ETS point mutation constructs and subjected to a ChIP assay (Fig. S1). Interestingly, point mutations within ELF3 DNA binding domain did not block AR recruitment to AREs within the PSA, NKX3.1 and TMPRSS2 genes in R1881 treated cells suggesting that repression of AR function by ELF3 does not involve competition for DNA binding. This finding is consistent with our observation that the ETS domain when expressed on its own acts as a dominant negative inhibitor.

ELF3 depletion increases androgen-dependent migration of PC cells and ectopic expression of ELF3 inhibit cell proliferation *in vitro*

AR has been shown to play a critical role in PC cell migration. To assess the potential role of ELF3 in this process LNCaP PC cells were transfected with either of two siRNAs targeting ELF3 and the impact of this manipulation on migration was evaluated. As expected, addition of R1881 increased LNCaP cell migration and this activity was not affected by a control siRNA. However, introduction of the siRNAs targeting ELF3 led to a significant increase in LNCaP cells migration (Fig. 5A and 5B). Further, to explore the effect of ELF3 ectopic expression on PC proliferation, TetOn-Flag-ELF3-LNCaP cells were grown in CS-RPMI1640 and subjected to a proliferation assay using xCELLigence technology (Fig. 5C). As predicted, R1881 treated cells showed an increase in LNCaP cell proliferation. Conversely, the induction of ELF3 expression by Dox abrogates the proliferation of LNCaP cells treated with R1881.

ELF3 expression inhibits PC cell growth in a xenograft model of PC

To examine the biological function of ELF3 in PC *in vivo*, the TetOn-Flag-ELF3-LNCaP cell line (described above) was propagated as a xenograft in mice. Cells were injected s.c into the right flank of non-castrated SCID male mice and assigned into two groups, one fed with doxycycline containing diet to induce ELF3 expression, the other fed with a regular diet. As shown in Fig. 6A, tumors grew significantly faster in control compared to the doxycycline-induced group. The growth velocity of the tumors in the control group was significantly higher than in the doxycycline fed group and the average tumor weight at the end of the experiment in control was also higher when compared to tumors that were grown in mice fed doxycycline (Fig. 6B). Importantly, overexpression of ELF3 did not affect the expression levels of AR (Fig. 6A, inset). However, the expression of selected AR target genes was inhibited in the ELF3 expressing tumors (Fig. 6C). Cumulatively, these findings highlight the functional importance of ELF3 in PC pathogenesis.

DISCUSSION

The androgen-signaling axis is a primary target for therapeutics used for the treatment of advanced prostate cancer. Whereas most of these drugs function by directly interfering with AR function, it has become clear of late that approaches that target the expression and or activity of AR coregulators may also yield useful pharmaceuticals³¹. There is, therefore, a considerable amount of interest in identifying those coregulators that are essential for androgen action in prostate cancer and in follow-on studies, which evaluate the likely impact of interfering with these factors on processes of pathological importance in cancer. Thus, in this study we have undertaken a biochemical and genetic approach to define the role of the AR-interactor, ELF3, a member of the ETS family of transcription factors, on AR signaling and on prostate cancer pathology.

The majority of prostate cancers have been shown to express at least one gene fusion comprised of an ETS family member together with an AR target gene. Why these particular fusions dominate over others in this disease is unclear but it may relate to the high transcriptional activity of AR target genes in prostate cancer. Regardless, the high

prevalence of ETS proteins in these fusions suggests that dysregulation of the activity of transcription factors in this class contributes to cancer pathogenesis. The functional relationship between AR and the ETS proteins was further substantiated by studies, which demonstrated that overexpression of AR, and the ETS factor ERG in murine prostate facilitates the development of invasive adenocarcinoma^{26, 32}. Furthermore, using ChIP-chip and ChIP-seq approaches it was determined that ETS-binding sites were enriched near sites of AR-occupancy and indeed direct co-occupancy of AR and ERG at regulatory sequences was observed on 44% of target genes. In agreement with these observations, ERG was found to interact directly with AR through via its ETS domain but the mechanism(s) that enable these proteins to bind in a cooperative manner to DNA remain elusive³³. A functional role for ERG as a corepressor was highlighted by studies, which revealed that its overexpression in PC cells resulted in the inhibition of the expression of AR responsive genes associated with differentiation to which binding sites for this transcription factor had been mapped. Here we show that ELF3, like ERG, inhibits AR signaling. However, the molecular mechanisms underlying the functional antagonism between ELF3 and AR signaling are distinct. Of note we have determined that the physical interaction of ELF3 with AR blocks the interaction of the receptor with its regulatory sequences in target genes. Not surprisingly, therefore, in contrast to what was described for ERG, co-occupancy of AR and ELF3 at AR target genes was not observed in our studies. Together, these findings reveal that the mechanisms by which the ETS proteins impact AR function are different; a finding that may reflect their different roles in AR action in the normal prostate and in prostate cancer.

The currently available antiandrogens work by competitively blocking agonist binding to the receptor and inducing a conformational change that (a) prevents the intramolecular interaction between the amino and carboxyl termini of AR and (b) disrupts the interaction of AR with its attendant cofactors⁸. In addition, the specific conformational change induced by the most efficacious antiandrogens, MDV3100 and ARN509, reduce the affinity of AR for DNA³⁴. Thus, subtle differences in the structure of an AR ligand can facilitate functionally important alterations in the conformation of AR and results in the preferential recruitment of corepressors²⁷. Key to the development of such compounds is the identification and characterization of those AR-corepressors that are expressed in prostate cancer cells. The studies presented in this paper highlight the potential utility of developing compounds that enable a robust interaction of AR with ELF3. Given the dramatic effect of ELF3 overexpression on the growth of prostate tumors *in vivo* and on cell migration *in vitro* it is anticipated that compounds that facilitate AR/ELF3 interactions would have useful therapeutic activity in prostate cancer. These data also highlight the potential of compounds that increase ELF3 expression and effect an inhibition of AR activity in an indirect manner.

In summary, ELF3 is an AR-interacting corepressor the disruption of which results in enhances AR-signaling in prostate cancer and whose overexpression significantly impedes the androgen dependent growth of prostate cancer xenografts. A role for this protein in AR action has not been contemplated previously although our studies highlight its importance in prostate cancer pathogenesis.

MATERIALS AND METHODS

Chemicals and reagents and antibodies

DMEM, MEM, RPMI-1640, LipofectAMINE™ 2000, Opti-MEM reduced serum medium, fetal bovine serum (FBS), nonessential amino acids (NEAA), sodium pyruvate (NaPyr) and the penicillin/streptomycin/l-glutamine stock mix, anti-mouse antibody coupled to Alexa-555 and the anti-rabbit antibody coupled to Alexa-488 were purchased from Invitrogen (Carlsbad, CA). Methyltrienolone (R1881) was purchased from PerkinElmer (Waltham, MA). Bicalutamide (Casodex®), G418, fatty acid free BSA, puromycin and anti-Flag M2 antibody were purchased from Sigma (St. Louis, MO). Doxycycline and Tet-system approved FBS were purchased from Cloneteck (Mountain View, CA). Luciferase assay reagents were from Promega (Madison, WI). Custom oligonucleotide primers were from IDT (San Diego, CA). The reagents for real-time RT-qPCR were from Roche Diagnostic (Indianapolis, IN). AR (N-20)X was from Santa Cruz biotechnology (Santa Cruz, CA). ELF3 antibody was from Epitomics (Burlingame, CA) and V5-tag antibody was from Abcam (Cambridge, MA).

Plasmids

CMV-βgal, PGL4.26, pcDNA3.1-ARwt, pSG5-AR, PSA-Luc, KLF5-Luc, MMTV-Luc and 2XARE-Luc constructs have been previously described³⁵. Mouse pcDNA-Flag-ELF3 construct, its deletions and mouse GFP-point mutations were a generous gift from Dr. Angie Rizzino (University of Nebraska Medical Center) and previously described in³⁶. The mouse Flag-ELF3 (I279P) and Flag-ELF3 (A312P) mutants, were generated by subcloning of the ELF3 cDNA of GFP-ELF3 (I279P) and GFP-ELF3 (A312P) obtained above into the pcDNA3-Flag-SV40/NLS vector. pcDNA-nV5-ELF3 was generated by subcloning of the human ELF3 cDNA clone #3537846 obtained from ATCC into pcDNA-nV5 vector. The pcDNA-nV5-ELF3 (L143P) mutant was generated by the site directed mutagenesis Kit from stratagene using the pcDNA-nV5-ELF3 vector as a template. pLVX-Tight-Puro-Flag-ELF3 was generated by PCR amplification using the mouse pcDNA-Flag-ELF3 vector above as a template. The amplified cDNA was cloned into pLVX-Tight-Puro vector.

Cell culture

LNCaP, Hela and HepG2 were obtained from ATCC (Manassas, VA). The LNCaP cells were routinely cultured in RPMI-1640 Medium supplemented with 10% fetal bovine serum (FBS), 0.1 mM NEAA and 1 mM NaPyr. The HepG2 cells were maintained in MEM supplemented with 10% FBS, 0.1 mM NEAA, and 1 mM NaPyr. Hela cells were maintained in DMEM Medium supplemented with 10% of FBS, 0.1 NaPyr and 0.1 mM NEAA.

Western blot analysis, preparation of cell lysates and luciferase assay

Western blots were performed as previously described³⁷. Luciferase assays were also performed as described³⁵. For luciferase assays, each treatment was performed in triplicate. Results are expressed as mean ± SD. Experiments were repeated at least three times with a representative experiment shown.

Immunofluorescence and confocal microscopy

LNCaP cells were plated at density (3×10^5) per well onto coverslips in 6-well plates in phenol free charcoal stripped CS-RPMI-1640 medium and transfected with Flag-ELF3 plasmid using LipofectAMINE™ 2000. 48h later the cells were treated with either R1881 or vehicle for additional 16 hrs and the immunofluorescence was performed as described previously³⁸.

Co-immunoprecipitation and GST pulldown assay

LNCaP cells were plated in CS-RPMI1640 media for 48h. Then the cells were treated with either vehicle or R1881 for additional 24h. At the endpoint the whole cell lysate was extracted and subjected to CoIP as described³⁹. For the GST pull down assay, the GST and full-length ELF3-GST fusion proteins, radioactive AR full length or truncated proteins were prepared and subjected to pull down assay as previously described³⁹.

RNA Isolation, cDNA preparation, and quantitative RT-PCR

RNA isolation, cDNA preparation and qPCR were performed as previously described in ³⁵. Reverse and forward primers are listed in (Table S1). All samples were assayed in triplicate and normalized to an internal control RPLP0.

Chromatin immunoprecipitation assays

AR ChIP assays were performed as described previously⁴⁰. Antibody for AR (N-20)X and Anti-FLAG M2 were used for chromatin immunoprecipitation. The forward and reverse primers used for the various target sequences in the ChIP assays are shown in (Table S1). The intensity of the enrichment signals was normalized to a none ARE negative control region.

Inducible TetOn-Flag-ELF3 LNCaP stable cell line

Lentiviruses derived from the pLVX-TetOn advanced system vectors were purchased from Clontech (Mountain View, CA) were used to stably manipulate the expression of Flag-ELF3 in LNCaP cells. The 293T packaging system was used to generate the viruses and the resulting purified Lentiviruses were used to infect LNCaP cells. Sequential infection was used to generate the cell line needed for this study. The first round of infection was used to generate the LNCaP cells that constitutively express the tetracycline-controlled-transactivator (rtTA) by infecting the cells with viruses carrying pLVX-TetOn expression vector. Then the recombinant cells were selected using 0.8 mg/ml G418. The cells were exposed to a second round of infection with viruses expressing Flag-ELF3 cloned into a pLVX-Tight-Puro vector and selected with puromycin 2 µg/ml. The new cell line was maintained in antibiotic G418 and puromycin containing media. Protein and mRNA expression levels of ELF3 were confirmed by Western blot and qPCR analysis respectively in the presence and the absence of doxycycline treatment at 2 µg/ml.

Migration assays and siRNA transfection of cultured human prostate cell lines LNCaP cells were plated, and transfected with siRNAs against ELF3, AR coding sequences or siRNA

control (siC). The siRNA transfection and migration assay was performed as described previously³⁵. The siRNAs sequence list is shown in Table S1.

Proliferation Assay

Proliferation assay was done by Roche DP real time cell analyzer (RCTA) xCELLigence technology. TetOn-Flag-ELF3 LNCaP cells were grown in phenol free CS-RPMI 1640 medium. 48h later the cells were treated with $\pm$ Dox and with either vehicle or R1881 as indicated for additional 24h. Background impedance was determined after incubating the E16-plates with 100 μ l of CS-RPMI 1640 medium for 30 min at room temperature. Then the cells were plated in E16-plate at 2×10^4 cells per well and continue the treatments with $\pm$ Dox and with either vehicle or R1881 then placed in the xCELLigence machine for additional 50h. The Delta Cell Index for each time point (DCI_{ti}) was measured every 1h. DCI_{ti} was calculated as the cell index (CI_{ti}) at a given time point plus a Delta value. The Delta value is the difference between a DCI reference value (=1) and the cell index at the Delta time point as follow $DCI_{ti} = CI_{ti} + (1 - CI_{\Delta time})$. Delta Time point ($CI_{\Delta time}$) was set at 5h a time-point following cell adherence but prior to cell proliferation.

Murine tumor xenograft model

NOD.CB17-*Prkdc^{scid}*/J none castrated SCID male mice 4 weeks old were purchased from Jackson Laboratories. After a one week period of acclimation, the mice were injected with 6×10^6 TetOn-Flag-ELF3 LNCaP suspended in 200 μ l of a 50% mixture containing RPMI 1640 medium and Matrigel matrix basement membrane (BD Corporation, Bedford, MA) subcutaneously into the right flank region. The mice were either fed with doxycycline 200 mg/kg containing diet (BioServ, NJ) to induce the ELF3 expression or with a control regular diet. The sizes of the resultant tumors were measured weekly. At endpoint, the tumors were harvested surgically, flash-frozen for RNA and protein extraction.

Statistical analysis

Results are presented as mean $\pm$ SD. The significance of statistical differences (*P* values) between the indicated groups in each experiment was determined using student's *t*-test and ANOVA. **P* value 0.05 unless indicated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

ELF3 expression is down regulated in PC tissues and interacts with AR. **A**, Waterfall plot of ELF3 expression in paired-sample data. Whole-transcript data was downloaded from the Gene Expression Omnibus under accession GSE21034 and only the paired-sample data was used for analysis. ELF3 expression was downregulated in 20/29 patients in primary tumors compared to normal adjacent sites. **B**, Interaction between ELF3 and AR *in vivo*. Lysates from LNCaP cells treated with vehicle or R1881 immunoprecipitation (IP) and immunoblot (WB) with the indicated antibodies. **C**, Direct interaction between ELF3 and AR. *In vitro* translated full length AR or deletion mutants as indicated by the schematic representation (left) were subjected to pull-down analysis with full length GST-ELF3 (right). **D**, ELF3 colocalized with AR in LNCaP cells. Fluorescence microscopy images of LNCaP cells expressing endogenous AR (green) and Flag-ELF3 (red) and treated with either vehicle (top panel) or R1881 (bottom panel) for 16 hrs and probed with anti-rabbit AR or Flag anti-mouse followed by specific Alexa-conjugated secondary antibody.

Figure 2.

ELF3 represses AR-mediated transcription. **A**, Repression of 2XARE, PSA and MMTV luciferase reporter activity by ELF3 co-transfected with AR in HepG2 cells treated with vehicle or 10 nM R1881 with an immunoblot (inset) showing the expression levels of AR, Flag Elf3 and RPLP0 serving as a control. Error bars, SD; *, $P < 0.05$. **B**, Repression of PSA, KLF5 and MMTV luciferase reporter activity by ELF3 transfected in LNCaP cells treated with vehicle or 10 nM R1881 with an immunoblot (inset) showing the expression levels of AR, Flag Elf3 and RPLP0 as a loading control. Error bars, SD; *, $P < 0.05$. **C**, PSA promoter activity in HepG2 cells co-transfected with AR and ELF3 or control (C) expression vectors and treated with increasing doses of R1881. Error bars, SD; *, $P < 0.05$. **D**, Effect of CSX treatment on the PSA promoter activity in R1881-treated HepG2 cells co-transfected with AR and ELF3 or control (C) expression vectors. Error bars, SD; *, $P < 0.05$.

Figure 3.

Identification of ELF3 domains involved in repression of AR transcriptional activity. Left, schematic diagram displaying ELF3 functional PNT, TAD, SAR, AT-hook and ETS domains, the T7-ELF3 fragment obtained in the original screen and the ELF3 deletion and point mutation constructs used in this study. Right, bar graph showing the % repression of the PSA promoter activity in HepG2 cells co-transfected with control ELF3 or ELF3 derivatives described previously in material and methods and AR and treated with 10 nM R1881. Error bars, SD; *, $P < 0.05$. *B*, WB of showing the expression levels of AR, Flag-ELF3 full length (FL), Flag-ELF3 mutations and deletions, V5 tag ELF3 (FL) and its V5 tag mutation relative to RPLP0 loading control in HepG2 cells transfected and treated as described in A.

Figure 4.

ELF3 modulates the expression of AR target genes and prevent its recruitment to AR response elements. **A**, Western blot analysis showing the protein levels of AR and ELF3 in LNCaP cells transfected with either a control siRNA (siC), a siRNA targeting AR (siAR) or two different siRNAs targeting ELF3 (siELF3#1 and #2) and treated with vehicle (–) or 10 nM R1881. RPLP0 served as a control. **B**, Heatmap indicating the differential induction of endogenous AR target genes in LNCaP cells in response to 10 nM R1881 as monitored by qRT-PCR upon AR or ELF3 knockdown. **C**, Western blot analysis of the inducible TetOn-

Flag-ELF3-LNCaP stable cell line showing the expression level of ELF3 after 24h induction by Dox. GAPDH served as a control. *D*, Differential induction of AR target genes in R1881-treated TetOn-Flag-ELF3-LNCaP cells upon ELF3 induction by Dox as monitored by qRT-PCR. Error bars, SD; *, $P < 0.05$. *E*, Enrichment of AR and Flag-Elf3 binding to *PSA*, *NKX3.1* and *TMPRSS2* regulatory regions as assayed by standard ChIP upon induction of ELF3 expression by Dox in TetOn-Flag-ELF3-LNCaP cells. Error bars, SD; *, $P < 0.05$.

Figure 5.

ELF3 knockdown promotes androgen-induced LNCaP cell migration and ELF3 ectopic expression inhibits cell proliferation. **A**, Boyden chamber migration assays were used to assess the migration of LNCaP cells following ELF3 knockdown and treatment with either vehicle (top) or 10 nM R1881 (bottom). Migrated cells were stained with crystal violet and imaged at X100 magnification. **B**, Bar graphs representing average migrated cell numbers of four different microscopic fields of vehicle or R1881-treated cells following ELF3 knockdown. Error bars, SD; *, $P < 0.05$. **C**, TetOn-Flag-ELF3-LNCaP Real Time proliferations assay. Cells were grown in CS-RPMI1640 for 48h and treated with $\pm$ Dox and with vehicle or R1881 as indicated for additional 24h. Then cells were plated in E16-plates and kept with the same treatment for the entire period. The proliferation signals were recorded by xCELLigence machine 5h after cell plating, the time required for cell adhesion and before proliferation.

Figure 6.

ELF3 reduces tumor growth in a non-castrated mice xenograft assay. *A*, Inducible TetOn-Flag-ELF3-LNCaP stable cells were transplanted subcutaneously into the right flanks of none castrated SCID male mice. The animals were divided into two groups, one fed with Dox (n=7) and the other group fed with control diet C (n=6) for a total period of 7 weeks. The growth curve of the xenografts with a representative image (inset) of immunoblotting analysis showing the expression levels of ELF3 and AR in tumors of Dox fed mice versus controls. RPLP0 served as a control. *B*, Tumor weight at 7 weeks after of implantation (bottom) together with a representative photograph of tumors excised from Dox and C fed mice (top). *C*, Expression levels of AR target genes in tumors excised from Dox and C fed mice as monitored by qRT-PCR. Error bars, SD; *, $P < 0.05$.