Identification of a Novel Coregulator, SH3YL1, That Interacts With the Androgen Receptor N-Terminus

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Nuclear receptor (NR)-mediated transcriptional activity is a dynamic process that is regulated by the binding of ligands that induce distinct conformational changes in the NR. These structural alterations lead to the differential recruitment of coregulators (coactivators or corepressors) that control the expression of NR-regulated genes. Here, we show that a stretch of proline residues located within the N-terminus of androgen receptor (AR) is a bona fide coregulator binding surface, the disruption of which reduces the androgen-dependent proliferation and migration of prostate cancer (PCa) cells. Using T7 phage display, we identified a novel AR-interacting protein, Src homology 3 (SH3)-domain containing, Ysc84-like 1 (SH3YL1), whose interaction with the receptor is dependent upon this polyproline domain. As with mutations within the AR polyproline domain, knockdown of SH3YL1 attenuated androgen-mediated cell growth and migration. RNA expression analysis revealed that SH3YL1 was required for the induction of a subset of AR-modulated genes. Notable was the observation that ubinuclein 1 (UBN1), a key member of a histone H3.3 chaperone complex, was a transcriptional target of the AR/SH3YL1 complex, correlated with aggressive PCa in patients, and was necessary for the maximal androgen-mediated proliferation and migration of PCa cells. Collectively, these data highlight the importance of an amino-terminal activation domain, its associated coregulator, and downstream transcriptional targets in regulating cellular processes of pathological importance in PCa. (Molecular Endocrinology 29: 1426–1439, 2015)

Androgens act by binding to the androgen receptor (AR), a member of the steroid hormone receptor subfamily of nuclear receptors (NRs). The binding of androgens to AR causes its dissociation from heat shock protein complexes, translocation to the nucleus, homodimerization, binding with coregulators (commonly still referred to as cofactors) and recruitment to regulatory regions of AR target genes (1). It has been demonstrated that the pharmacology of AR agonists, antagonists and selective AR modulators (SARMs) is determined by the impact of the bound ligands on receptor structure and the effect that this has on coregulator recruitment (2–5). Thus, depend-

Abbreviations: AR, androgen receptor; ChIP-Seq, chromatin immunoprecipitation-sequencing; CRPC, castration-resistant PCa; DBD, DNA-binding domain; DOX, doxycycline; FDA, United States Food and Drug Administration; GAL4, galactose-responsive transcription factor 4; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; GST, glutathione S-transferase; IRES, internal ribosome entry site;
ing on the relative and absolute expression of functionally distinct coregulators the same AR-ligand complex can manifest different biological activities in different cells. Despite the beneficial physiological effects that androgens have on promoting sexual differentiation and increased bone and muscle mass, AR signaling also has deleterious pathological effects; promoting prostate and prostate cancer (PCa) growth (6).

When diagnosed early PCa can often be treated successfully with surgery and/or radiation alone (6). However, a significant number of patients progress to the advanced stages of PCa. Because AR is a primary driver of PCa growth and metastasis, patients with advanced disease are generally treated with systemic hormone therapy to prevent the spread of the disease (7). Although androgen ablation therapy is the standard of care for advanced PCa, most tumor cells develop resistance to this therapy. Interestingly, relapse of the disease is often associated with increased AR signaling (6).

Several mechanisms have been proposed to explain the development of resistance to endocrine therapy although the most prevalent are AR overexpression, aberrant expression and/or activity of coregulators, and the expression of constitutively active, C-terminally truncated AR splice variants (6–8). Hence, although the ligand-binding domain (LBD) is the target of existing endocrine therapeutics it now appears as if other regions of AR, particularly the N-terminal domain, are crucial for the malignant progression of PCa.

To date, the N-terminus of AR has been poorly understood. This is due in large part to the intrinsically disordered structure of this region which has precluded its crystallization (9). Within this region there exists a polyproline domain that is thought to be important in AR action (10–12). Although the role of the analogous domain in the progesterone receptor (PR) is well established, the role of this domain in AR function remains enigmatic (11–19). In the case of PR, the polyproline domain facilitates the interaction of the receptor with the Src homology 3 (SH3) domain of Src kinase, which has also been reported to interact with AR in a trimer complex with estrogen receptor-α (11, 12, 15, 17, 18). However, others have questioned such a role for the AR polyproline domain (10). The goal of this study, therefore, was to define the mechanism(s) by which the polyproline domain influences AR action and how this impacts androgen action in processes of pathological importance in cancer.

Materials and Methods

Cell culture and reagents

LNCaP, VCaP, 22Rv1, PC-3, HeLa, CV-1, and HEK293 cell lines were obtained from American Type Culture Collection. Androgen-sensitive LAPC4 cells were a gift from Charles L. Sawyers (Memorial Sloan Kettering Cancer Center). HEK293TS cells were a generous gift from Christopher Counter (Duke University School of Medicine). Cells were maintained and validated as previously described (20–23). Methyltrienolone (R1881) was purchased from PerkinElmer. Cycloheximide (catalog number C7698), doxycycline (DOX) hyclate (catalog number D9891), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (catalog number G8795), antitag (catalog number F3165), and anti-v5 tag antibodies (catalog number V8012) were obtained from Sigma. Anti-SH3-domain containing, Ysc84-like 1 (SH3YL1) (catalog number ab122141) and antiubinuclein 1 (anti-UBN1) (catalog number ab101282) antibodies were purchased from Abcam. Agarose A/G beads (catalog number sc-2003) and anti-AR antibody (catalog number sc-816) were from Santa Cruz Biotechnology, Inc.

Creation of inducible stable cell lines using retroviruses or lentiviruses

Stable cell lines were created with standard retroviral and lentiviral approaches as previously described (20).

For the retrovirus, murine stem cell virus (MSCV)-AR-inter- nal ribosome entry site (IRES)-enhanced green fluorescent protein (eGFP) constructs were made using standard cloning techniques. These constructs were cotransfected (FuGENE; Roche Applied Science) with a packaging vector pCL10A1 into the 293TS packaging cell line.

For the lentivirus, commercially available short hairpin RNAs (shRNAs) in the pGIPZ backbone were obtained from Thermo Scientific. An shRNA that we determined in preliminary experiments (data not shown) to give the best knockdown of SH3YL1 and a nonsilencing control were cloned into the pLNDUCER11 backbone, a generous gift from Thomas Westbrook (Baylor College of Medicine) and used to produce lentivirus as previously described (24).

In both cases, viral supernatants were filtered, supplemented with 8-μg/mL polybrene, and used to replace the media on top of the target cells for 2 serial 24-hour infections. Green fluorescent protein (GFP)-positive cells were sorted through 3 rounds of flow cytometry. Each cell line was validated with quantitative PCR (qPCR) and Western blotting.

Cell proliferation and migration assays

Cell proliferation assays were carried out as previously described by measuring the cellular DNA content using a FluoroReporter Blue fluorometric double-stranded DNA Quantitation kit (Life Technologies) (24).

Boyden dual-chamber migration assays were carried out as previously described (25). For the high-throughput scratch-
wound assays, LNCaP cells were seeded in 96-well plates 72 hours after doxycycline treatment. Twenty-four hours after seeding, cells were scratched as previously described (26) and the media changed. With the new media, cells were given a second dose of doxycycline. Analysis of the migration was performed using an IncuCyte Zoom and done as previously described (26).

Small interfering RNA (siRNA) transfection

Stealth and Silencer Select siRNA (Life Technologies) transfections were carried out as previously described (23), with the exception that Silencer Select siRNAs were transfected at a final concentration of 10nM. The sequences of the siRNAs are listed in Supplemental Table 1, and qPCR confirmation of siSH3YL1 knockdown is shown in Supplemental Figure 1.

Plasmid transfection and reporter gene assays

Unless otherwise noted, for all experiments cells were first steroid starved for 72 hours in phenol red-free medium containing 8% charcoal-stripped fetal bovine serum. Plasmids were then transfected using Lipofectamine 2000 (Life Technologies) according to the manufacturer’s instructions. Cells were treated with hormones approximately 16 hours before the assay. Luciferase and β-galactosidase (transfection normalization) activities were measured as previously described (25). Each treatment was performed in triplicate, and results are expressed as mean ± SE. Each experiment was repeated at least 3 times, with a representative experiment shown.

Immunoblot analysis

Immunoblotting was conducted as previously described (24). Densitometry was performed using ImageJ software (National Institutes of Health, NIH) and samples were normalized to GAPDH (loading control).

RNA isolation, cDNA preparation, and quantitative RT-PCR (qPCR)

RNA isolation, cDNA preparation, and qPCR were carried out as previously described using 36B4 as an internal control (20). The sequences of the primers are listed in Supplemental Table 2.
T7 phage display

Protein interactions with full-length, ligand-bound AR tethered to DNA was identified using a high-throughput T7 phage display screen as previously described (5, 27).

Coimmunoprecipitation

LNCaP cells were transfected with v5-SH3YL1 or flag-SH3YL1 48 hours before androgen treatment. Sixteen hours after treatment, cells were harvested in radioimmunoprecipitation assay buffer as previously described (28). Lysates were incubated in radioimmunoprecipitation assay at 4°C followed by centrifugation at 14,000 rpm for 15 minutes. Protein supenatant was transferred to a new prechilled tube, measured for protein concentration, and 500 µg incubated with 2-µg anti-v5, antiflag or IgG control antibody and 20-µL agarose A/G beads at 4°C. Beads were washed and subjected to Western blotting as previously described (28).

Glutathione S-transferase (GST) pull-down assay

The GST pull-down assay was performed as previously described using full-length human SH3YL1 cloned into the pDEST15 vector to be expressed as a GST-fusion protein and whole-cell extracts of LNCaP cells treated with R1881 (29). MemCode (Thermo Scientific) stained blots were used as a control.

Microarray

LNCaP cells stably expressing the pINDUCER11-shSH3YL1 construct were treated with vehicle (ethanol), 100pM R1881, or 10nM R1881 for 24 or 72 hours with DOX before RNA collection. Samples were hybridized to Illumina Human HT-12 arrays and scanned on an Illumina HiScan BeadArray. We then preprocessed the data with the IlluminaExpressionFileCreator module in GenePattern (30). We checked for data integrity issues by generating boxplots that show the distribution of expression values, as well as principle component analysis plots to identify potential outlier samples (data not shown). We normalized the data by quantiles. Using a t test, we found genes that were differentially expressed between pairs of conditions with a 1.5-fold change and P < .05. We tested a total of 12 pairs of conditions: vehicle vs 100pM R1881, vehicle vs 10nM R1881, 100pM R1881 vs 10nM R1881, vehicle vs DOX, 100pM R1881 vs 100pM R1881 + DOX, and 10nM R1881 vs 10nM R1881 + DOX; each comparison at 24 and 72 hours. We generated the heatmap using custom-developed software and leveraged Cluster 3.0 for clustering the genes (using default parameters). SH3YL1 levels were significantly decreased with DOX treatment as was verified by microarray (Supplemental Figure 2) and qPCR (data not shown). GEO accession number is GSE64885.

Chromatin immunoprecipitation-sequencing (ChIP-Seq)

ChIP-Seq analysis was performed as previously described (31, 32).

Analysis of UBN1 association with clinical variables using The Cancer Genome Atlas (TCGA) data

A Kaplan-Meier survival plot was generated in March 2015 using data from TCGA available at www.cancergenome.nih.gov.

Figure 2. The polyproline domain of AR controls the expression of a subset of androgen-mediated genes. A, CV-1 cells were transfected with pCDNA empty vector, pCDNA-ARwt, or pCDNA-AR Δpro expression vectors in combination with an mouse mammary tumor virus-Luciferase reporter plasmid. After transfection, cells were treated with vehicle or increasing concentrations of R1881 (0.01nM, 0.1nM, 1nM) for 24 hours. Cells were harvested and assayed for luciferase activity. All luciferase values were normalized to β-galactosidase transfection controls. Data are expressed as mean relative light units (RLUs) ± SE. B, LNCaP cells expressing GAL4, v5-ARwt, v5-AR Δpro, or v5-AR(C562S) were transfected with siControl or siAR 3‘-UTR followed by treatment with vehicle or 10nM R1881 for 16 hours. RNA was then collected and transcript levels of the AR target genes FKBP51 and KLK3 (PSA) were assessed using qPCR. Data are normalized to 36B4 and expressed as mean ± SE.
Prostate adenocarcinoma tumors with mRNA and UBN1 expression greater than 1.5-fold above the mean were compared with the remaining samples. We evaluated the association of mRNA levels for the UBN1 gene with a number of reported clinical variables for the Prostate Cancer patient cohort collected and profiled by TCGA (https://tcga-data.nci.nih.gov/tcga/). Gene expression profiles were downloaded for the entire patient cohort and quantile normalized; we then evaluated the association between each clinical variable and UBN1 mRNA levels using an ANOVA test and further applied multiple hypothesis testing correction (q < 0.1) using the R statistical system.

Results

The polyproline domain of AR is required for maximal androgen-mediated PCa cell proliferation and migration

Studies of AR structure/function have indicated that sequences within both the amino-terminus and the carboxyl-terminus of the receptor are required for maximal transcriptional activity (35, 36). Although the canonical coregulator binding site denoted as activation function-2 (AF-2) within the carboxyl-terminus of AR has been studied extensively, considerably less effort has been focused on defining the roles of the amino-terminal regions in receptor function. Of particular interest to us was a polyproline region located in the amino-terminus of the receptor that exhibits the structural features of an SH3-interacting domain and which we considered was likely to function as a protein-protein interaction surface (Figure 1A). Thus, the first goal of these studies was to define the impact of disrupting this domain within AR on the biology of androgens in cellular models of PCa.

As a first step, we developed a strategy to study the activity of mutations within the polyproline region of AR in relevant PCa cells without interference from the endogenous, wild-type (wt) receptor. To this end, a retroviral approach was used to create LNCaP cells stably overexpressing a galactose-responsive transcription factor 4 (GAL4) control protein, v5-tagged wt AR (v5-ARwt), polyproline-deleted AR (v5-ARpro), or a DNA-binding domain (DBD) mutant (v5-AR(C562S); a v5-tagged AR with a cysteine to serine point mutation at amino acid 562) (Figure 1B). Simultaneously, the levels of endog-

![Image](97x176 to 517x440)
enous AR were depleted using siRNAs directed towards the 3′-untranslated region (UTR) of the receptor mRNA (Figure 1B). Knockdown of the endogenous receptor mRNA, and appropriate expression of the exogenously expressed receptor variants, was confirmed by qPCR and immunoblot (Supplemental Figure 3 and Figure 1C). As expected, depletion of endogenous AR attenuated androgen-stimulated cell proliferation in the GAL4 control cell line, an effect that could only be reversed by the expression of v5-ARwt but not v5-ARΔpro (Figure 1D). However, similar to what others have previously reported and highlighting the fidelity of our complementation approach, overexpression of the v5-ARΔpro construct (or the GAL4 or v5-ARwt) in the presence of the endogenous AR (siControl) had no significant effect on LNCaP cell proliferation. These results indicate that the polyproline domain within AR is required for maximal androgen-dependent PCa cell proliferation.

We next wanted to determine the impact of disrupting the AR polyproline domain on androgen-regulated LNCaP cell migration. Using a previously described Boyden dual-chamber migration assay (see Materials and Methods), we confirmed that androgen treatment led to a significant increase in LNCaP migration and that this was attenuated upon AR knockdown (Figure 1E). Reexpression of v5-ARwt restored the migratory capacity of cells in which endogenous wt AR had been depleted. Such complementation was not accomplished by expression of a DNA-binding deficient AR mutant (v5-AR[C62S]) and importantly, was only partially rescued by expression of the v5-ARΔpro mutant. Thus, as with cell proliferation, an intact polyproline domain is needed for maximal AR-mediated cell migration.

Disruption of the polyproline domain within AR impacts its transcriptional activity

A comparative assessment of the activity of wt AR and AR variants in a cotransfection assay was performed to define the importance of the polyproline domain in transcriptional activation. Interestingly, in AR-negative CV-1 cells, the activity of exogenously expressed, wt AR or v5-ARΔpro were comparable when evaluated on the mouse mammary tumor virus (MMTV) promoter (Figure 2A). Using the complementation assay described above, we next assessed the relative activity of wt AR and the polyproline mutant AR on the expression of the endogenous AR target genes, FKBP51 and KLK3 (prostate-specific antigen [PSA]), in LNCaP cells (Figure 2, B and C). No differences were noted between the ability of the wt and polyproline mutant-containing receptors to regulate FKBP51 (Figure 2B). However, we did observe a reduction in PSA transcript levels in cells expressing v5-ARΔpro when...
compared with the cells expressing wt AR (Figure 2C). Together, these data indicate that the polyproline domain of AR is necessary for the transcription of a subset of androgen-mediated genes, a likely consequence of the differential requirement for coregulators that interact with this specific region of the receptor.

Identification of a novel coregulator that binds to the polyproline domain of AR

Previously, we described the use of a high-throughput protein-protein interaction screen using T7 phage display to identify, in an unbiased manner, proteins interacting with full-length, ligand-bound AR tethered to DNA (5). Among the proteins identified in this manner was SH3YL1, an SH3 domain-containing protein that we hypothesized may have the ability to interact with the AR polyproline domain (Figure 3A). Using both coimmunoprecipitation and GST pull-down assays we confirmed that indeed SH3YL1 interacts with AR (Figure 3, B and C). Further, a mammalian 2-hybrid assay was used to demonstrate that the androgen-dependent interaction of SH3YL1 with AR requires an intact polyproline domain (Figure 3D). This requirement for the polyproline domain was further validated using coimmunoprecipitation (Figure 3E). Not surprisingly, SH3YL1 also interacted with agonist-bound PR, the only other NR with a polyproline domain (Supplemental Figure 4), suggesting that SH3YL1 may also be involved in PR action and modulate some progesterone-mediated effects, an area of ongoing investigation.

SH3YL1 is necessary for maximal androgen-mediated PCa cell proliferation and migration

The data generated thus far suggest that SH3YL1 may be a mediator of the functional activities of the AR polyproline domain. To address this possibility, we evaluated the impact of SH3YL1 knockdown on androgen-mediated proliferation in 2 different hormone-sensitive PCa cell lines. Here, siRNA-mediated depletion of SH3YL1 resulted in a significant decrease in the androgen-mediated proliferation of both LNCaP and LAPC4 cells (Figure 4, A and B). This activity was confirmed in cells engineered to express an inducible shRNA directed against SH3YL1 (Figure 4, C and D). To explore potential roles for SH3YL1 in advanced PCa, we generated a model in which conditional knockdown of SH3YL1 could be accomplished in the castration-resistant PCa (CRPC) cell line 22Rv1. This cell line expresses AR splice variants that retain the amino-terminus and DBD and exhibit constitutive activity (8). Importantly, knockdown of SH3YL1 in this cell line also resulted in a significant decrease in proliferation (Figure 4E). Interestingly, in the AR-negative PCa cell line, PC-3, siRNA-mediated depletion of SH3YL1 resulted in variable decreases in basal cell proliferation (Supplemental Figure 5, A and B), suggesting that like many other NR coregulators, SH3YL1 may have additional roles beyond AR, an area we are actively pursuing.

We next evaluated the role of SH3YL1 in androgen-mediated LNCaP migration. Using the Boyden dual-chamber migration assay described above, it was observed that knockdown of SH3YL1 dramatically reduced the number of migrating cells under basal conditions and after a 12-hour androgen treatment (Figure 5A). This activity was confirmed using a high-throughput...
put microscopy imaging system (IncuCyte Zoom) to analyze the role of SH3YL1 on LNCaP migration in a scratch test assay (Figure 5B and Supplemental Figure 6). Notably, only cells expressing an shRNA directed against SH3YL1 (shSH3YL1 + DOX) exhibited impaired androgen-mediated cell migration. Inducible knockdown of SH3YL1 in the CRPC cell line 22Rv1, or chemical siRNA-mediated knockdown in the AR-negative cell line PC-3, reduced the number of cells migrating under basal conditions, further highlighting the importance and possible multiple roles of SH3YL1 in PCa (Supplemental Figures 5C and 7).

UBN1 is a transcriptional target of AR and SH3YL1

Given that SH3YL1 modulated the effects of androgens on cancer cell proliferation and migration, we reasoned that SH3YL1 could be controlling a subset of androgen-regulated genes involved in these processes. To identify these genes, we took advantage of our inducible LNCaP system to knock down SH3YL1 in the presence or absence of androgens and looked for changes in gene expression using a microarray (Figure 6A and Supplemental Figure 2 and Supplemental Table 3). Similar to what we observed with the deletion of the AR polyproline domain, microarray analysis revealed that FKBP51 levels were not altered by knockdown of SH3YL1, whereas KLK3 (PSA) levels were reduced, although not significantly (data not shown). However, siRNA-mediated knockdown in combination with a shorter androgen treatment (16 h) revealed that knockdown of SH3YL1, similar to deletion of the AR polyproline domain, resulted in a significant reduction of PSA mRNA levels (Supplemental Figure 8A). This was in contrast to FKBP51 mRNA
levels, which were again not significantly altered by the knockdown of SH3YL1 (Supplemental Figure 8B). Although here it appeared at first that AR polyproline disruption decreased FKBP51 expression, suggesting a broader role for this domain in AR-dependent, SH3YL1-independent transcription, this effect was likely due to the fact that the v5-ARs in general could not fully rescue the effects of our endogenous AR knockdown on FKBP51 expression (Figure 2B, compare v5-ARwt and v5-ARΔpro). Interestingly, we did still observe a significant fold androgen induction of PSA compared with vehicle in SH3YL1 knockdown cells ± AR polyproline disruption (15-fold [−AR polyproline mutation] and 235-fold [+AR polyproline mutation], respectively). However, we suspect this may be a mathematical artifact that resulted from the nearly complete loss of detectable basal PSA mRNA levels after SH3YL1 knockdown and/or AR polyproline domain disruption (i.e., leaving a minuscule denominator to calculate fold induction, a scenario susceptible to wide variations in fold induction calculations). Regardless, at this time we cannot rule out the possibility that additional SH3YL1-dependent and possibly AR-independent regulatory mechanisms, stimulatory or inhibitory, could be regulating this gene.

Within the subset of genes that were jointly regulated by AR and SH3YL1, UBN1, a member of the histone H3.3 chaperone complex (37–39), was sensitive to androgen treatment and was down-regulated in the absence of SH3YL1. The change in gene expression observed in the microarray was confirmed using both qPCR (Figure 6B) and by Western immunoblot (Figure 6C and Supplemental Figure 9A). We also confirmed that UBN1 expression was increased in response to androgens in another hormone-sensitive cell model, VCaP (Supplemental Figure 9B). Further, the androgen-mediated increase in UBN1 expression was blocked by the competitive inhibitor enzalutamide (Figure 6D), confirming that its expression was AR-dependent. Increases in UBN1 expression 4 hours after androgen treatment or in the presence or absence of cyclohexamide suggest that UBN1 is a direct target of AR (Supplemental Figure 9C and D). In support of this, the results of AR ChIP-Seq experiments in several PCa cell lines indicate that AR binds, albeit weakly, to an intronic region of UBN1 (Figure 6E). AR's binding peak in UBN1 was dwarfed in comparison with a well-defined direct transcriptional target, CAMKK2, potentially explaining why this AR target had not been described until now (20). Nonetheless, several androgen-response element half sites within the UBN1 AR-binding region were identified. Importantly, knockdown of either SH3YL1 or both the full-length and constitutively active splice variants of AR in the CRPC 22Rv1 cell model resulted in decreased UBN1 protein expression (Figure 6F; quantification in Supplemental Figure 9E). Moreover, LNCaP cells expressing polyproline domain-mutated AR, unlike wt AR, could not rescue UBN1 expression, similar to cells lacking endogenous AR (Gal4 control) or expressing the DBD-mutated AR (v5-AR[C562S]) (Figure 6G).

Figure 7. UBN1 expression correlates with disease progression and poor patient prognosis. A, Kaplan-Meier analysis of TCGA demonstrating that UBN1 mRNA levels predict poor prognosis in PCa patients. B, High UBN1 levels correlate significantly with Gleason score. C, UBN1 levels increase in patients with detectable PSA levels after targeted molecular therapy. D, Patients incurring new tumors after initial treatment correlate with increased UBN1 expression. E and F, Increased UBN1 levels associate with tumor grade (E) and lymph node metastasis (F).
UBN1 expression correlates with disease progression and poor prognosis in patients with PCa

Analysis of clinical data derived from TCGA demonstrated that high UBN1 transcript levels correlated with poor patient prognosis (Figure 7A). Correspondingly, UBN1 levels also significantly correlated with Gleason score (Figure 7B), biochemical (PSA) recurrence (Figure 7C), new tumor occurrence after initial treatment (Figure 7D), tumor stage (Figure 7E) and metastasis (Figure 7F). Taken together, these observations indicate that UBN1 may play a role in the pathobiology of advanced PCas.

UBN1 is necessary for maximal androgen-mediated cell proliferation and migration

To determine if the impact of AR/SH3YL1 signaling on PCa cell proliferation and migration could be mediated through UBN1, we next assessed the role of UBN1 in these processes. Of note, knockdown of UBN1 expression (Figure 8A and Supplemental Figure 10) resulted in a significant decrease in androgen-mediated LNCaP proliferation (Figure 8B). Moreover, UBN1, like SH3YL1, was also necessary for maximal androgen-mediated migration in a Boyden dual-chamber assay (Figure 8C), confirming its functional role in multiple AR-mediated processes of pathological significance in PCa. Importantly, transient overexpression of UBN1 rescued the impaired androgen-mediated proliferation of LNCaP cells expressing only the AR/pro mutant (Figure 8D), indicating that UBN1 is a downstream mediator of the effects of the AR/SH3YL1 complex on PCa cell proliferation and migration.

Discussion

The results of the studies outlined in this report confirmed the importance of the AR polyproline domain in processes of pathological importance in PCa. To date, several approaches have been used to study the polyproline domain of AR, but have yielded conflicting results. For example, NR domains are often studied by exogenously transfecting various wt or mutant NR constructs into NR-negative cell lines (ex. transfecting AR-negative PC-3 cells with AR). This approach thus makes the assumption that the newly created NR transcriptional complex will be identi-
cal to that which forms in its native environment. However, this assumption carries caveats in that the NR-negative cell types have clearly evolved to no longer depend on that NR. Hence, this could be a highly artificial approach. To study the AR polyproline domain, some have transfected cells with SH3-domain containing peptides (SH3 domains canonically interact with proline-rich regions) to block its function (17). Although this would indeed block AR polyproline signaling, it also inhibits the activity of other polyproline domain-containing cellular proteins, of which there are many. Other studies have been reported which have failed to observe a significant role of the AR polyproline domain in PCa (10). However, in most of these studies the AR mutants used to evaluate the activity of the polyproline domain have been overexpressed in PCa cells and evaluated in the background of high levels of the wt receptor. Hence, unless the mutant AR had a strong dominant-negative phenotype or functioned as a significant hypermorph, its activity would be masked by the endogenous, wt AR. To circumvent this problem, we used an AR-replacement strategy to demonstrate the importance of the polyproline domain within AR on PCa cell proliferation, migration, and on the transcription of a subset of AR-mediated genes (Figure 9).

In this study, we determined that SH3YL1 interacted with the AR-polyproline domain and functioned as a bona fide AR coregulator. Although relatively little is known about SH3YL1, it, through its SH3 domain, interacts with the polyproline domains of several proteins (40, 41). Thus, it is likely that SH3YL1 has activities beyond its role as a mediator of AR action. However, in PCa cells, our data reveal a necessary role for SH3YL1 in AR-mediated growth and migration. Interestingly, SH3YL1 is expressed in several different PCa cell lines and in some cell lines examined we have determined that it migrates in Western immunoblots as a doublet, suggesting that its expression and activity may be regulated by splicing events and/or posttranslational modifications (Figure 6F). Follow-up studies are currently underway to determine how SH3YL1 activity is regulated. We have noted that SH3YL1 is a highly conserved protein with homologs identified in fungi, plants, and vertebrates (42). It has previously been shown to play important roles in meiosis, hair follicle formation, and dorsal ruffle formation (41–43). Further, it has recently been shown that SH3YL1 also interacts with the proline-rich region of dedicator of cytokinesis 4, promoting Ras-related C3 botulinum toxin substrate 1 (Rac1) activation and cell migration in the breast cancer cell line, MDA-MB-231 (40). This could explain why SH3YL1 also affected the migration of AR-negative PC-3 cells. Little is currently known about the other binding partners of SH3YL1; however, we suspect that there are several that enable AR-independent phenotypes. Nonetheless, the impact of SH3YL1 knockdown on proliferation and migration in hormone-sensitive, castration-resistant and AR-negative PCa cells further highlights the importance of this molecule in PCa. Taken together with our data presented here, this may indicate that SH3YL1 could have an important role in multiple cancer types.

In this study, we also identified UBN1 as a transcriptional target of AR/SH3YL1 in PCa cells and that its expression correlates with clinical outcome. UBN1 is a multifunctional protein involved in several important cellular processes and, not surprisingly, is widely expressed in different cell types and throughout development (44). UBN1 has been found to compete for binding to activator protein-1 (AP-1) consensus sites by interacting with the basic domains of the transcription factors Epstein-Barr virus transcription factor and c-Jun (44). Furthermore, UBN1 forms a complex with histone cell cycle regulator (HIRA), calcineurin binding protein 1 (CABIN1), and antisilencing function 1a histone chaperone (ASF1a); a
histone H3.3 chaperone complex that is localized to active promoters as well as active and weak/poised enhancers (Figure 9) (38, 39, 45). The localization of this so-called “HIRA, UBN1, CABIN1, ASF1a (HUCA)” complex at promoters correlates with gene expression (38). Hence, our work here may have uncovered the AR-mediated regulation of a new transcriptional network via control of UBN1. However, it should be noted that UBN1 may also manifest its regulatory activities in a non-genomic manner. To that end, UBN1 has been described as a component of the nuclear and adherent junction complex protein family that interacts with the tight junction protein zonula occludens-1, suggesting that it could also function through transcription-independent mechanisms to facilitate its role in androgen-mediated migration (46, 47). Certainly, future studies focused on the UBN1-me- diated transcriptional and nontranscriptional processes, in the context of AR signaling in PCa, are warranted.

Because of our interest in transcriptional regulation, we focused on defining the role of AR/SH3YL1 on the regulation of UBN1 expression. However, as shown in Figure 6A and Supplemental Table 3, this specific AR complex appeared to regulate other genes that could also contribute towards the observed phenotypes. For instance, another AR/SH3YL1-regulated gene was the prolactin receptor. Prolactin has been shown to exhibit mitogenic activities in the prostate (48, 49). Hence, it is likely that the ultimate impact of the AR/SH3YL1 complex on PCa biology is an amalgam of multiple signaling events.

Recently, the ability to selectively decrease androgen signaling in the prostate while maintaining it throughout the rest of the male body, particularly in the bone and muscle, has led to the pursuit of SARMs as potential pharmaceuticals for the treatment of PCa, cachexia, sarcopenia, and other muscle wasting syndromes (3, 50, 51). However, to date, no SARMs have attained United States Food and Drug Administration (FDA) approval. The data presented here could help guide the next generation of SARMs. For example, SARMs that lead to the recruitment of SH3YL1 would not be desirable because they would then promote PCa cell growth and migration. As such, it would be prudent to screen against AR ligands that facilitate SH3YL1 recruitment. However, whether this interaction can be uncoupled from the desired anabolic effects in the muscle and bone remains to be seen.

In PCa, the continued importance of AR in the advanced stages of the disease is reflected by the recent FDA approvals of several new drugs (ex; enzalutamide and abiraterone acetate) targeting AR’s LBD activity. Although patients treated with these promising new drugs live slightly longer, they too eventually succumb to disease relapse and subsequent mortality. These extremely resistant disease states are again largely due to residual AR activity. One emerging mechanism of continued resistance is altered AR mRNA splicing (52). This aberrant splicing leads to the generation of constitutively active AR variants that lack the C-terminal LBD and, hence, are completely insensitive to all existing AR-targeted drugs. As such, there is a major need to identify which regions of the truncated receptor facilitate pathological processes and determine whether these regions represent new therapeutic targets. We think that our findings here could have significant therapeutic implications given the increased interest in AR’s N-terminus, an area we are actively pursuing.

In summary, our data support the concept that AR conformation, coregulator recruitment and biology are intimately linked. Through this work, we have a better understanding of specifically how the AR polypeptide domain affects androgen-mediated PCa cell proliferation, migration and transcription. This study adds fundamental new knowledge to the field of AR-coregulator biology by focusing on a specific domain and elucidating its role. Importantly, this work could also aid in the rational development of improved SARMs and highlights potential new targets for the treatment of PCa.

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