USP22 Regulates Oncogenic Signaling Pathways to Drive Lethal Cancer Progression

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Abstract

Increasing evidence links deregulation of the USP22 deubiquitylase to cancer development and progression in a select group of tumor types, but its specificity and underlying mechanisms of action are not well-defined. Here we show that USP22 is a critical promoter of lethal tumor phenotypes that acts by modulating nuclear receptor and oncogenic signaling. In multiple xenograft models of human cancer, modeling of tumor-associated USP22 deregulation demonstrated that USP22 controls androgen receptor (AR) accumulation and signaling, and that it enhances expression of critical target genes co-regulated by AR and MYC. USP22 not only reprogrammed AR function, but was sufficient to induce the transition to therapeutic resistance. Notably, in vivo depletion experiments revealed that USP22 is critical to maintain phenotypes associated with end-stage disease. This was a significant finding given clinical evidence that USP22 is highly deregulated in tumors which have achieved therapeutic resistance. Taken together, our findings define USP22 as a critical effector of tumor progression which drives lethal phenotypes, rationalizing this enzyme as an appealing therapeutic target to treat advanced disease.
Introduction

Developing new means to treat aggressive tumors is of critical clinical importance. To date, there are few metrics that identify the subset of tumors that will progress to therapeutic resistance, and means to treat later stages of disease remain elusive. However, a gene signature was identified that predicts death from disease across multiple solid tumors; this ‘Death-from-Cancer’ signature is comprised of 11 genes and predicts for disease recurrence, formation of metastasis, and/or response to therapy (1). Most of the signature impinges on known cancer-associated pathways (e.g. mitosis, growth factor signaling), and have established roles in promoting pro-tumorigenic phenotypes. Intriguingly, the deubiquitylating (DUB) enzyme USP22, one of >50 Ubiquitin-Specific Proteases (USPs) that cleave ubiquitin (Ub) moieties from target substrates (2), is a key element of the signature with poorly defined functions in human cancer.

While understanding of USP22 function remains scant, recent studies provide preliminary insight into the cellular activities. Initially, USP22 was identified as conserved component of human SAGA (Spt–Ada–Gcn5 acetyltransferase) transcriptional regulatory complex (3, 4) through which USP22 modulates transcription of genes through mono-deubiquitylation of histones H2A and H2B (5, 6). Additionally, USP22 promotes stability of multiple cancer-associated protein targets through deubiquitylation, (e.g. TRF1 (7) and SIRT1 (8)), and influences oncogene accumulation (e.g. BMI1) (10). Alternatively, USP22 utilizes deubiquitylation as a means to modulate protein function and promote cell proliferation (9), and increased USP22 expression is associated with poor outcomes in multiple cancers (11-16). Importantly, USP22 proved to be required for c-MYC (referred to here as MYC) function and potentiates MYC-mediated oncogenic cell transformation in a subset of cancers (4). Breast cancer (BCa) and prostate adenocarcinoma (PCa) frequently display altered MYC signaling throughout disease progression (17, 18), and in a subset of these tumor types, MYC deregulation acts in concert with hormone-regulated transcription factors (e.g. the estrogen receptor, ER, and the androgen receptor, AR) to promote malignant phenotypes. In BCa, MYC deregulation occurs in ~40% of tumors and is a downstream effector of ER signaling that promotes estrogen-induced cell proliferation and endocrine-therapy resistance (19, 20). In models of PCa, high MYC
drives tumorigenesis (21), while in human disease, elevated MYC (mRNA and protein) occurs early, and MYC gene amplification correlates with both disease progression and poor survival (22). MYC is positioned downstream of AR and can promote PCa cell growth in the absence of androgens, termed castration-resistance (23). Importantly, based on mouse model studies, MYC-dependent prostate tumor formation requires AR signaling (21). Thus, MYC is a known effector of BCa and PCa progression, frequently acting in concert with ER and AR.

While resistance to hormone therapy in BCa can occur intrinsically by lack of ER expression (20), PCa is exquisitely dependent on AR signaling at all stages; thus, ablation of AR activity (known as androgen deprivation therapy, ADT) is the first line of therapeutic intervention for disseminated disease. Resistance to ADT occurs as a result of inappropriately restored AR activity (termed castration-resistant PCa, CRPC) through multiple mechanisms, most frequently via enhanced accumulation of AR (24). Heightened AR expression alone is sufficient to drive CRPC in xenograft models, and high AR is robustly associated with increased risk of death from PCa (25). Based on genome-wide analysis in PCa, AR binding patterns are enriched for MYC binding motifs in CRPC (26), suggesting that AR and MYC act in concert to promote CRPC progression. Thus, developing means to cooperatively target AR and MYC would be of significant clinical benefit.

Given these observations and the need to develop additional targets to manage advanced disease, it was hypothesized that USP22 may be positioned to control fundamental oncogenic signaling nodes implicit to PCa initiation and/or progression. Findings herein demonstrate that USP22 predicts for PCa disease outcome, promotes CRPC phenotypes, and is necessary for CRPC tumor maintenance by controlling signaling events dually regulated by AR and MYC. Key observations demonstrate, for the first time, that USP22 expression promotes activation of targets genes coordinately regulated by AR and MYC, which is maintained in the absence of androgens or the presence of AR antagonists, through enhanced AR protein accumulation. Most strikingly, USP22 deregulation induces androgen-independent AR recruitment to target gene regulatory loci and subsequent expression of a CRPC-associated gene profile, and supports cell growth and proliferation in the absence of androgens. Conversely, depletion of USP22 dramatically down-regulates AR protein
levels and abrogates basal and DHT-stimulated AR activity in both ADT-sensitive and CRPC cells. Finally, USP22 is significantly upregulated in CRPC tumor samples compared to primary tumors, and is requisite for CRPC xenograft tumor growth. In sum, these studies identify USP22 as a regulator of oncogene expression and activity; a novel driver of progression to CRPC by virtue of the ability to regulate AR levels, AR output, and coordination of AR/MYC signaling.

Material and Methods

Cell culture and treatments

LNCaP and C4-2 cells were maintained in IMEM supplemented with 5% FBS (heat-inactivated fetal bovine serum). 22Rv1 cells were maintained in DMEM supplemented with 10% FBS. Media was supplemented with 2 mmol/L L-glutamine and 100 units/mL penicillin-streptomycin. For hormone-deficient conditions, phenol red-free media was supplemented with charcoal dextran-treated serum (CDT). Dihydrotestosterone (DHT) was used at 1nM for 16 hours, unless otherwise noted. Casodex was used at 20µM, cycloheximide at 10µg/µl (Fisher), epoxomicin at 10µM (Santa Cruz), MG132 at 25µM (Santa Cruz). Cell lines were not cultured longer than 6 months after receipt from the original source of American Type Culture Collection.

Gene expression analysis

mRNA was analyzed by quantitative RT-PCR (qPCR) as described (27) using primers described in Supplementary Table S1.

Cell growth assays

Cells were treated with 1 µg/ml doxycycline or USP22 inhibitor then seeded at equal densities in hormone proficient or depleted conditions, as indicated. Cells were harvested at indicated time points and cell number was determined using trypan blue exclusion and a hemacytometer. Media and treatments were refreshed every 72 hours.
Flow cytometry

Cells were treated and seeded in hormone proficient or depleted conditions, as indicated, and labeled with BrdU (Invitrogen) 2 hours prior to harvest. Cells were fixed in 100% ethanol, stained with FITC-conjugated anti-BrdU antibody (BD Biosciences) and processed using FACS Calibur (BD Biosciences).

Chromatin Immunoprecipitation (ChIP) analysis

Cells were cultured in hormone depleted conditions for 72 hours and ChIP analyses and qPCR were performed as previously described (27) using primers described in Supplementary Table S1.

Immunoblotting and protein stability

Cells were seeded in hormone proficient or hormone deficient conditions and treated as specified. Protein isolation and immunoblotting were conducted as previously described. Antibodies used to detect proteins were: AR-N20 (previously described [27]), USP22 (3), MYC (Cell Signaling), GAPDH (Santa Cruz), β-actin (Santa Cruz). Protein stability was analyzed by seeding equivalent cell number and 24 hours later treating with 10µg/µl cycloheximide (Fisher Scientific) for indicated time course. To analyze contributions of proteasomal degradation, LNCaP cells were infected with indicated shRNA-containing lentivirus and selected with puromycin for 5 days, including culture in hormone-deficient media for 72 hours, stimulation with 1nM DHT, and 25µM MG132 or 10µM epoxomicin treatment for final 8 hours.

USP22 depletion

Lentiviral shRNA plasmids corresponding to USP22-1 (XM_042698.6-914s1c1, Sigma), USP22-2 (XM-042698.6-2196s1c1, Sigma) and Luciferase (SHC007, Sigma) were obtained from TRC library and used as previously published [4]. For siRNA-mediated depletion, USP22 SMARTpool oligonucleotides (M-006072-01) were transfected with Dharmafect (Dharmacon) and incubated for 72 hours. For inducible USP22 depletion, shRNA sequences targeting USP22 used for transient
depletion were annealed and cloned into pENTR/d-topo and packaged into virus using the techniques mentioned above. Tet-Repressor (TetR) expression constructs were purchased from Invitrogen as part of the Virapower system and positive populations were selected using Blasticidin.

**Immunohistochemistry**

TMAs were stained for USP22 by using USP22 polyclonal antibody diluted 1:150 (NBP1-49644 Novus) with detection via LEICA polyvision+ (PV6119, LEICA Microsystems). Briefly, unstained 5μm sections were cut from paraffin TMA blocks; slides were deparaffinized by standard techniques, steamed for 25 min in sodium citrate buffer, cooled for 5 min, blocked with peroxidase blocking solution for 5 min, incubated with the primary antibody for 45 min at room temperature, and incubated with secondary PowerVision+ rabbit antibody for 30 min.

**Xenograft analysis**

All procedures involving mice were performed in accordance with Thomas Jefferson University IUCUC protocols. 7 week old SCID mice (NCI Frederick) were surgically castrated and seven days later 2.75x10^6 cells in 100ul total saline/Matrigel (BD Biosciences) were injected subcutaneously into the flank. When tumors reached ~100-150 mm^3 mice were administered 2mg/ml doxycycline in sucrose-supplemented water. Doxycycline water was refreshed every 4 days. Tumor volume was measured with calipers.

**Statistical Analysis**

All results were analyzed using the 2-tailed Student’s t test (adjusted for variance) or Mann-Whitney test. For all analyses, $P < 0.05$ was deemed significant.
Results

In ‘Death-from-Cancer’ gene signature, USP22 selectively predicts for PCa patient survival.

The ‘Death-from-Cancer’ 11-gene signature predicts for disease recurrence, metastasis, and therapeutic failure in multiple cancers; in PCa, the signature predicted for recurrence after therapy and decreased 5-year survival (1). Analysis of individual components revealed that 10 of the 11 genes are altered in PCa (28), thus, patient survival was stratified for individual components. As shown in Fig. 1A, 6/10 genes demonstrate no significant, independent predictive power; by contrast, elevated levels of BUB1, KNTC1, mKi67, or USP22 alone associated with poor survival. Functional analyses of these four reveal a distinct pattern, in that BUB1 (a regulator of mitotic spindle checkpoints), KNTC1 (involved in chromosome segregation), and Ki67 (a known marker of active mitotic cycling) are each associated with enhanced cellular proliferation. These findings are consistent with previous reports linking enhanced cellular proliferation to poor outcome (29). However, USP22, which was also a robust marker of poor outcome, has no known role in the cell cycle.

To assess putative consequences, cooperative events were investigated. Analysis of primary and metastatic prostate tumors indicated that USP22 deregulation co-occurred with both AR and MYC upregulation within the same tumor (Fig. 1B, left, p=0.04 and 0.002, respectively). When survival of patients within this data set was analyzed, USP22 combined with AR perturbation statistically predicted for poor outcome (Fig. 1B, right). These data demonstrate that a significant proportion of PCa specimens harbor aberrant USP22, AR, and MYC expression. This is significant, as AR upregulation drives the CRPC phenotype, MYC is a known PCa oncogene, and USP22 regulates MYC transcription. Therefore, it was critical to gain further insight into the role of USP22 in hormone-dependent cancer.

USP22 Enhances AR Activity and Promotes Bypass of AR Antagonists

Since high USP22 predicted for poor outcome and was altered in concert with AR and MYC, this disease state was modeled by stable upregulation of USP22 (LN-USP22) in ADT-sensitive PCa cells. Compared to control (LN-Vac) cells, AR activity in the absence of androgen was low, as
expected. By contrast, USP22 deregulation induced marked enhancement of ligand-independent AR activity, determined by analyses of multiple, clinically relevant AR target genes (Fig. 2A). Levels of induction were similar to that observed with DHT in control cells (Fig. 2A). Moreover, USP22 and DHT acted cooperatively to further enhance AR activity (Fig. 2A). These data demonstrate that USP22 potentiates both ligand-dependent and ligand-independent AR function.

Based on previous findings that increased AR activity is sufficient to bypass the response to AR antagonists (30), the ability of the direct AR antagonist Casodex to inhibit androgen-mediated AR activity in the context of USP22 deregulation was determined. Casodex abrogated androgen-mediated AR activity in control cells (compared to DHT-stimulated conditions). However, AR activity was resistant to Casodex in LN-USP22 cells (1.28, 4.05, and 6.21-fold increases of KLK3, TMPRSS2, and FKBP5 expression, respectively, compared to control, Fig. 2A). These data demonstrate that tumor-associated USP22 elevation has the capacity to activate AR in the absence of ligand, and render AR function refractory to Casodex. Thus, USP22 not only enhances AR activity in the absence of ligand, but USP22 thwarts the effects of AR antagonists.

Since USP22 is necessary for MYC function (4), the ability of USP22 modulation to influence genes co-regulated by both AR and MYC was determined. Ornithine decarboxylase (ODC) gene expression is induced by MYC (31) and AR (32), and is overexpressed in PCa (33). Accordingly, ODC mRNA increased 2-fold in response to DHT in control cells, and was abrogated by Casodex (Fig. 2B). LN-USP22 cells expressed significantly higher ODC in the absence of DHT. Additionally, DHT stimulation in USP22 upregulated cells promoted a ~4-fold increase in ODC expression above DHT-stimulated conditions (Fig. 2B), which was significantly sustained in LN-USP22 cells upon Casodex treatment (although compared to the AR targets examined in Fig. 2A, Casodex showed a relatively more pronounced inhibitory effect). Given these results, the impact on additional MYC target genes that are unaffected by androgens, MTA1 and BAG1, was assessed. As expected, DHT had no significant impact on expression; however, little effect on gene expression was observed by USP22 upregulation (Supplementary Fig. S1A), indicating that the effects of USP22 on MYC in this tumor...
type may be restricted to genes co-regulated by AR and MYC, and that USP22 may be required for but not sufficient to alter MYC activity in PCa cells.

Next, the putative mechanisms by which USP22 alters transcriptional output were determined. Consistent with previous reports, LN-USP22 cells expressed similar levels of MYC (compared to control) in the absence or presence of DHT (Supplementary Fig. S1B). As expected, AR protein levels in control cells were enhanced by DHT, attributed to the known capacity of androgen to stabilize AR (Fig. 2C, left, compare lanes 1,3). Strikingly, USP22 upregulation in the absence of exogenous DHT enhanced AR levels similar to that observed with DHT in control cells (Fig. 2C, left, compare lanes 1, 2 and 2, 3). Furthermore, DHT and USP22 acted in concert to further increase AR expression above that seen in DHT-stimulated or USP22 high cells alone (Fig. 2C, left, compare lanes 3, 4). This increased AR was not a result of increased AR gene expression, as mRNA levels were not increased by USP22 (Fig. 2C, right). These data put forth the provocative hypothesis that USP22 increases the activity of AR through altering AR protein levels, thus identifying a novel role for USP22 in modulating steroid receptor function.

**USP22 Promotes Castration Resistant Phenotypes**

The observations that USP22 upregulation is sufficient to promote ligand-independent AR expression/activity, and induce Casodex resistance are clinically relevant, as these attributes reflect key biochemical characteristics of CRPC. To determine if USP22 is sufficient to promote the transition to CRPC, additional molecular readouts were assessed. First, recruitment of AR to well-characterized AR occupied regions (ARORs) of clinically-relevant AR target genes in the absence of hormone was quantified by ChIP-qPCR. As expected, androgen-deprivation of control cells was coincident with low AR occupancy (~0.3% input; Fig. 2D). Strikingly, USP22 deregulation significantly increased AR occupancy at known ARORs (1.15-2.6% input; Fig. 2D), but not in control regions of the KLK3/PSA ('EF' region, Supplementary Fig. S1C). These results indicate that enhanced USP22 promotes AR binding and AR-dependent transcription in the absence of ligand, suggesting that USP22 mediates castrate-resistant AR activity.
Second, the ability of USP22 to promote gene signatures strongly associated with CRPC was assessed. While \textit{UBE2C} expression was unchanged, \textit{CDC20} and \textit{CDK1} \((34)\) were both significantly increased to levels seen in other models of CRPC (Fig. 2E). In addition to upregulation of known AR target genes, the CRPC specific signature also includes androgen-repressed genes associated with polycomb group protein pathways involved in development and differentiation \((35)\). Consistent with the premise that USP22 drives CRPC-associated AR activity, expression of \textit{OPRK1}, \textit{SI}, \textit{MET}, and \textit{DDC} were significantly down-regulated under castrate conditions in LN-USP22 cells, relative to control conditions \((\text{Fig. 2E, } \text{right})\). Together, these data demonstrate that USP22 regulates ligand-independent AR residence at target gene loci and promotes AR-driven CRPC gene profiles, which may have specificity for USP22 perturbation, further implicating USP22 as an independent effector of aggressive tumor phenotypes.

\textbf{USP22 Regulates Proteasome-dependent AR Degradation}

Since the deubiquitylase function of USP22 can putatively protect substrates from degradation, the impact of USP22 expression on AR stability was initially measured using cycloheximide (CHX). AR stability was enhanced by USP22, whereby the AR half-life was extended from 14 hours \((\text{LN-Vec})\) to 18 hours \((\text{LN-USP22})\) \((\text{Fig. 3A})\). To further define the mechanism by which USP22 regulates AR, previously characterized shRNAs against USP22 \([4]\) or control \((\text{luciferase, shLUC})\) were employed in the presence or absence of proteasome inhibitors. First, depletion of USP22 significantly reduced AR protein \((\sim 73\%)\) compared to control \((\text{Fig. 3B, compare lanes 3,4 and 7,8})\). Additionally, treatment with two different proteasome inhibitors MG132 \((\text{Fig. 3B, left})\) or epoxomicin \((\text{Fig. 3B, right})\) rescued AR levels by \(\sim 3\text{-fold}\). These data suggest that USP22 functions to enhance AR stability and promote inappropriate castration-resistant AR signaling through proteasome-dependent regulation of AR levels.
USP22 Depletion Suppresses Ligand-Dependent and Castration-Resistant AR Expression and Activity

As AR signaling is required for disease maintenance and progression, the ability to reduce AR expression and activity would be of clinical benefit. As expected, in control cells, DHT promoted significant AR accumulation (Fig. 4A, lanes 1,3). In contrast, USP22 depletion reduced AR protein levels in the absence of androgen, and inhibited DHT-induced AR expression, within in models of therapy-sensitive disease (Fig. 4A, compare lanes 2, 4). Additionally, siRNAs targeting USP22 resulted in loss of AR protein, (Supplementary Fig. S2) and reduced AR function. In androgen-deprived conditions, USP22 depletion modestly decreased basal AR activity. Androgen stimulation of ADT-sensitive cells increased KLK3, TMPRSS2, FKBP5, and ODC gene expression 2.5, 4.7, 8.6, and 4-fold, respectively; however, USP22 depletion significantly blunted DHT effects (decrease of 30%, 72%, 72%, 82%, respectively, Fig. 4B). It was further queried whether USP22 suppression could alter AR activity in the castrate setting. As shown, in CRPC cells, USP22 depletion suppressed AR protein accumulation in both the androgen-stimulated and deprived conditions (Fig. 4C, compare lanes 1,2 and 3,4). Thus, the effect of USP22 downregulation on AR levels is retained in CRPC. With regard to function, CRPC cells exhibited robust AR activity in the absence of ligand. Consistent with the impact on AR levels, AR activity was suppressed (Fig. 4D), indicating that USP22 is both sufficient and necessary for CRPC-derived AR activity. While CRPC cells retain substantive AR activity in the absence of androgen, the receptor does remain responsive to DHT stimulation. Exemplifying this, control cells stimulated with DHT showed increased KLK3, TMPRSS2, FKBP5, and ODC gene expression (1.6, 2.0, 17.2, and 11.9-fold, Fig. 4D). Similar to results in ADT-sensitive cells (Fig. 4B), USP22 depletion abrogated DHT-induced AR activity in CRPC (Fig. 4D). Combined, these data illustrate the potential for targeting AR in both early-stage and advanced disease, underpinned by the dramatic requirement of USP22 to maintain AR levels and activity.

Another mechanism capable of contributing to transition to CRPC is expression of constitutively active AR splice variants (AR-SVs), which lack the ligand-binding domain and, as such, are unresponsive to AR antagonists (36-38). Notably, when USP22 was upregulated in CRPC model
systems expressing both AR full length (AR-FL) and AR-SVs, levels of both species were significantly enhanced (Fig. 5A). USP22 further increased ligand-independent AR transcriptional activity, based on KLK3/PSA expression, compared to control (Fig. 5B). To determine if loss of USP22 could impact AR-SV expression, and represent a novel mechanism to target constitutively active AR, USP22 expression was depleted by shRNA (shUSP22-2), which resulted in significantly reduced protein expression of both AR-FL and AR-SVs in androgen-deprived and DHT-stimulated conditions (Fig. 5C). Additionally, in both culture conditions, USP22 depletion diminished AR activity (Fig. 5D). Combined, these data suggest that USP22 expression is required for AR expression in ADT-sensitive PCa and CRPC models, and represents a novel target that can modulate the expression of both full length and constitutively active AR.

**USP22 Promotes Ligand-dependent and Castrate-resistant PCa Cell Growth**

To determine the biological impact of USP22 deregulation, the impact on cellular outcomes was determined. First, using hormone-proficient conditions, USP22 enhanced the rate of cell cycle progression, evidenced through increased BrdU incorporation (Fig. 6A, left). Second, USP22 significantly increased cell growth in the presence of androgen (Fig. 6A, right). Third, and most critically, USP22 robustly promoted cell growth and proliferation in the absence of androgen. As shown in Figure 6B, control cells significantly reduced BrdU incorporation upon hormone deprivation (compare Fig. 6A), whereas, USP22 upregulation induced a 2.7-fold increase in BrdU incorporation and substantially enhanced cell proliferation rates in the absence of androgen. Thus, USP22 promotes biological and biochemical castration resistance, thus defining a novel mechanism of CRPC progression.

Given the ability of USP22 to enhance AR accumulation, AR activity, and CRPC, the biological impact of a model of tetracycline-inducible shUSP22 was developed in therapy-sensitive PCa cells. As shown, doxycycline (Dox) decreased USP22, resulting in marked loss of AR (Fig. 6C, top), attenuated cell cycle progression (determined by BrdU incorporation Fig. 6C, top right, middle), and...
significantly suppressed of cell doubling (Fig. 6C, bottom). These data collectively demonstrate that USP22 is critical for cell cycle progression and cell growth in ADT-sensitive PCa.

**USP22 Expression is Elevated in CRPC and is Required for CRPC Growth**

Based on the data above, suppression of USP22 in models of ADT-sensitive PCa and aggressive CRPC decreased the AR signaling axis (Fig. 4 and 5). Additionally, based on human gene expression and cell models, USP22 upregulation is associated with decreased patient survival (Fig. 1 and 2). To further interrogate the profile of USP22 alterations in during tumor progression, USP22 expression was analyzed using clinical specimens of primary PCa and CRPC. As shown, USP22 was detected in both the cytoplasm and nuclei, and was expressed in surrounding stroma, in low grade Gleason 6 PCa (Fig. 7A). In higher grade Gleason 8 PCa, USP22 expression was enriched in the nuclei of epithelia, in addition to cytoplasm and fibroblasts, although the overall staining intensity was not significantly increased between primary PCa groupings (Gleason 5/6 and 7/8/9, Fig. 7A). In CRPC, USP22 expression was highly detectable and enriched in the nucleus (Fig. 7A). When quantified, USP22 detection was significantly increased in CRPC, compared to primary tumors grouped by Gleason score (Fig. 7A). These findings suggest that USP22 increases as a function of disease progression and represents a critical mediator of progression to CRPC.

To determine whether USP22 could serve as a putative therapeutic target in advanced disease, CRPC xenografts expressing Dox-inducible shRNA directed against USP22 were utilized. As shown, Dox treatment resulted in decreased USP22 expression and corresponding reduction in AR expression (Fig. 7B, left), reduced the proliferative capacity (40% control to 0.6% BrdU-positive shUSP22 cells Fig. 7B, middle), and suppression of cell doubling (Fig. 7B, right). Given the robust in vitro biological response, the ability of USP22 depletion to inhibit CRPC growth in vivo was assayed by recapitulating the castration-resistant environment. For these studies, immunocompromised mice were castrated and seven days later injected with CRPC cells expressing Dox-inducible shUSP22 or parental controls. As expected, given the ability of CRPC cells to proliferate in the absence of androgen, tumors were established and monitored for growth to 100 – 150 mm³. At that time, mice
were randomized to Dox-supplemented drinking water or control. Prior to Dox administration, both cell models demonstrated similar percentage of tumor rate take (≈70%), and grew at similar rates. As shown, USP22 depletion resulted in tumor growth suppression (Fig. 7C). These findings strongly support the contention that not only is USP22 a driver of CRPC, but that established CRPC tumors are reliant on sustained USP22 expression and activity. Combined, these data herein identify USP22 as a master regulator of AR stability and activity that drives prostate cancer progression.

Discussion

Given that the AR signaling axis is paramount for early stage PCa and development of lethal CRPC, understanding novel mechanisms of AR maintenance and uncovering new targets to thwart AR-mediated tumor growth is critical in advancing patient treatment options. This study presents novel data demonstrating the ability of a single ‘Death-from-Cancer’ Signature gene, USP22, to control signaling pathways requisite for prostate cancer cells. Key findings show that (i) increased USP22 predicts for poor patient outcome, (ii) USP22 is required for AR accumulation and activity, and maintained activity in the presence of antagonist treatment, (iii) USP22 promotes a castration-resistant transcriptional profile and associated therapeutic resistance, and (iv) USP22 expression is increased in CRPC tumor samples and is necessary for CRPC AR activity and tumor growth. Together, these results support the provocative hypothesis that USP22 activity is essential for AR expression, activity, and CRPC tumor growth.

USP22 Controls AR Stability and Resultant PCa Progression

The findings that USP22 regulates AR levels and activity, and promotes CRPC phenotypes are of significance, as restored AR activity is the major mechanism for transition to incurable CRPC (24). Findings herein demonstrate that USP22 is indispensable for castration-resistant AR expression and activity, cell proliferation, and tumor growth. USP22 promotes CRPC through enhanced ligand-independent and androgen-stimulated AR transcriptional activity and sustained AR activity in the
presence of antagonists, concomitant with AR protein accumulation. These results are divergent with a previous report that suggesting that USP22 functions as an AR transcriptional coactivator but does not influence protein expression (3). One important distinction is that previous findings are largely based on reporter assays analyzing USP22-mediated AR transactivation in Drosophila and human embryonic kidney (HEK) cells, and AR expression perturbation under conditions of ectopic overexpression of AR and USP22 in HEK cells. By contrast, the studies herein clearly demonstrate the importance of USP22 as a modulator of endogenous AR in human PCa. Further, USP22 mediates AR expression through a proteasome-dependent mechanism, since modeling clinically-relevant USP22 upregulation results in an increased AR protein half-life and proteasome inhibition rescued the decreased AR expression following USP22 depletion. Using multiple approaches to monitor changes in AR ubiquitylation, levels of this modification were not reproducibly changed in response to USP22 knockdown (Supplementary Figure S3), suggesting that AR may be an indirect target. Alternatively, degradation could be driven by USP22-mediated mono-deubiquitylation and not detectable in the experimental assays. Similarly, several proteins (e.g. PAX3 and cyclinB1), can be monoubiquitylated and sufficiently targeted to the proteasome for degradation (39, 40). It is also possible that USP22 is indirectly regulating degradation in a proteasome-dependent, ubiquitin-independent mechanism. Such modes of regulation have precedent; for example, REG activates the proteasome and directly recruits substrates for degradation in the absence of ubiquitin, (e.g. p21Clp1, p16Ink4a (41), and SRC/AIB1 (42)). NQO1 associates with proteasome subunit 20S and p53 to regulate access to the proteasome in an NADH-dependent mechanism, and subsequently controls p53 degradation (43). Identification of potential endogenous USP22 targets is currently in progress.

These findings are amongst the first to identify cancer-associated molecules that alter AR stability. Selected E3 ligases (e.g. MDM2, CHIP, and Siah2) can control AR protein levels through addition of ubiquitin moieties and subsequent proteasomal degradation, but the link to human disease is uncertain. MDM2 can target AR for degradation in an AKT-dependent manner (44), but there is limited evidence demonstrating MDM2 deregulation in PCa. CHIP (C-terminus of Hsp70-interacting protein) ubiquitylates AR (45), but it is unclear if CHIP expression is altered in PCa. Lastly, Siah2
targets only chromatin-bound AR for degradation (46), consistent with previous studies demonstrating the requirement of ubiquitylation and turnover of nuclear receptors for efficient transcription. The link between DUBs and AR is even less well characterized. USP26 can deubiquitylate AR in the presence of androgen (47), while USP10 and 2A-DUB both regulate AR transcriptional activity but do not alter protein stability (48, 49). Thus, the studies here identify USP22, which is predictive for PCa disease outcome and is increased in advanced incurable patient samples, as a DUB that promotes AR accumulation and PCa progression.

**USP22 Regulates AR Splice Variants**

Multiple mechanisms induce resurgent AR activity and resultant CRPC formation following hormone therapy, including AR overexpression/amplification, somatic AR mutations, post-translational modification and cofactor perturbations, intracrine androgen synthesis, and AR-SVs (24). Here, constitutively active AR-SVs, which lack a ligand-binding domain and are not inhibited by clinically-approved AR antagonists, also require USP22 for accumulation (Fig. 5). Given the clinical implications for AR-SV-positive tumors, intensive efforts are ongoing elucidate AR-SV regulation. The AR ligand binding domain contains two lysine residues (845 and 847) that promote degradation upon ubiquitylation, suggesting that regulation of AR-SV expression is independent of these canonical proteasomal degradation targets (50). Since USP22-mediated DUB activity can modulate transcriptional elongation (5) and H2B monoUb can affect exon skipping (51), it is possible that USP22 could be involved in mediating AR alternative splicing. Regardless of the mechanism, studies here show that disrupting USP22 expression or activity dramatically reduces AR-SV production and/or accumulation, thus providing one mechanism by which USP22 may control CRPC transition and presenting a potential means to target the heretofore non-targetable AR-SVs.

**USP22 selectively controls MYC in PCa**

The concept that USP22 controls AR activity is complemented by observations that USP22 is required for expression of genes co-regulated by both AR and MYC. Prior reports showed that USP22
is recruited via SAGA to MYC binding sites and therein modulates MYC output. Further studies reveal that USP22 function is highly selective, such that it is required for activation of a subset of p53 target genes (4, 8). Studies herein suggest that USP22-mediated MYC regulation in PCa is likely gene selective, based on USP22 increasing gene expression of AR/MYC co-regulated target (ODC) but not of multiple MYC targets. Whether MYC occupancy is altered at co-regulated sites in response to tumor-associated USP22 deregulation remains an open question that will be revealed by genome-wide analyses. The concept that USP22 is required for genes co-regulated by AR and MYC is intriguing, given the clinical importance of AR-MYC crosstalk. It is of interest that AR and MYC both function to promote expression of target genes required for growth and proliferation, and are intricately interconnected. In mouse models of PCa, MYC-driven tumor formation is reduced by castration or AR antagonists (52). Androgen stimulation increases MYC mRNA levels, which is suppressed in response to Casodex (23), and AR promotes MYC activation indirectly through mediating expression of the ETS fusion protein, TMPRSS2:ERG (53). ODC is one characterized gene dually regulated by AR and MYC. However, MYC binds to promoters of numerous AR target genes (54), suggesting that USP22 could regulate a unique, cell-specific gene signature that is critical for AR-MYC coordination.

**USP22 Deregulation in Human Malignancies**

While these studies identify a key role for USP22 in PCa, USP22 expression is predictive for poor outcome in numerous malignancies. For example, USP22 is required for hepatocellular carcinoma (HCC) and bladder cancer growth (55, 56), is elevated in advanced melanoma (57), and independently predicts for poor prognosis in colorectal carcinoma (12), invasive BCa (11), gastric cancer (58), esophageal and oral squamous cell carcinoma (15, 16), and papillary thyroid carcinoma (13). While these outcomes could occur via USP22-mediated MYC/CyclinD2 and/or BMI-1-mediated modulation (10), AR activity promotes tumorigenesis in several of these tumor types, including bladder (59), HCC (60) and breast carcinoma (61). In contrast to the studies reported here for PCa, previous studies stopped short of identifying molecular targets that mediate USP22-dependent tumor
phenotypes. In BCa, AR is a driver of disease in the molecular apocrine subtype, wherein AR drives a subset of classical ER-responsive genes mediated by FOXA1 (62), and promotes a signaling pathway that activates β-catenin, HER3, and HER2 (61). Intriguingly, a feed-forward pathway was found within this tumor type, whereby AR activates MYC, which binds to promoters of androgen-responsive genes to promote sustained AR activity (54). Similar to PCA, cell growth is responsive to Casodex treatment (62) and USP22 depletion reduces AR expression (data not shown), suggesting that targeting USP22 in this BCa subset could be advantageous. Further investigation into the role of USP22 in molecular apocrine BCa is ongoing. In sum, USP22 represents a biomarker for advanced malignancies and can influence AR expression in multiple tumor scenarios, suggesting that USP22 inhibition could have implications in numerous tumor types.

**USP22 as a Therapeutic Target**

Despite a growing body of literature demonstrating that USP-family members are implicated in multiple diseases, including cancer, development of active site inhibitors is challenging (63). USP7 destabilizes multiple tumor suppressors, and as such is associated with deregulating signaling pathways altered in cancer (64). Several inhibitors have been generated that selectively inhibit USP7 in a reversible manner. However, no agents that specifically target USP enzymatic activity are in clinical trials. The potential benefit of inhibiting USP22 is of putative clinical relevance, as restored AR activity is the major mechanism for transition to incurable CRPC. As shown herein, USP22 promotes and is required for the maintenance of CRPC; conversely, USP22 depletion blunts cell growth in models expressing mutated AR, constitutively active AR, and AR amplification. USP22 depletion acted in concert with castration to elicit an enhanced biochemical and cellular response, and based on data presented herein, targeting USP22 should also act in concert with the recently approved CYP17A inhibitor abiraterone acetate, which targets intracrine androgen synthesis (65, 66). On balance, USP22 would be predicted to enhance therapeutic options for treatment of CRPC.

In summary, the present first-in-field observations identify USP22 as a major effector of AR levels, AR output, AR-MYC coordination, and the transition to CRPC. Robust *in vitro* and *in vivo* data
support the concept that USP22 is not only a major driver of disease progression, but that therapeutic targeting of USP22 would be of likely high clinical benefit.

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Figure Legends

Figure 1. In ‘Death-from-Cancer’ gene signature, USP22 selectively predicts for PCa patient survival. A, Prostate cancer patient survival plots of ‘Death-from-Cancer’ genes. Genes are functionally grouped and demonstrate BUB1, Ki67, KNTC1 and USP22 are univariably predictive. B, Regulation of USP22, AR, and Myc RNA expression in metastatic castrate prostate cancer samples. USP22, AR, and Myc are altered concurrently in metastatic PCa. Data set obtained from cBio Cancer Genomics Portal (http://www.cbioportal.org/public-portal/).

Figure 2: USP22 Increases AR Activity, Promotes Bypass of AR Antagonists, and Drives Expression of CRPC Gene Signature. A, LNCaP with stable USP22 upregulation (LN-USP22) and control cells (LN-Vec) were cultured in androgen-deprived media then stimulated with 1 nM DHT or vehicle for 16 hours, and/or 20µM Casodex for 24 hours. mRNA transcript levels of AR target genes PSA, TMPRSS2, and FKBP5 were analyzed by qRT-PCR. B, Similarly treated cells were analyzed by qRT-PCR for mRNA levels of ODC, a Myc target and androgen-responsive gene. C, left, LN-USP22 and control cells were androgen deprived and stimulated with DHT or vehicle and cell lysates were immunoblotted with AR, USP22 and GAPDH antibodies. right, LN-USP22 and control cells AR gene transcript was monitored by qRT-PCR. D, LN-USP22 and control cells were cultured in hormone-depleted media for 72 hours. Samples were harvested for ChiP analysis and AR was immunoprecipitated with AR-N20 antibody and analyzed using primers targeting KLK3/PSA enhancer III, TMPRSS2 enhancer V region, and FKBP5 enhancer 6/7 region. E, LN-USP22 and control cells were cultured in androgen-deprived media for 72 and mRNA of UBE2C, CDC20, CDK1, OPRL1, SI, MET, and DDC were analyzed by qRT-PCR. * p<0.05, **p<0.01

Figure 3. USP22 Alters AR Degradation via Proteasome Bypass. A, To assess AR stability, LN-USP22 and control cells were cultured in FBS-containing media and treated with 10 µg/µl cycloheximide (CHX) for the indicated times. Cell lysates were immunoblotted with AR and GAPDH antibodies. Representative AR expression levels presented. B, LNCaP cells were infected with shUSP22-1 or shLuciferase lentivirus for 120 hours, and androgen-deprived for the final 72 hours. Cells were then treated with 1nM DHT for 16 hours, with or without 25µM MG132 (left) or 10µM epoxymicin (right). Cell lysates were immunoblotted with AR, USP22, and GAPDH antibodies.

Figure 4. USP22 Depletion Suppresses Ligand-Dependent and Castrate-Resistant AR Expression and Activity. A, LNCaP cells were infected with shUSP22-1 or control (shLuc)-encoding lentivirus for a total of 120 hours, including androgen deprivation during the final 72 hours with 1nM DHT or vehicle stimulation for 16 hrs. Cells lysates were immunoblotted with AR, USP22, and actin antibodies. B, Cells were treated as in A and mRNA transcript levels of AR targets PSA, TMPRSS2, FKBP5, ODC were analyzed by qRT-PCR. C, C4-2 CRPC cells were infected and treated as in A and immunoblotted with AR, USP22, and actin antibodies. D, C4-2 cells were treated as in A and mRNA transcript levels of AR targets PSA, TMPRSS2, FKBP5, ODC were analyzed by qRT-PCR. * p<0.05, **p<0.01

Figure 5. USP22 promotes AR splice variant accumulation. A, 22Rv1 cells stably expressing USP22 or vector were androgen-deprived for 72 hours and lysates were immunoblotted for AR (which recognizes full length AR and the lower molecular weight splice variant), USP22, and GAPDH. B, 22Rv1 cells were treated as in A and mRNA expression of PSA was analyzed by qRT-PCR. C, 22Rv1 cells were infected with USP22 (shUSP22-2) or control (shLuc)-encoding lentivirus shRNA for a total of 120 hours, including androgen deprivation during the final 72 hours with 1nM DHT or vehicle stimulation for 16 hours. Cells lysates were immunoblotted with AR, USP22, and actin antibodies. D, Cells were treated as in C and mRNA expression of KLK3/PSA, TMPRSS2, and FKBP5 was quantified by qRT-PCR. * p<0.05, **p<0.01
Figure 6. USP22 Promotes Ligand-dependent and Castrate-resistant PCa Cell Growth. A, LN-USP22 and control cells were cultured in hormone-proficient media and analyzed for BrdU incorporation. Representative scatter plot shown, bar graph is relative percent BrdU incorporation. Cell growth was determined by trypan blue exclusion (top right). B, LN-USP22 and control cells were cultured in hormone-depleted media for 72 hours and analyzed for BrdU incorporation. Representative scatter plot shown and bar graph is relative percent BrdU incorporation. Cell growth was determined over 120 hours. C, LNCaP Tet-inducible shUSP22-1 cells cultured in FBS-containing media were treated with 1µg/ml doxycycline and cell lysates were immunoblotted with AR, USP22, and GAPDH antibodies (top left). Treated cells were analyzed for BrdU incorporation (top, middle) cell growth (bottom). * p<0.05, **p<0.01

Figure 7. USP22 Expression is Elevated in CRPC Patient Samples and Required for CRPC Tumor Growth. A, Representative USP22 IHC staining images from TMA specimen of increasing disease progression (left). USP22 protein expression increases from primary to CRPC (right). B, Tet-inducible shUSP22-1 C4-2 cells cultured in complete media were treated with 1µg/ml doxycycline or vehicle for 72 hours for depletion of USP22 (left), then replated for 24 hrs for BrdU analysis, representative scatter plots shown (middle). Cell growth was determined by trypan-blue exclusion over time (right). C, SCID mice were castrated and 7 days later injected with 2.75x10^6 C4-2 tet inducible shUSP22 cells in flank. When tumors reached 100 – 150 mm^3, mice were stratified into 2 mg/ml doxycycline or sucrose water treatment groups. Graph represents tumor volumes of individual tumors at day 20 post-treatment, relative to day 0 volume. **p<0.01
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B) Similarly treated cells were analyzed by qRT-PCR for mRNA levels of ODC, a Myc target and androgen-responsive gene. (C) \( ^* \) p<0.05, **p<0.01 on April 14, 2017. © 2013 American Association for Cancer Research. cancerres.aacrjournals.org Downloaded from cancerres.aacrjournals.org on April 14, 2017. © 2013 American Association for Cancer Research.
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