Androgen Receptor Is the Key Transcriptional Mediator of the Tumor Suppressor SPOP in Prostate Cancer

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Abstract

Somatic missense mutations in the substrate-binding pocket of the E3 ubiquitin ligase adaptor SPOP are present in up to 15% of human prostate adenocarcinomas, but are rare in other malignancies, suggesting a prostate-specific mechanism of action. SPOP promotes ubiquitination and degradation of several protein substrates, including the androgen receptor (AR) coactivator SRC-3. However, the relative contributions that SPOP substrates may make to the pathophysiology of SPOP-mutant (mt) prostate adenocarcinomas are unknown. Using an unbiased bioinformatics approach, we determined that the gene expression profile of prostate adenocarcinoma cells engineered to express mt-SPOP overlaps greatly with the gene signature of both SRC-3 and AR transcriptional output, with a stronger similarity to AR than SRC-3. This finding suggests that in addition to its SRC-3–mediated effects, SPOP also exerts SRC-3–independent effects that are AR-mediated. Indeed, we found that wild-type (wt) but not prostate adenocarcinoma–associated mutants of SPOP promoted AR ubiquitination and degradation, acting directly through a SPOP-binding motif in the hinge region of AR. In support of these results, tumor xenografts composed of prostate adenocarcinoma cells expressing mt-SPOP exhibited higher AR protein levels and grew faster than tumors composed of prostate adenocarcinoma cells expressing wt-SPOP. Furthermore, genetic ablation of SPOP was sufficient to increase AR protein levels in mouse prostate. Examination of public human prostate adenocarcinoma datasets confirmed a strong link between transcriptomic profiles of mt-SPOP and AR. Overall, our studies highlight the AR axis as the key transcriptional output of SPOP in prostate adenocarcinoma and provide an explanation for the prostate-specific tumor suppressor role of wt-SPOP.

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Introduction

The androgen receptor (AR) is a critical driver of prostate adenocarcinoma pathophysiology, regulating proliferation, metabolism and migration, and is also a validated therapeutic target (1). The importance of the AR axis in prostate adenocarcinoma is further illustrated by the frequent overexpression, especially in the state of castration-resistant prostate cancer (CRPC), of steroidogenic enzymes that lead to persistence of intratumoral androgens (2–7), as well as AR itself (2, 8–12), and its coactivators (8, 10, 13, 14). Additional mechanisms of noncanonical AR activation, including AR mutations (15–19), ligand-independent AR splice variants (20–24) and cytokine-induced ligand-independent activation (25), as well as epigenetic dysregulation of miRNAs that control AR homeostasis (26), contribute to CRPC progression and further highlight the critical importance of the AR axis in prostate adenocarcinoma. There is compelling evidence that even taxanes, the only family of cytotoxic chemotherapeutics that has ever demonstrated an overall survival benefit in prostate adenocarcinoma, exert anticancer activity in CRPC by inhibiting the AR axis (27–29).

Whole-exome sequencing studies recently discovered that the E3 ubiquitin ligase adaptor speckle-type POZ protein (SPOP) is frequently affected by somatic nonsynonymous point mutations in prostate adenocarcinoma (30–32). SPOP harbors an N-terminal MATH (Meprin and Traf Homology) domain that recruits substrate proteins, and a C-terminal BTB (Bric-a-brac/Tramtrack/Broad complex) domain that interacts with Cullin 3 (Cul3) and Rbx1 to promote substrate ubiquitination. SPOP, via its MATH domain, binds to and promotes the
ubiquitination of several substrates, including the death domain–associated protein Daxx (33), the phosphatase Puc, the transcriptional regulator Ci/Gli (34), the variant histone MacroH2A (35), and the key AR coactivator Steroid Receptor Coactivator (SRC)-3 (36, 37). All SPOP mutations reported in prostate adenocarcinoma affect conserved residues in the substrate-binding pocket (30–32), suggesting that they modify substrate specificity and can drive the accumulation of several proteins with roles in prostate adenocarcinoma pathophysiology. We previously reported that wt-SPOP plays a critical tumor suppressor role in prostate adenocarcinoma cells and promotes the turnover of SRC-3 protein, thus suppressing its capacity to function as an AR coactivator. This tumor suppressor effect is abrogated by the prostate adenocarcinoma–associated SPOP mutations (36). SRC-3 promotes prostate adenocarcinoma cell proliferation and survival (38), cell migration and invasiveness (39), and development of CRPC (40). However, the relative contribution of the various reported SPOP substrates to the pathophysiology of SPOP–mutant (mt) prostate adenocarcinoma has not been fully elucidated.

In the present study, we examined the global gene expression profiles of AR(+) prostate adenocarcinoma cells engineered to express wild-type (wt)–SPOP or three different mt–SPOPs. The gene signatures of all mt–SPOPs exhibited high overlap with each other and with the gene signature of androgen-treated prostate adenocarcinoma cells. Gene Set Enrichment Analysis (GSEA) revealed that while the transcriptomic footprint of mt-SPOP enriches for signatures of both SRC-3 and AR transcriptional output, it matches more closely to AR than to SRC-3. This suggested that, in addition to SRC-3–mediated effects, SPOP also exerts important SRC-3–independent/AR-mediated functions. Our biochemical studies revealed that wt-SPOP can bind AR directly and promote its ubiquitination and degradation. This activity is abrogated by the prostate adenocarcinoma–associated SPOP mutations, leading to AR stabilization and increased cell proliferation. Xenografts of prostate adenocarcinoma cells expressing mt-SPOP expressed more AR protein and grew faster in immunocompromised mice than those expressing wt-SPOP. Genetic ablation of SPOP resulted in increased AR protein levels in the mouse prostate. In addition, examination of several publicly available human prostate adenocarcinoma datasets confirmed the link between the transcriptomic outputs of mt–SPOPs and AR. Our studies identify the AR axis as the key transcriptional output of the tumor suppressor SPOP in prostate adenocarcinoma and provide an explanation why mutations in the substrate-binding pocket of SPOP occur frequently and in a prostate adenocarcinoma–specific manner.

Materials and Methods
Reagents and antibodies
Enzalutamide (MDV3100) was kindly provided from Medivation. The antibodies used were: mouse monoclonal anti-Flag M2 (Sigma), anti-SPOP (Abcam), rabbit polyclonal anti-AR (Cell Signaling Technology), rabbit anti-AR (Santa Cruz Biotechnology), anti-β-actin (Sigma), mouse anti-Flag–HRP (Sigma), rat anti-HA–HRP (Roche), anti-rabbit IgG–HRP, and anti-rat IgG–HRP (Sigma), respectively.

Cell culture
Human cell lines were obtained from the ATCC via the Tissue and Cell Culture Core Laboratory at Baylor College of Medicine (Houston, TX), where they are regularly submitted for cell line authentication (by STR profiling) and mycoplasma testing, and passaged for fewer than 6 months. Human Embryonic Kidney 293T cells, cervical carcinoma HeLa cells, and PC DU145 cells were cultured in DMEM high glucose (Life Technologies) with 10% FBS (Life Technologies) in a 5% CO₂ incubator at 37°C. LNCaP and 22Rv1 cells were cultured in RPMI1640 (Life Technologies) supplemented with 10% FBS; LAPC4 cells were cultured in Iscove Modified Dulbecco's Medium (Life Technologies) plus 15% FBS, 1 mmol/L R1881, and 2 mmol/L of L-glutamine; PC3 cells were cultured in DMEM/F12 (F-12 Nutrient Medium, Life Technologies) with 10% FBS; and VCaP cells were maintained in DMEM high glucose (Life Technologies) with 10% FBS and 1 mmol/L R1881. The human CRPC cell line Abl [characterized by and obtained from Dr. Zoran Culig, Innsbruck Medical University, Tyrol, Austria (19)], was maintained in RPMI1640 medium (Life Technologies) supplemented with 10% charcoal-stripped FBS. SRC-3 knockout (KO) cells (SRC-3–/–), generated using a zinc finger nuclease (ZFN) to knockout both SRC-3 alleles and documented to lack SRC-3 (41), were a generous gift from Dr. Bert W. O'Malley, Baylor College of Medicine (Houston, TX). Previously described (36) doxycycline-inducible Abl stable transfectants (Abl-control vector, Abl-SPOPwt, Abl-SPOP-Y87N, Abl-SPOP-F102C, Abl-SPOP-S119N, Abl-SPOP-W131G, Abl-SPOP-F133L, and Abl-SPOP-F133V) were maintained in RPMI1640 supplemented with 10% tetracycline-tested FBS (Atlanta Biotech, Inc.) and 300 μg/mL G-418 (Life Technologies). Additional prostate adenocarcinoma cell lines expressing, under doxycycline-inducible promoter, wt or mutant SPOP, were established from LNCaP and VCaP cells, as previously (36).

Additional methods for bioinformatics analysis, in vitro and in vivo studies are presented in the Supplementary Methods.

Results
The F102C, F133V, and F133L SPOP mutants result in similar transcriptomic responses in prostate adenocarcinoma cells
We performed gene expression profiling of Abl prostate adenocarcinoma cells engineered to express the prostate adenocarcinoma–associated SPOP mutants F102C, F133V, and F133L, or SPOP WT (or control vector). Gene expression signatures were derived for each individual mutant against SPOP WT and for all three mutants combined against SPOP WT (P < 0.05, fold change exceeding 4/3), as presented in Fig. 1A. By comparing the three signatures with respect to each other, we determined that the three SPOP mutants tested produce highly similar transcriptomic responses (Fig. 1A and Supplementary Fig. S1), which are very distinct (essentially inverted) from the effects of the wt–SPOP (Fig. 1A).
Global gene expression profiling identifies the AR transcriptional output as the top enriched gene set in prostate adenocarcinoma cells expressing mutant SPOPs. A, hierarchical clustering of gene expression profiles of Abl prostate adenocarcinoma cells transfected with control vector, wt-SPOP, or the SPOP mutants F102C, F133V, F133L (genes differentially expressed, t test $P < 0.05$, fold change exceeding 4/3 times) demonstrates that all three SPOP mutants have highly similar effects on the prostate adenocarcinoma transcriptome, which are very distinct (essentially inverted) from the effects of the wt-SPOP. B, the top Molecular Signature Database (MSigDB) match for the mt-SPOP gene signature, out of 10,295 available gene sets analyzed in an unbiased fashion, is the "NELSON_RESPONSE_TO_ANDROGEN_UP." Also, three of the top five chemical and genetic perturbation (CGP) gene sets in prostate adenocarcinoma correspond to androgen-induced transcriptomic responses. C, the AR activity score was calculated on the basis of a previously published gene signature of androgen-stimulated LNCaP cells (42), for Abl cells expressing control vector, wt-SPOP, or mt-SPOP. Compared with control vector, cells expressing wt-SPOP exhibit a lower AR activity score, whereas mt-SPOPs increase the AR activity score. D, GSEA revealed that SRC-3–upregulated genes (genes downregulated by SRC-3 siRNA) are significantly enriched in the mt-SPOP gene signatures. The values on the y-axis for each graph represent enrichment scores (corresponding to the magnitude of the enrichments for each analysis). For each graph, the normalized enrichment score (NES, computed via the GSEA analysis) and the significance of the enrichment ($q = $ false discovery rate also computed via the GSEA analysis) are indicated. The NES scores range from 1.35 to 1.47 (all $q < 0.02$). However, we found that the mt-SPOP gene signatures show stronger enrichment, with NES ranging from 1.88 to 2.71 ($q < 0.001$), for androgen-induced genes (genes downregulated by AR siRNA or induced by androgen treatment of prostate adenocarcinoma cells).
GSEA using the Molecular Signatures Database identifies the AR transcriptional output as the top enriched gene set in prostate adenocarcinoma cells expressing mutant SPOPs

We evaluated the transcriptomic responses induced by mt-SPOPs (for each mutant SPOP, genes were ranked by the fold change between the mutant and the WT SPOP samples) in an unbiased comparison with the entire Molecular Signatures Database (MSigDB, containing 10,295 gene signatures), using adjusted q < 0.05 as our filtering criteria. The overall best match was "NELSON_RESPONSE_TO_ANDROGEN_UP," which corresponds to genes upregulated by androgen in prostate adenocarcinoma cells (42). We further focused on the Chemical and Genetic Perturbations gene set collection (over 3,400 signatures), and filtered the results for experiments in prostate adenocarcinoma cells. Three of the top five gene sets correspond to androgen-induced signatures (Fig. 1B). Utilizing the Nelson signature (42) to calculate an AR activity score for each transfected prostate adenocarcinoma sample, we confirmed that, compared with control vector, cells expressing wt-SPOP exhibit a lower AR activity score, whereas mt-SPOPs (F102C, F133V, and F133L) increase the AR activity score (Fig. 1C). Similar results were obtained using another androgen-induced signature as well (43) (Supplementary Fig. S2). We also expanded our analysis using a wider panel of SPOP mutants (including also Y87C, Y87N, S119N, F125V, and W131G) and demonstrated by quantitative reverse-transcriptase polymerase chain reaction (q-PCR) that they, too, have partially or completely lost the capacity of wt-SPOP to suppress the expression of the AR-dependent genes SGK1, CAMKK2, ABCG4, HOMER2, SPINT, and NXXL1 (Supplementary Fig. S3). In combination with our prior report that wt-SPOP, but not these 8 prostate cancer–associated mutants, suppresses the expression of the AR-dependent genes KLK3 (PSA) and FKBP5 (36), we have confirmed that all 8 SPOP mutants result in similar gene expression profiles, specifically lacking the capacity of wt-SPOP to suppress AR transcriptional activity.

The gene signatures of mt-SPOPs enrich for genes regulated by direct AR antagonists

We next investigated, using the GSEA method, the enrichment of SPOP-regulated gene signatures for genes regulated by a collection of drugs (FDA-approved or experimental) used or proposed, respectively, for prostate adenocarcinoma treatment. The genes upregulated by mt-SPOPs (over wt-SPOP samples) were highly enriched for genes downregulated by direct AR antagonists, such as enzalutamide, bicalutamide, ARN-509, and compound 30. Inversely, the genes downregulated by mt-SPOPs (for each mutant SPOP, genes were ranked by the fold downregulation by wt-SPOP siRNA) are significantly enriched in the mt-SPOP gene signatures, with normalized enrichment scores (NES) ranging from 1.35 to 1.47 (all q < 0.02, Fig. 1D). This confirms our previous report that the estrogen receptor–mediated SPOP interaction is critically dependent on the SPOP substrate-binding cleft of the MATH domain. In agreement, while the C-terminal fragment of wt-SPOP (a.a. 172–374, containing the BTB domain) failed to bind to the AR protein, the N-terminal fragment (a.a. 1–172) of wt-SPOP, containing the MATH domain and its substrate-binding pocket, efficiently coimmunoprecipitated with AR protein in vitro (Fig. 2C). These observations indicate that SPOP-wt can bind to and that the AR–SPOP interaction is critically dependent on the SPOP substrate-binding cleft of the MATH domain. To further dissect the impact of the tumor suppressor SPOP on AR expression in prostate adenocarcinoma cells, we examined AR protein expression in Abl prostate adenocarcinoma cells engineered to express, under a tetracycline-inducible promoter, SPOP-wt or the prostate adenocarcinoma–associated SPOP mutants. Immunoblot analyses revealed that, upon induction with doxycycline, SPOP-wt, but not the prostate adenocarcinoma–associated SPOP mutants, significantly suppressed AR protein expression in Abl prostate adenocarcinoma cells (Fig. 3A). Of note, a subset of mutants (including F102C) increased AR protein expression above baseline (i.e., no exogenous SPOP) levels, suggesting a possible gain-of-function "dominant-negative effect" of these SPOP mutants on the function of endogenous (wt) SPOP. Real-time qRT-PCR
To further explore the impact of SPOP on AR protein stability, we used cycloheximide treatment and immunoblot analyses to quantify the half-life of the AR protein in Abl cells induced to express SPOP-wt or the prostate adenocarcinoma–associated SPOP mutants (SPOP-Y87N, SPOP-Y87C, SPOP-F102C, SPOP-S119N, SPOP-F125V, SPOP-W131G, SPOP-F133L, and SPOP-F133V), respectively. In Abl cells transfected with the control vector, the half-life of AR is approximately 500 minutes (Fig. 3B). Expression of SPOP-wt destabilized and dramatically shortened the half-life of AR protein to approximately 200 minutes. On the contrary, in Abl cells expressing mt-SPOPs, the half-life of AR protein is significantly extended, in particular in the case of SPOP-F102C (Fig. 3B). We also confirmed the interaction of AR protein and wt-SPOP in Abl cells using coimmunoprecipitation. In the immune complex precipitated by the anti-AR antibody, both AR protein and wt-SPOP were detected (Fig. 3C). Similarly, AR protein was detected in the immune complex precipitated by the SPOP-specific antibody in Abl cells transfected with wt-SPOP (Fig. 3C). Importantly, in Abl cells transfected with SPOP-F102C, SPOP did not coimmunoprecipitate with AR in either condition, i.e., immunoprecipitation with anti-AR or with anti-SPOP (Fig. 3C). Taken together, these data provide direct evidence for the association between AR protein and the E3 ubiquitin ligase adaptor SPOP (wt) in prostate adenocarcinoma cells.

**SPOP promotes ubiquitination of AR protein and this activity is abrogated by the prostate adenocarcinoma–associated SPOP mutations**

SPOP functions as an adaptor protein that facilitates the recruitment of substrates to the Cullin-3/BRX-1 E3 ligase complex to promote ubiquitination and degradation of its substrate proteins (36). To further dissect the functional role of binding of SPOP on ubiquitination and degradation of AR protein, we coexpressed AR (Flag-tagged) and HA-tagged ubiquitin (Ub) together with SPOP-wt or prostate adenocarcinoma–associated SPOP mutants (SPOP-F102C, SPOP-F133V, SPOP-F125V, SPOP-S119N, SPOP-Y87C, and SPOP-Y87N) or SPOP-C-terminal fragment (lacking the MATH domain) in 293T cells, and examined the levels of ubiquitin-conjugated AR protein. As shown in Fig. 4A, the levels of ubiquitinated AR protein (immunoprecipitated by anti-Flag antibody) were significantly increased when SPOP-wt was also expressed, whereas expression of any prostate adenocarcinoma–associated SPOP mutant effectively inhibited the accumulation of Ub-AR. Furthermore, the SPOP C-terminal fragment (a.a. 172–374) had no effect on the ubiquitination state of AR protein (Fig. 4A). These data are consistent with the fact that this fragment lacks the MATH domain and did not bind to the AR protein (Fig. 2C). We also coexpressed a Cullin-3 dominant negative (DN) construct (CUL-DN, truncated Cullin-3 containing the SPOP binding domain but defective in RBX-1 recruitment) together with SPOP-wt and AR in 293T cells and observed that CUL-DN efficiently rescued the depletion of AR protein levels caused by SPOP-wt (Fig. 4B). Collectively, these observations suggest that SPOP serves as an adaptor protein for the Cullin-3 E3 ubiquitin ligase complex to promote ubiquitination of AR protein.

![Figure 2](image_url)
SPOP directly binds the AR protein through an SBC motif in the hinge region of AR

Because wt-SPOP (but not its prostate adenocarcinoma–associated mutants) can bind the AR coactivator SRC-3 (36), we determined whether SRC-3 mediates or facilitates the interaction of wt-SPOP with AR. We coexpressed AR and SPOP-wt in 293T cells and HeLa cells (both express SRC-3) and in a HeLa SRC-3 KO subclone, that lacks SRC-3 expression (41). We found that SPOP-wt could effectively suppress AR protein levels and coimmunoprecipitate with AR in all three cell lines (Supplementary Fig. S7), suggesting that the interaction between SPOP-wt and AR can occur even in the absence of SRC-3 protein. This led us to examine whether SPOP-wt can bind AR directly. SPOP recognizes and binds to its substrates at
a specific serine/threonine-rich peptide motif (SPOP Binding Consensus motif, SBC; ref. 35). Our bioinformatics analysis of AR protein sequence identified an SBC motif within its hinge region, 646-ASSTT-650 (Supplementary Fig. S8A), which is located in the PEST sequence previously identified (44). Mutations in this motif segment have been frequently reported in vitro and this activity is abrogated by the prostate adenocarcinoma–associated SPOP mutations. A, 293T cells were cotransfected with pcDNA3-HA-human ubiquitin (1 μg) and pcDNA3-AR-Flag (1 μg), together with same amount (1 μg) of pcDNA3.1 expression vectors for Wt-SPOP or its prostate adenocarcinoma–associated mutated variants (SPOP-F102C, SPOP-F133V, SPOP-F125V, SPOP-119N, SPOP-Y76G, SPOP-Y87N) or SPOP-C terminal fragment (a.a. 172–374, lacking the MATH domain), respectively. Anti-Flag antibody was used to immunoprecipitate AR protein and anti-HA-HRP antibody was used to visualize the ubiquitinated AR by immunoblot analysis. To detect the AR protein (input) in cell lysate samples, anti-AR antibody (Santa Cruz Biotechnology) was used. The levels of ubiquitinated AR protein were significantly increased when SPOP-wt was also expressed, whereas expression of any prostate adenocarcinoma–associated SPOP mutant effectively inhibited the accumulation of Ub-AR. Furthermore, the SPOP C-terminal fragment (a.a. 172–374) had no effect on AR ubiquitination. B, overexpression of Cullin-3 dominant negative (DN) efficiently rescued the depletion of AR protein levels caused by SPOP-wt. 293T cells were cotransfected with pcDNA3-AR-Flag (0.5 μg), and pcDNA3.1 (empty vector control) or pcDNA3.1-HA-SPOPwt, together with pcDNA3 (empty vector control) or pcDNA3-HA-CUL3-DN vector, respectively. The cells were collected and lysed for immunoblot analysis to detect the expression of AR protein (Flag-tagged AR), Cullin-3 DN (HA-tagged CUL3-DN), and β-actin.

We confirmed that point mutations in this AR SBC motif, specifically A646D, S647F, S648N, and STT648/649/650AAA, can abolish the affinity of AR for SPOP-wt upon coexpression in 293T cells (Supplementary Fig. S8B). Moreover, ARv7, a naturally occurring C-terminal–truncated AR variant that lacks the SBC motif, did not bind SPOP-wt upon coexpression in 293T cells (Supplementary Fig. S8C). These data strongly support the functional involvement of this AR SBC motif to the recognition and binding of AR protein by SPOP-wt.

**Endogenous SPOP regulates AR protein levels in prostate adenocarcinoma cells**

SPOP is ubiquitously expressed in prostate adenocarcinoma cell lines (36). We next examined the interaction of endogenous SPOP (wt) with AR protein and the role of SPOP in regulating AR expression in a panel of prostate adenocarcinoma cells. We found that in LNCaP, Abl, VCaP, and LAPC4 prostate adenocarcinoma cells, AR protein coimmunoprecipitated with endogenous SPOP (wt) by anti-SPOP–specific antibody (Supplementary Fig. S9A). As expected, no signal was found in AR-negative PC-3 and DU145 control prostate adenocarcinoma cells. Moreover, by silencing endogenous SPOP by siRNA transfection (three different SPOP siRNAs) in LNCaP and Abl cells, we induced significant increase of AR protein levels in these cells (Supplementary Fig. S9B). The AR mRNA levels did not change to a degree that could explain the increase in AR protein (Supplementary Fig. S9C and S9D), suggesting that the increase in AR protein expression is mainly posttranslational. These observations confirm that endogenous SPOP functions as an adaptor for the E3 ligase Cullin-3/RBX-1 complex to promote AR protein ubiquitination and eventually, degradation. We also examined the impact of silencing SPOP on AR protein levels in 22Rv1 cells, that endogenously express both full-length (FL) AR and its splice variant ARv7. All three SPOP siRNAs increased both the FL AR protein form (as expected) as well as, unexpectedly, ARv7 (Fig. 5A). Neither AR FL nor ARv7 mRNA was increased by silencing SPOP (Supplementary Fig. S10), again supporting a posttranslational mechanism of action. Moreover, exogenous expression of SPOP-wt in 22Rv1 cells suppressed the protein expression of both FL AR and ARv7 (Fig. 5B). As these results apparently contradicted our prior findings from transient expression of ARv7 in 293T cells (Supplementary Fig. S8C), we hypothesized that, in 22Rv1 cells, an indirect mechanism may allow the interaction of SPOP-wt with ARv7, despite the absence of the SBC that we characterized above (Supplementary Fig. S8). Specifically, we hypothesized that ARv7 may be able to interact with SPOP-wt via heterodimerization with AR-FL. To explore this hypothesis, we examined the in vitro recruitment of ARv7 to SPOP-wt with or without concurrent presence of AR-FL in 293T cells. In 293T cells (that lack endogenous AR), when ARv7 is expressed in the absence of AR-FL, its expression in unaffected by SPOP-wt (Fig. 5C) and it cannot interact with SPOP-wt (Fig. 5D). However, ARv7 protein could interact with SPOP-wt when coexpressed with AR-FL (Fig. 5D). This observation helps explain our findings in 22Rv1 cells and provides an alternative, indirect mechanism for SPOP-wt to regulate ubiquitination and post-translational degradation of ARv7 in prostate adenocarcinoma.
Figure 5. Endogenous SPOP exerts posttranslational regulation of AR-FL and ARv7 expression levels in prostate adenocarcinoma cells. A, 22Rv1 cells were transfected with nontargeting siRNA (si-NT) and three different SPOP siRNAs (A–C) and incubated for 48 hours. At the end of treatment, cell lysates were prepared and immunoblot analyses were conducted for the expression levels of AR-FL, AR-v7, SPOP, and β-actin in the lysates. Compared with the nontargeting siRNA control (siNT), all three SPOP siRNAs induced significant increase of AR-FL and AR-v7 protein levels in these cells. B, 22Rv1 cells, engineered to express, under a tetracycline-inducible promoter, wt-SPOP, were induced with 500 ng/mL of doxycycline (Dox) for 24 hours. Cell lysates were prepared and immunoblot analyses were conducted for the expression levels of AR-FL, ARv7, SPOP, and β-actin in the lysates. Ectopic expression of SPOP-wt in 22Rv1 cells suppressed the expression of both full-length AR and AR-v7 proteins. C–G, SPOP-wt can indirectly regulate ubiquitination and proteasomal degradation of ARv7 through AR-FL/ARv7 heterodimers. C, 293T cells were cotransfected in 6-well plates with 1 μg pcDNA3-AR-FL-Flag or pcDNA3-ARv7-Flag, with increasing amount of pcDNA3.1-HA-SPOP wt (0, 0.8, 1.5 μg), respectively. Thirty-six hours after transfection, cells were harvested and immunoblot analysis was conducted as indicated. We conclude that in 293T cells (that lack endogenous AR), when ARv7 is expressed in the absence of AR-FL, its expression is unaffected by SPOP-wt. D, 293T cells were cotransfected with 10 μg pcDNA3.1-HA-SPOPwt and (i) 10 μg pcDNA3-AR-FL-Flag; or (ii) 10 μg pcDNA3.1-ARv7-Flag; or (iii) 5 μg pcDNA3-AR-FL (no Flag) together with 5 μg pcDNA3-ARv7-Flag. The transfected cells were treated with 250 nmol/L of bortezomib (PS-341) for another 8 hours and the lysates were used for coimmunoprecipitation/immunoblot analysis as in Fig. 2B. In 293T cells (that lack endogenous AR), when ARv7 is expressed in the absence of AR-FL, it cannot interact with wt-SPOP. However, AR-v7 protein interacted with wt-SPOP when coexpressed with AR-FL, suggesting that the interaction between ARv7 and wt-SPOP may be mediated through the formation of AR-FL/ARv7 heterodimers. E and F, models of posttranslational regulation of AR via ubiquitination by the SPOP/Cullin-3/RBX-1 E3 ligase complex. F, direct model: The substrate-binding pocket of the MATH domain of SPOP-wt binds to the SBC motif (a.a. 646–651) within the hinge region of AR-FL. Eventually, the ubiquitinated AR protein is routed for degradation through the proteasome pathway. This interaction is abrogated by the prostate adenocarcinoma-associated SPOP mutations. G, indirect model: ARv7 (lacking the SBC due to alternative splicing) can bind to SPOP-wt indirectly, by forming heterodimers with AR-FL.
cells, despite the absence of the SBC, and possibly through the formation of AR-FL/ARv7 heterodimers (Fig. 5E and F).

**Mutant SPOP promotes in vivo prostate adenocarcinoma growth in immunocompromised mice**

We xenografted subcutaneously, in the flank of SCID-Beige male mice, Abl cells engineered to express, under a tetracycline-inducible promoter, wt-SPOP or SPOP-F102C. The mice were fed with water containing doxycycline (200 µg/mL) starting the day after cell injection, and kept on doxycycline for the duration of the experiment. We observed that Abl-SPOP-F102C xenografts grew significantly faster (Fig. 6A, \( P < 0.0001 \)) and expressed more AR protein (Fig. 6B) than Abl-SPOP-WT xenografts, supporting the role of mt-SPOP as a prostate adenocarcinoma oncogene. There was no difference in the % tumor take rate between the SPOP alleles (100% in both cases); there was only delay in the growth of the xenografts expressing wt compared with the mt-SPOP.

**Arbitrary effect of mt-SPOP in the context of AR axis inhibition**

LNCaP cells expressing the prostate adenocarcinoma-associated SPOP mutants proliferated faster than cells expressing wild-type SPOP. This was observed under regular growth conditions (medium supplemented with 10% FBS, which contains basal androgen levels), as well as under androgen deprivation (medium supplemented with 10% CSS). However, the separation of the growth curves was more pronounced under androgen deprivation conditions, consistent with a more significant contribution of mt-SPOP to cell proliferation under androgen deprivation conditions (Supplementary Fig. S11A and S11B). Similarly, our data suggest that the presence of SPOP mutations may contribute to partially decreased sensitivity to enzalutamide (Supplementary Fig. S11C).

**Correlation of SPOP signature score with AR activity score in prostate adenocarcinoma patient cohorts**

Finally, we investigated the relationship between the transcriptional output of the SPOP pathway and the androgenic activity in prostate adenocarcinoma specimens, using previously published datasets from four large patient cohorts. We applied the established method of computing a gene signature \( z \)-score for each sample, as previously described (8), by adding \( z \)-scores for upregulated genes and subtracting \( z \)-scores for downregulated genes. Specifically, we computed a sum of \( z \)-scores for all the genes in the SPOP mutation gene signatures (F133V, F133L, F102C, and combined mutants), and for all the genes in two androgen-induced signatures: Hieronymus and colleagues (AR activity score 1; ref. 43) and Nelson and colleagues (AR activity score 2; ref. 42). In all four patient cohorts, we found a strong positive correlation between the SPOP signature score and the AR activity scores (Fig. 7 and Supplementary Fig. S12).

**Discussion**

The somatic heterozygous missense mutations in the substrate-binding pocket of SPOP, that frequently occur in human prostate adenocarcinomas, are rare to absent in other malignancies (endometrial carcinomas harbor SPOP mutations, but not in the substrate-binding pocket). This observation suggests that the role of SPOP mutations in prostate adenocarcinoma involves a change in SPOP affinity for a prostate-specific substrate. Several substrates have been reported for SPOP, including the death domain–associated protein Daxx (33), the phosphatase Puc, the transcriptional regulator Ci/Gli (34), the variant histone MacroH2A (35), and the key AR coactivator Steroid Receptor Coactivator (SRC)-3 (36–37), yet none of them is restricted to the prostate and it is unclear how they could...
explain a prostate-specific mechanism of action for SPOP mutations. Through its participation in the AR axis, SRC-3 is a plausible candidate for this role, but it also is an important oncogene in other malignancies (including breast carcinomas; refs. 46, 47) where the SPOP substrate-binding pocket is not mutated. Therefore, the relative contribution of the reported SPOP substrates to the pathophysiology of SPOP-mutant (mt) prostate adenocarcinoma remains unknown.

For this reason, we interrogated, by microarray, the transcriptomic profiles of AR(+) prostate adenocarcinoma cells engineered to express wild-type (wt)-SPOP or three different mt-SPOPs (F133V, F133L, and F102C). First, the gene signatures of all mt-SPOPs overlapped highly with each other, suggesting that the transcriptional footprint of the three mutants tested is fairly identical. This is in agreement with experimental data from our group (Figs. 2 and 3 and ref. 36) that all prostate adenocarcinoma–associated SPOP mutations disrupt SPOP binding to substrate, and with bioinformatics predictions of the impact of these mutations on SPOP function (30). An unbiased search for gene sets that are enriched in genes upregulated by mt-SPOP revealed that the gene signatures most closely related to those of mt-SPOPs were signatures derived from androgen treatment of prostate adenocarcinoma samples from the Cai and colleagues cohort (GSE32269; R range: 0.87–0.94, all comparisons P < 0.001; ref. 51). B, relative distribution of SPOP mutation scores and androgen activity scores over metastatic prostate adenocarcinoma samples from the Cai and colleagues cohort (GSE32269; R range: 0.75–0.87, all comparisons P < 0.001; ref. 51).

Figure 7. SPOP gene signature scores in human prostate adenocarcinoma specimens correlate strongly with androgen receptor activity. A and B, we computed a sum of z-scores for all the genes in the SPOP mutation gene signatures (F133V, F133L, F102C, and combined mutation signature) and for all the androgen-dependent genes [per two androgen-response datasets: Hieronymus and colleagues (AR activity score 1; ref. 43) and Nelson and colleagues (AR activity score 2; ref. 42)]. A, relative distribution of SPOP gene signature scores and androgen activity scores over primary prostate adenocarcinoma samples from the Cai and colleagues cohort (GSE32269; R range: 0.87–0.94, all comparisons P < 0.001; ref. 51). B, relative distribution of SPOP mutation scores and androgen activity scores over metastatic prostate adenocarcinoma samples from the Cai and colleagues cohort (GSE32269; R range: 0.75–0.87, all comparisons P < 0.001; ref. 51).
(approved for prostate adenocarcinoma treatment or experimental agents) again confirmed that the transcriptional footprints of mt-SPOPs are highly anticorrelated with the signatures of AR antagonists: bicalutamide, enzalutamide (MDV3100), and ARN-509 (48). Specifically, genes upregulated by the AR antagonists are downregulated by mt-SPOPs, whereas genes downregulated by the AR antagonists are upregulated by mt-SPOPs. The gene signatures of mt-SPOPs had only limited or no overlap with the signatures of drugs that are not direct AR antagonists. Collectively, these results provide the first evidence that ranks the AR axis as the top transcriptional output of the tumor suppressor SPOP in prostate adenocarcinoma.

We previously reported that wt-SPOP binds to and promotes the turnover of SRC-3 protein, which suppresses the capacity of SRC-3 to function as an AR coactivator, and the tumor suppressor effects of wt-SPOP are abrogated by the prostate adenocarcinoma–associated SPOP mutations (36). In the present studies, we determined the relative contribution of SRC-3 versus AR to the transcriptional output of mt-SPOPs. We silenced SRC-3 or AR in LNCaP cells via siRNA and generated corresponding gene expression profiles. We also utilized two publicly available signatures of androgen-treated prostate adenocarcinoma cells (42, 43). GSEA analysis revealed that the transcriptional footprint of mt-SPOPs enriches for SRC-3–dependent genes, confirming the importance of SRC-3 that we documented in our prior study (36). Interestingly, we also identified that the gene signatures of mt-SPOPs showed higher enrichment for genes regulated by AR and androgen than for genes regulated by SRC-3. This raised the hypothesis that, in addition to its SRC-3–mediated effects, SPOP also exerts important SRC-3–independent/AR-mediated functions. This led us to evaluate the impact of SPOP on AR itself.

Our biochemical studies revealed that wt-SPOP can bind AR directly and promote its ubiquitination and degradation. We confirmed these results in both androgen-dependent (LNCaP, VCaP, LAPC4) and androgen-independent (Abl, 22Rv1) cells. We identified a SBC motif in the hinge region of AR that is recognized directly by SPOP. This binding activity is abrogated by the prostate adenocarcinoma–associated SPOP mutations, leading to AR stabilization, AR-mediated signaling, and increased cell proliferation. In agreement, we found that the alternatively spliced variant AR-v7, that lacks the SPOP-binding hinge region, cannot directly bind wt-SPOP when expressed into 293T cells. While our manuscript was in its final stage of preparation, An and colleagues. (49) also reported the presence of an SBC motif in the hinge region of AR and found that wt-SPOP can regulate the expression of full-length AR but not truncated variants that lack the hinge region. However, in our extensive studies of 22Rv1 prostate adenocarcinoma cells, that express both full-length AR and constitutively active variant AR-v7, we documented that three different SPOP siRNAs increased the protein levels of both full-length and variant AR-v7. Moreover, ectopic expression of wt-SPOP suppressed the protein levels of both full-length AR and variant AR-v7 in 22Rv1 cells. This apparent contradiction was explained when we cotransfected both full-length AR and variant AR-v7 cDNA, together with SPOP-wt cDNA, into 293T cells. We found that AR-v7 can be coimmunoprecipitated with wt-SPOP only if full-length AR is also present. Our findings suggest a model where wt-SPOP can interact with AR-v7 via AR full-length/AR-v7 heterodimers (which have been previously proposed to exist in prostate adenocarcinoma; ref. 50). Moreover, our animal studies demonstrated that xenografts of prostate adenocarcinoma cells expressing mt-SPOP express more AR protein and grow significantly faster in immunocompromised mice than prostate adenocarcinoma cells expressing wt-SPOP. We also found that the prostate of hemizygous Spop knockout mice express more AR protein compared to age-matched wild-type mice. Finally, examination of several publicly available human prostate adenocarcinoma specimen datasets further illustrated the strong link between the transcriptomic outputs of mt-SPOPs and AR. Collectively, our data establish the role of mt-SPOP in AR regulation and prostate adenocarcinoma growth in vivo. We conclude that wt-SPOP is an important tumor suppressor in prostate cells, and mt-SPOP is an oncogenic driver in prostate adenocarcinoma. SPOP regulates the stability of two key components of the AR axis: AR itself and its coactivator SRC-3. We determined that the AR axis is the main transcriptional output of the tumor suppressor SPOP in prostate adenocarcinoma. Our studies provide an explanation why mutations in the substrate-binding pocket of SPOP occur frequently and in a prostate adenocarcinoma–specific manner and enhance our understanding of the pathophysiology of this common prostate adenocarcinoma genotype. Induction of expression of wt-SPOP in prostate adenocarcinoma cells would deplete AR (full-length and constitutively active splice-variants) as well as its coactivator SRC-3, extinguish AR signaling and inhibit prostate adenocarcinoma growth, leading to significant therapeutic implications. Taken together, these data support the rationale to further explore the regulation of expression of the tumor suppressor SPOP in prostate adenocarcinoma.

Finally, our experimental results have raised the possibility that, while all prostate adenocarcinoma–associated SPOP mutants are associated with a loss of AR suppression (loss-of-function effect), certain SPOP mutants (e.g., F102C) may actually enhance AR signaling above baseline (i.e., no exogenous SPOP) levels, thus exerting a “gain-of-function” oncogenic effect. This phenomenon, which could be attributed to a putative “dominant-negative” effect of mutant SPOP on the function of wt-SPOP, may acquire particular importance because SPOP mutations are always heterozygous in prostate cancer specimens. Therefore, our data hint to possible functional differences between the various SPOP mutants regarding their oncogenic potential and even prognostic significance, which, obviously, will need to be validated in clinically annotated human prostate cancer specimens.

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

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References


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