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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 (PC), 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 factor SRC-3. However, the relative contributions that SPOP substrates may contribute to the pathophysiology of SPOP-mutant (mt) PC is unknown. Using an unbiased bioinformatics approach, we determined that the gene expression profile of PC cells engineered to express mt-SPOP overlaps greatly with the gene signature of both SRC-3 and AR transcriptional output, with a stronger effect on 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 PC-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 PC cells expressing mt-SPOP expressed higher AR protein levels and grew faster than tumors composed of PC cells expressing wt-SPOP. Further, genetic ablation of SPOP was sufficient to increase AR protein levels in mouse prostate. Examination of public human PC 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 PC, and they provide an explanation for the prostate-specific tumor suppressor role of wt-SPOP.

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Keywords

SPOP; prostate cancer; androgen receptor; SRC-3

INTRODUCTION

The androgen receptor (AR) is a critical driver of prostate adenocarcinoma (PC) pathophysiology, regulating proliferation, metabolism and migration, and is also a validated therapeutic target (1). The importance of the AR axis in PC is further illustrated by the frequent overexpression, especially in the state of castration-resistant PC (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 non-canonical 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 PC. There is compelling evidence that even taxanes, the only family of cytotoxic chemotherapeutics that has ever demonstrated an overall survival benefit in PC, exert anti-cancer 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 non-synonymous point mutations in PC (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 PC 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 PC pathophysiology. We previously reported that wt-SPOP plays a critical tumor suppressor role in PC 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 PC-associated SPOP mutations (36). SRC-3 promotes PC 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) PC has not been fully elucidated.

In the present study, we examined the global gene expression profiles of AR(+) PC 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 PC 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

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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 PC-associated SPOP mutations, leading to AR stabilization and increased cell proliferation. Xenografts of PC 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 PC 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 PC and provide an explanation why mutations in the substrate-binding pocket of SPOP occur frequently and in a PC-specific manner.

MATERIALS AND METHODS

Reagents and Antibodies

Enzalutamide (MDV3100) was kindly provided from Medivation (San Francisco, CA). The antibodies used were: mouse monoclonal anti-Flag M2 (Sigma), anti-SPOP (Abcam, Cambridge, MA), rabbit polyclonal anti-AR (Cell Signaling), rabbit anti-AR (Santa Cruz), 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 cells lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA) via the Tissue and Cell Culture Core Laboratory at Baylor College of Medicine, 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 Dulbecco's modified Eagle's medium (DMEM) high glucose (Life Technologies, Carlsbad, CA) with 10% fetal bovine serum (FBS, Life Technologies) in a 5% CO_2 incubator at 37°C; LNCaP and 22Rv1 cells were cultured in Roswell Park Memorial Institute medium 1640 (RPMI1640, Life Technologies) supplemented with 10% FBS; LAPC4 cells were cultured in Iscove's Modified Dulbecco's Media (IMDM, Life Technologies) plus 15% FBS, 1 nM R1881 and 2 mM 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 nM R1881. The human CRPC cell line Abl (characterized by and obtained from Dr. Zoran Culig, Innsbruck Medical University (19)), was maintained in RPMI 1640 medium (Life Technologies) supplemented with 10% charcoal-stripped fetal bovine serum. 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. 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., Atlanta, GA) and

300 μg/ml G-418 (Life Technologies). Additional PC 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 Supplemental Methods.

RESULTS

The F102C, F133V, and F133L SPOP mutants result in similar transcriptomic responses in PC *cells*

We performed gene expression profiling of Abl PC cells engineered to express the PC-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 Supp. Fig. 1), which are very distinct (essentially inverted) from the effects of the wt-SPOP (Fig. 1A).

Gene set enrichment analysis using the Molecular Signature Database identifies the AR transcriptional output as the top enriched gene set in PC 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 to 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_RESPOSE_TO_ANDROGEN_UP”, which corresponds to genes up-regulated by androgen in PC 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 PC cells. Three out 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 PC sample, we confirmed that, compared to control vector, cells expressing wt-SPOP exhibit a lower AR Activity Score, while mt-SPOPs (F102C, F133V, and F133L) increase the AR Activity Score (Fig. 1C). Similar results were obtained using another androgen-induced signature (43) as well (Suppl. Fig. 2). We also expanded our analysis using a wider panel of SPOP mutants (including also Y87C, Y87N, S119N, F125V, and W131G) and demonstrated by RTqPCR that they, too, have partially or completely lost the capacity of wt-SPOP to suppress the expression of the AR-dependent genes SGK1, CAMKK2, ABCC4, HOMER2, SEPP1 and NKX3.1 (Suppl. Fig. 3). 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.

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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 PC treatment. The genes up-regulated by mt-SPOPs (over wt-SPOP samples) were highly enriched for genes down-regulated by direct AR antagonists, such as enzalutamide, bicalutamide, ARN-509, and compound 30. Inversely, the genes down-regulated by mt-SPOPs were highly enriched for genes up-regulated by the AR antagonists (Suppl. Fig. 4). Other drugs, including several that have been proposed to have indirect effects on AR (such as HDAC inhibitors, cardiac glycosides and docetaxel), enriched less robustly.

The gene signatures of mt-SPOPs enrich for genes induced by SRC-3 and androgen

We investigated the enrichment of SRC-3-regulated genes in our mt-SPOP signatures using the GSEA method. SRC-3-dependent genes (genes down-regulated by SRC-3 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 PC-associated SPOP mutants post-translationally stabilize SRC-3 (36). We also evaluated the enrichment of androgen-induced genes, utilizing our own gene expression signature from PC cells treated with AR siRNA (genes down-regulated by AR siRNA) and previously published gene signatures from androgen-treated PC cells (42–43). We discovered that androgen/AR-induced genes are highly enriched in the SPOP mutant signatures, with NES ranging from 1.88 to 2.71 ($q<0.001$). The higher NES observed for the AR-dependent genes, compared to the SRC-3-dependent genes, indicate that SPOP mutants promote AR-mediated signaling that extends beyond what can be explained purely by their impact on SRC-3 stabilization (Fig. 1D). This led us to examine in more detail the impact of SPOP on regulation of AR itself.

SPOP directly regulates AR protein stability and this activity is abrogated by the PC-associated SPOP mutations

We transiently co-expressed in 293T cells AR and SPOP (WT or the PC-associated SPOP mutants: SPOP-Y87N, SPOP-Y87C, SPOP-F102C, SPOP-S119N, SPOP-F125V, SPOP-W131G, SPOP-F133L, and SPOP-F133V). Immunoblot analyses revealed that the expression of AR protein itself was strongly suppressed in the presence of SPOP-wt, but not by the PC-associated SPOP mutants (Fig. 2A). We next determined whether wt-SPOP can interact with AR protein. Co-immunoprecipitation experiments revealed that SPOP-wt, but not the PC-associated SPOP mutants, can interact with the AR protein (Fig. 2B). This suggested that AR is a substrate for SPOP-wt, and that the mutations in the substrate-binding pocket of the MATH domain of SPOP abolish its interaction with AR. In agreement, while the C-terminal fragment of wt-SPOP (a.a.172–a.a.374, containing the BTB domain) failed to bind to the AR protein, the N-terminal fragment (a.a.1–a.a.172) of wt-SPOP, containing the MATH domain and its substrate-binding pocket, efficiently co-immunoprecipitated with AR protein *in vitro* (Fig. 2C). These observations indicate that SPOP-wt can bind to AR and that the AR-SPOP interaction is critically dependent on the SPOP substrate-binding cleft of the MATH domain. In order to further dissect the impact of the tumor suppressor SPOP on AR

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expression in PC cells, we examined AR protein expression in Abl PC cells engineered to express, under a tetracycline-inducible promoter, SPOP-wt or the PC-associated SPOP mutants. Immunoblot analyses revealed that, upon induction with doxycycline, SPOP-wt, but not the PC-associated SPOP mutants, significantly suppressed AR protein expression in Abl PC 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 RT-qPCR revealed that SPOP-wt did not suppress the expression of AR mRNA in these cells, suggesting that the impact of SPOP on AR protein levels is most likely post-translational (Suppl. Fig. 5). Similar results were obtained from ligand-dependent LNCaP and VCaP cells (Suppl. Fig. 6).

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 PC-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 ~500 min (Fig. 3B). Expression of SPOP-wt destabilized and dramatically shortened the half-life of AR protein to ~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 co-immunoprecipitation. 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 co-immunoprecipitate 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 PC cells.

SPOP promotes ubiquitination of AR protein and this activity is abrogated by the PC-associated SPOP mutations

SPOP functions as an adaptor protein that facilitates the recruitment of substrates to the Cullin-3/RBX-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 co-expressed AR (Flag-tagged) and HA-tagged ubiquitin (Ub) together with SPOP-wt or PC-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 ubiquinated AR protein (immunoprecipitated by anti-Flag antibody) were significantly increased when SPOP-wt was also expressed, whereas expression of any PC-associated SPOP mutant effectively inhibited the accumulation of Ub-AR. Furthermore, the SPOP C-terminal fragment (a.a.172–a.a.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

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not bind to the AR protein (Fig. 2C). We also co-expressed 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.

SPOP directly binds the AR protein through an SBC motif in the hinge region of AR

Because wt-SPOP (but not its PC-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 co-expressed 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 co-immunoprecipitate with AR in all three cell lines (Suppl. Fig. 7) 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) (35). Our bioinformatics analysis of AR protein sequence identified an SBC motif within its hinge region, 646-ASSTT-650 (Suppl. Fig. 8A), which is located in the PEST sequence previously identified (44). Mutations in this motif segment have been frequently reported in patients and are associated with various conditions including partial androgen insensitivity syndrome (PAIS, A646D and S648N), complete androgen insensitivity syndrome (CAIS, A646D), mild androgen insensitivity syndrome (MAIS, A646D) and PC (S647F, S648N and T650A) (45). 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 co-expression in 293T cells (Suppl. Fig. 8B). Moreover, ARv7, a naturally occurring C-terminal truncated AR variant which lacks the SBC motif, did not bind SPOP-wt upon co-expression in 293T cells (Suppl. Fig. 8C). 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 PC cells

SPOP is ubiquitously expressed in PC 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 PC cells. We found that in LNCaP, Abl, VCaP and LAPC4 PC cells, AR protein co-immunoprecipitated with endogenous SPOP (wt) by anti-SPOP specific antibody (Suppl. Fig. 9A). As expected, no signal was found in AR negative PC-3 and DU145 control PC 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 (Suppl. Fig. 9B). The AR mRNA levels did not change to a degree that could explain the increase in AR protein (Suppl. Fig. 9C–D), suggesting that the increase in AR protein expression is mainly post-translational. 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

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both the FL AR protein form (as expected) as well as, unexpectedly, AR-v7 (Fig. 5A). Neither AR FL nor ARv7 mRNA was increased by silencing SPOP (Suppl. Fig. 10), again supporting a post-translational mechanism of action. Moreover, exogenous expression of SPOP-wt in 22Rv1 cells suppressed the protein expression of both FL AR and AR-v7 (Fig. 5B). As these results apparently contradicted our prior findings from transient expression of ARv7 in 293T cells (Suppl. Fig. 8C), 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 (Suppl. Fig. 8). 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 co-expressed 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 proteasomal degradation of ARv7 in PC cells, despite the absence of the SBC, and possibly through the formation of AR-FL/ARv7 heterodimers (Fig. 5E–F).

Mutant SPOP promotes in vivo PC 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 PC 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 to the mt-SPOP.

Genetic ablation of Spop results in increased AR protein levels in the mouse prostate

Prostates of nine-month old hemizygous (SPOP+/−) C57/BL6 mice expressed more AR than SPOP wild-type littermates, providing further evidence that endogenous SPOP regulates AR expression *in vivo* (Fig. 6C).

Oncogenic effect of mt-SPOP in the context of AR axis inhibition

LNCaP cells expressing the PC-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 (Suppl. Fig. 11A–B). Similarly, our data suggest that the presence of SPOP mutations may contribute to partially decreased sensitivity to enzalutamide (Suppl. Fig. 11C).

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Correlation of SPOP signature score with AR activity score in PC patient cohorts

Finally, we investigated the relationship between the transcriptional output of the SPOP pathway and the androgenic activity in PC 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 up-regulated genes and subtracting z-scores for down-regulated 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 the all the genes in two androgen-induced signatures: Hieronymus et al. ((43), AR activity score 1) and Nelson et al. ((42), AR activity score 2). In all four patient cohorts, we found a strong positive correlation between the SPOP signature score and the AR activity scores (Fig. 7 and Suppl. Fig. 12).

DISCUSSION

The somatic heterozygous missense mutations in the substrate-binding pocket of SPOP, that frequently occur in human PCs, 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 PC 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 (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) PC remains unknown.

For this reason, we interrogated, by microarray, the transcriptomic profiles of AR(+) PC 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 ((36) and Fig. 2 and 3 of the current manuscript) that all PC-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 up-regulated by mt-SPOP revealed that the gene signatures most closely related to those of mt-SPOPs were signatures derived from androgen treatment of PC cells (42). Moreover, a secondary, candidate-based GSEA analysis across a panel of gene signatures derived from treating PC cells with various drugs (approved for PC treatment or experimental agents) again confirmed that the transcriptional footprints of mt-SPOPs are highly anti-correlated with the signatures of AR antagonists: bicalutamide, enzalutamide (MDV3100) and ARN-509 (48). Specifically, genes up-regulated by the AR antagonists are down-regulated by mt-SPOPs, while genes down-regulated by the AR antagonists are up-regulated by mt-SPOPs. The gene

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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 PC.

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 PC-associated SPOP mutations (36). In the present studies, we determined the relative contribution of SRC-3 vs 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 PC cells (42–43). GSEA analysis revealed that the transcriptomic 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 PC-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 et al. (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 PC 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 AR 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 co-transfected both full-length AR and variant AR-v7 cDNA, together with SPOP-wt cDNA, into 293T cells. We found that AR-v7 can be co-immunoprecipitated 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 PC (50)). Moreover, our animal studies demonstrated that xenografts of PC cells expressing mt-SPOP express more AR protein and grow significantly faster in immunocompromised mice than PC cells expressing wt-SPOP. We also found that the prostates of hemizygous *Spop* knockout mice express more AR protein compared to age-matched wild-type mice. Lastly, examination of several publicly available human PC 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 PC growth *in vivo*. We conclude that wt-SPOP is

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an important tumor suppressor in prostate cells, and mt-SPOP is an oncogenic driver in PC. 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 PC. Our studies provide an explanation why mutations in the substrate-binding pocket of SPOP occur frequently and in a PC-specific manner and enhance our understanding of the pathophysiology of this common PC genotype. Induction of expression of wt-SPOP in PC cells would deplete AR (full-length and constitutively active splice-variants) as well as its coactivator SRC-3, extinguish AR signaling and inhibit PC 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 PC.

Lastly, our experimental results have raised the possibility that, while all PC-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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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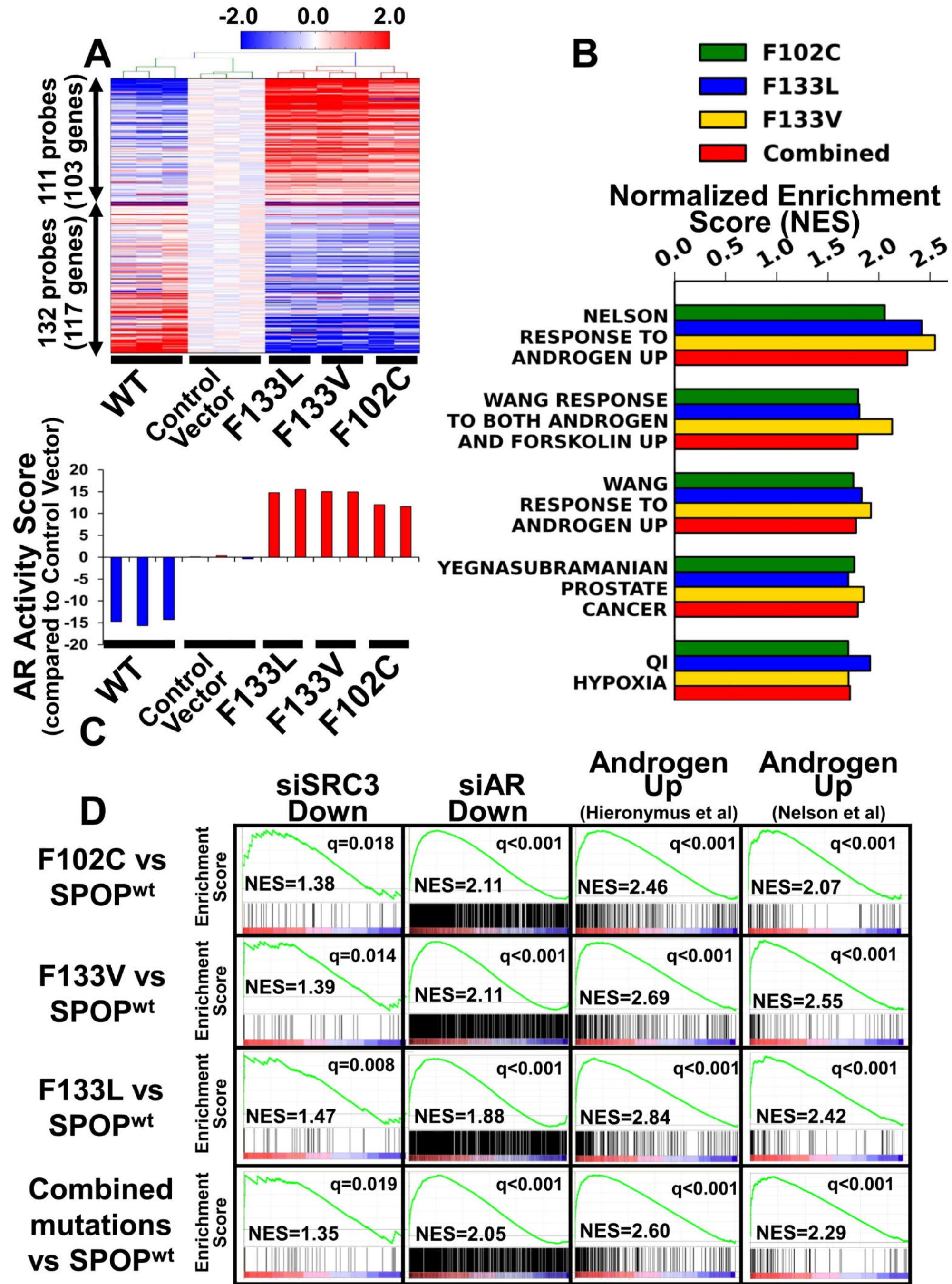

Fig. 1. Global gene expression profiling identifies the AR transcriptional output as the top enriched gene set in PC cells expressing mutant SPOPs

A. Hierarchical clustering of gene expression profiles of Abl PC 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×) demonstrates that all three SPOP mutants have highly similar effects on the PC 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

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sets analyzed in an unbiased fashion, is the “NELSON_RESPOSE_TO_ANDROGEN_UP”. Also, three out of the top five chemical and genetic perturbation (CGP) gene sets in PC correspond to androgen-induced transcriptomic responses. **C.** The AR Activity Score was calculated, based on a previously published gene signature of androgen-stimulated LNCaP cells (42), for Abl cells expressing control vector, wt-SPOP, or mt-SPOP. Compared to control vector, cells expressing wt-SPOP exhibit a lower AR Activity Score, while mt-SPOPs increase the AR Activity Score. **D**. Gene Set Enrichment Analysis (GSEA) revealed that SRC-3-upregulated genes (genes down-regulated 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). 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 down-regulated by AR siRNA or induced by androgen treatment of PC cells).

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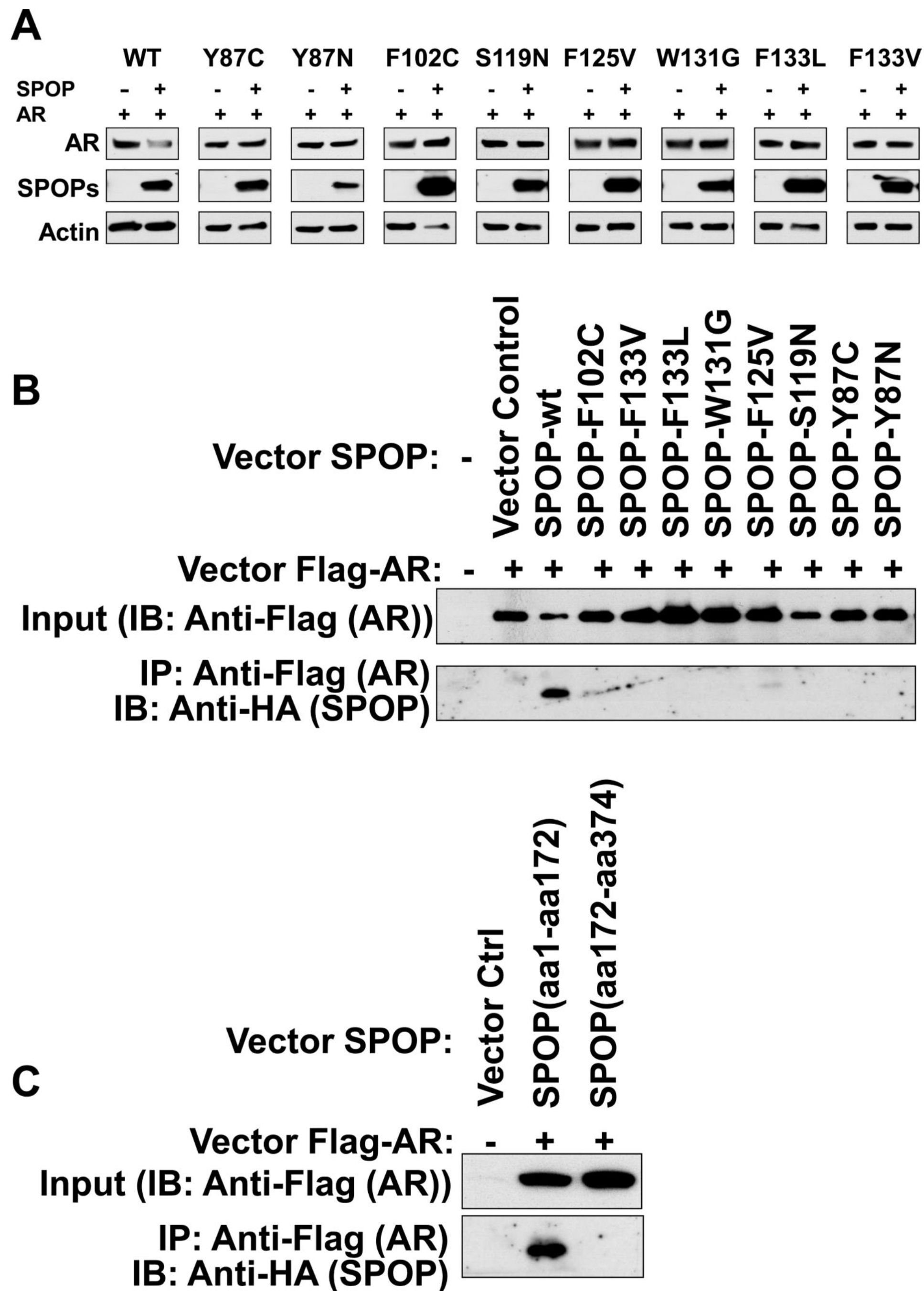

Fig. 2. Wt-SPOP, but not its PC-associated mutants, binds to and promotes degradation of AR protein

A. 293T cells were co-transfected with pcDNA3-AR-FLAG and pcDNA3.1-HA-SPOP-wt or pcDNA3.1-HA-SPOP-mutant expression vectors Forty-eight hours post transfection, cells were collected and the cell lysates were prepared and analyzed by immunoblotting for the expression of Flag-tagged AR (anti-Flag-HRP), HA-tagged SPOP (anti-HA-HRP) and β-Actin. The expression of AR protein was strongly suppressed in the presence of SPOP-wt, but not by its PC-associated mutants. **B**. 293T cells were co-transfected with pcDNA3-AR-

Flag and pcDNA3.1 (vector control) or pcDNA3.1-HA-SPOPwt, pcDNA3.1-HA-SPOP-F102C, pcDNA3.1-HA-SPOP-F133V, pcDNA3.1-HA-SPOP-F133L, pcDNA3.1-HA-SPOP-W131G, pcDNA3.1- HA-SPOP-F125V, pcDNA3.1-HA-SPOP-S119N, pcDNA3.1-HA-SPOP-Y87C, or pcDNA3.1-HA-SPOP-Y87N. The transfected cells were treated with 250 nM of the proteasome inhibitor bortezomib (PS341) for 8 more hrs and the lysates were used for co-IP/immunoblot analysis. Immunoblot analysis revealed that SPOP-wt, but not the PC-associated SPOP mutants, can interact with AR protein. **C**. 293T cells were co-transfected with pcDNA3-AR-Flag and pcDNA3.1 (vector control), pcDNA3.1-HA-SPOP N-terminal (a.a.1–a.a.172) residues, or pcDNA3.1-HA-SPOP C-terminal (a.a.172–a.a.374) residues. Immunoprecipitation with anti-Flag antibody and immunoblotting analysis were conducted as described in (B) and revealed that, while the C-terminal fragment of wt-SPOP (a.a.172–a.a.374, containing the BTB domain) failed to bind AR protein, the N-terminal fragment (a.a.1–a.a.172) of wt-SPOP, containing the MATH domain and its substrate-binding pocket, efficiently co-immunoprecipitated with AR protein *in vitro*.

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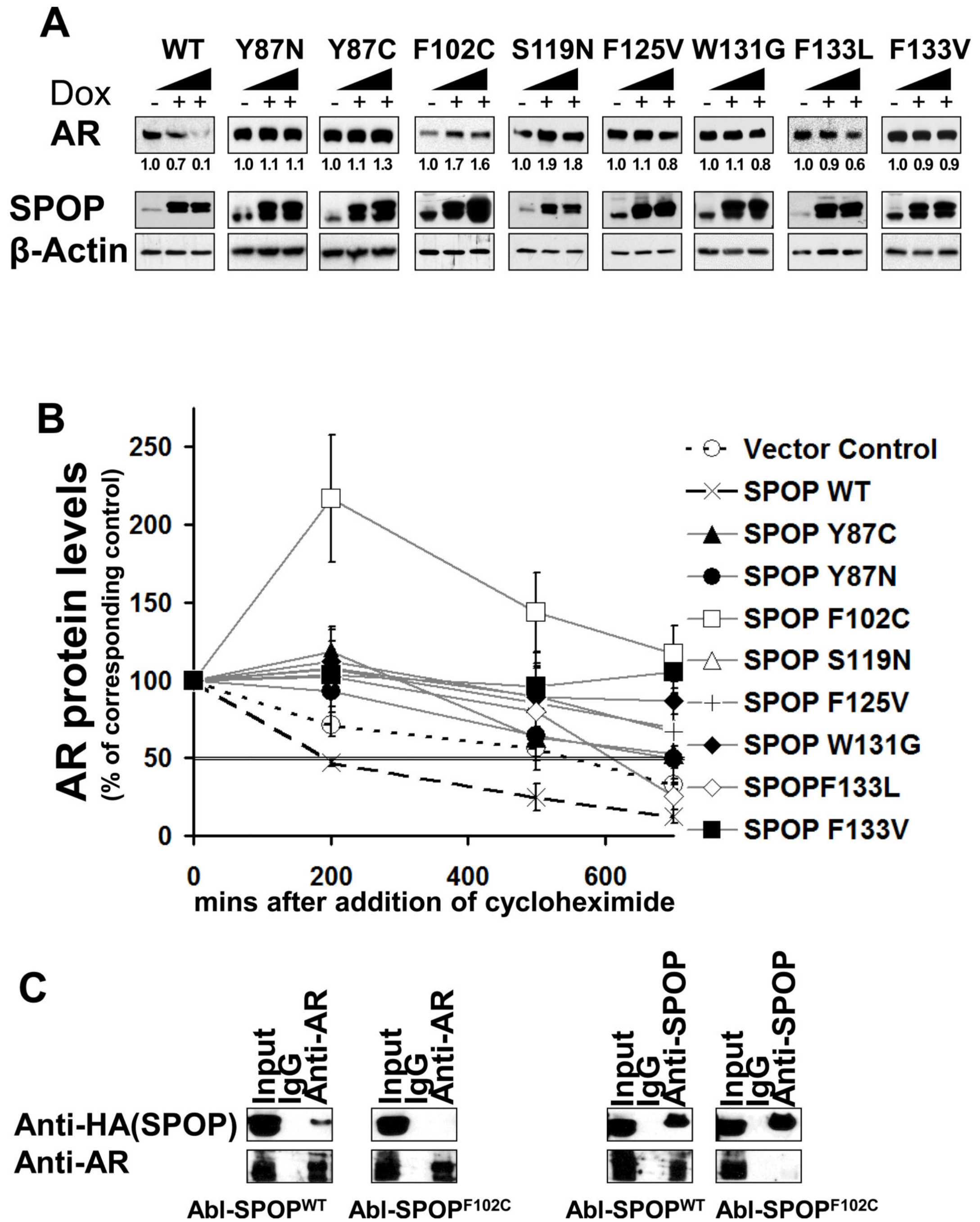

Fig. 3. Ectopic expression of wt-SPOP post-translationally regulates AR in PC cells

A. Abl PC cells engineered to express, under a tetracycline-inducible promoter, SPOP-wt or its PC-associated mutants, were treated with 0, 50 or 500 ng/ml of doxycycline (Dox) for 24 hours. Following this, cells were collected and cell lysates were prepared. Immunoblot analyses were conducted for the expression of AR, SPOP and β-Actin in the cell lysates. SPOP-wt, but not its PC-associated mutants, significantly suppressed AR protein expression in Abl PC cells. The numbers beneath the bands represent densitometry analysis performed on representative blots from each cell line. **B**. Abl PC cells engineered to express, under a

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tetracycline-inducible promoter, SPOP-wt or the PC-associated SPOP mutants, were treated with 200 ng/ml of doxycycline (Dox) for 24 hours. After Dox induction, cells were treated with 100 μg/ml cycloheximide (CHX) for 12 more hours. Cells were collected at 0, 200, 500 and 700 minutes following CHX treatment and cell lysates were prepared. Immunoblot analysis was conducted for the expression levels of AR and β-actin in the cell lysates. The immunoblot signals of AR proteins were quantified and plotted as described in Materials and Methods. AR expression is plotted as % of the respective (time zero) controls. **C**. Abl-SPOPwt and Abl-SPOP-F102C cells were induced with Dox for 24 hours and treated with 250 nM of bortezomib (PS-341) for another 8 hours. At the end of treatment, cell lysates were prepared and utilized for immunoprecipitation with anti-HA antibody or anti-AR antibody. Immunoblot analyses were conducted for the expression levels of HA-SPOP or AR protein in the immunoprecipitates, and detected association between AR protein and SPOP-wt (but not SPOP-F102C) in PC cells.

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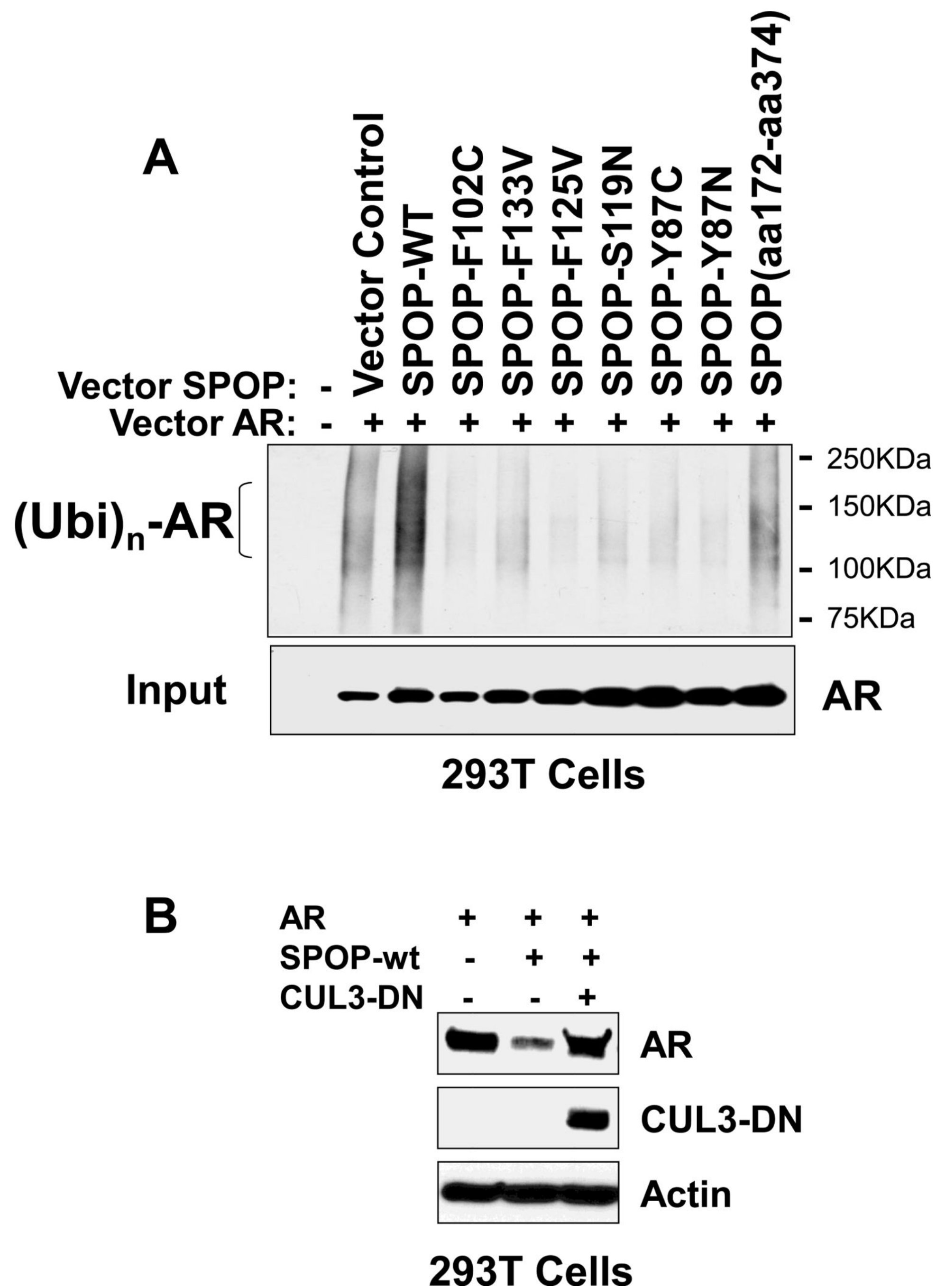

Fig. 4. Wt-SPOP promotes ubiquitination of AR Protein *in vitro* and this activity is abrogated by the PC-associated SPOP Mutations

A. 293T cells were co-transfected 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 PC-associated mutated variants (SPOP-F102C, SPOP-F133V, SPOP-F125V, SPOP-S119N, SPOP-Y87C, SPOP-Y87N) or SPOP-C terminal fragment (a.a.172–a.a.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 Biotech. Inc) was used. The levels of ubiquitinated AR protein were significantly increased when SPOP-wt was also expressed, whereas expression of any PC-associated SPOP mutant effectively inhibited the accumulation of Ub-AR. Furthermore, the SPOP C-terminal fragment (a.a.172–a.a.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 co-transfected with pcDNA3-AR-Flag (0.5μg), and pcDNA3.1 (empty vector control) or pcDNA3.1-HA-SPOPwt, together with pcDNA 3 (empty vector control) or pcDNA 3-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.

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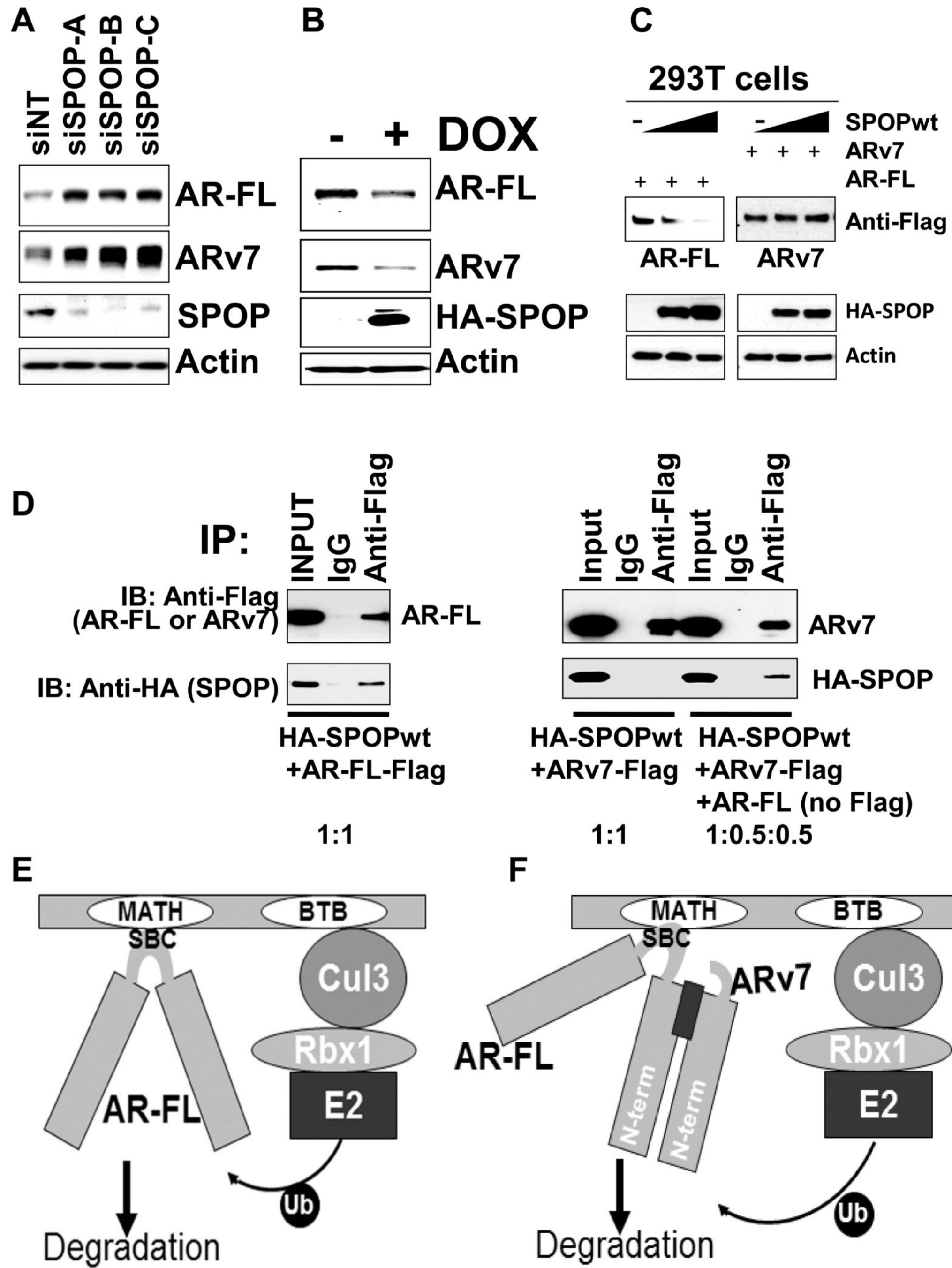

Fig. 5. Endogenous SPOP exerts post-translational regulation of AR-FL and ARv7 expression levels in PC cells

A. 22Rv1 cells were transfected with non-targeting siRNA (si-NT) and three different SPOP siRNAs (A, B, and 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 to the non-targeting 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,

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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 co-transfected 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 post 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 in unaffected by SPOP-wt. **D.** 293T cells were co-transfected with 10 μg pcDNA3.1-HA-SPOPwt and a) 10 μg pcDNA3-AR-FL-Flag; or b) 10 μg pcDNA3.1-ARv7-Flag; or c) 5 μg pcDNA3-AR-FL (no Flag) together with 5 μg pcDNA3-ARv7-Flag. The transfected cells were treated with 250 nM of bortezomib (PS-341) for another 8 hours and the lysates was used for co-IP/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 co-expressed with AR-FL, suggesting that the interaction between ARv7 and wt-SPOP may be mediated through the formation of AR-FL/ARv7 heterodimers. **E–F**. Models of post-translational 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–a.a.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 PC-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.

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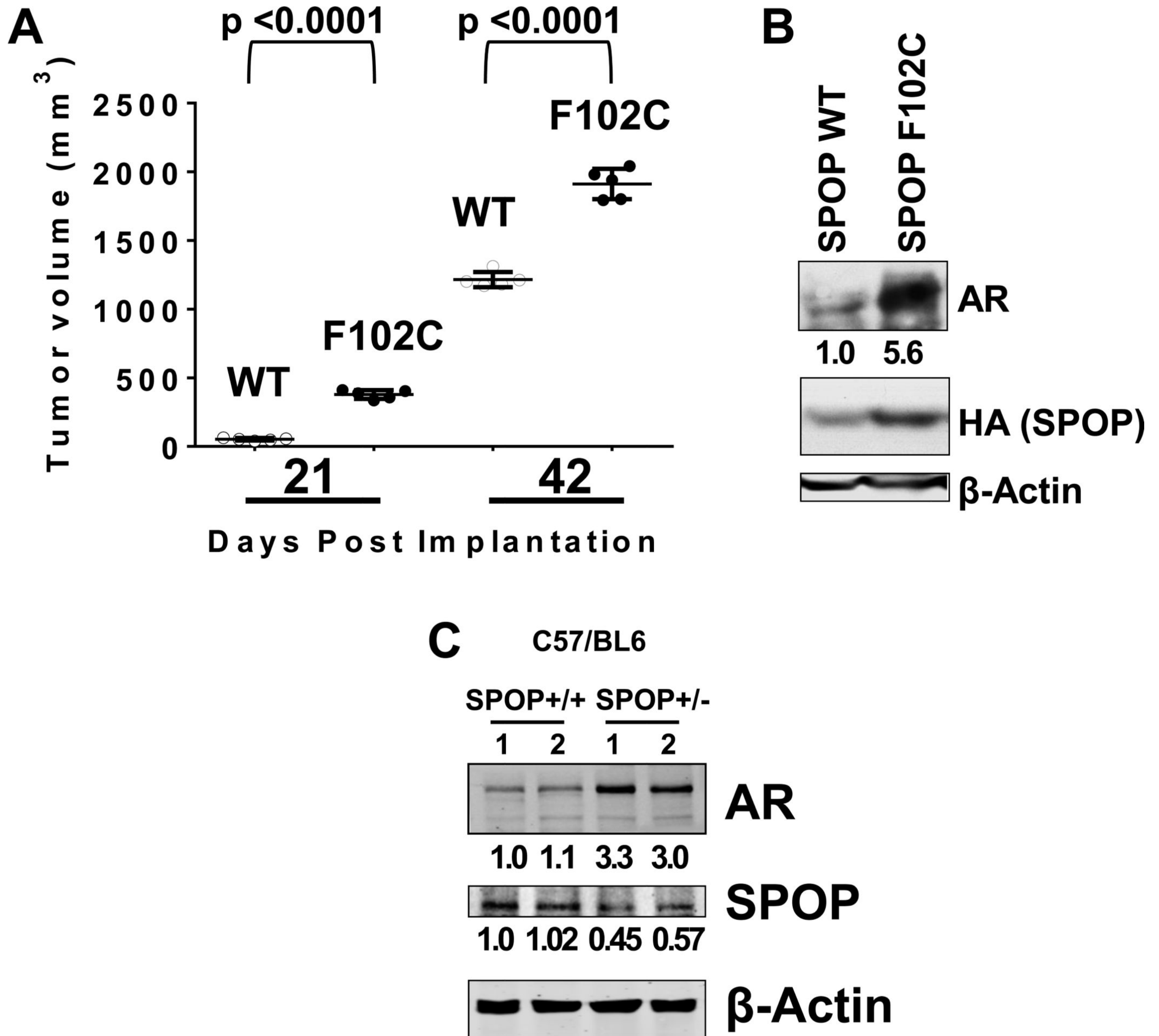

Fig. 6. SPOP regulates AR protein expression *in vivo*: (A–B) Mutant SPOP promotes AR protein and PC growth *in vivo* in immunocompromised mice

A. Two million Abl cells engineered to express, under a tetracycline-inducible promoter, wt-SPOP or SPOP-F102C were mixed with Matrigel and subcutaneously injected in the flank of male SCID-Beige mice (5 mice per cohort). The mice were provided with water containing doxycycline (200 μg/ml) starting one day after cell injection and kept for the duration of the experiment. The y axis depicts tumor volume in mm^3. Comparison between wt-SPOP and SPOP-F102C was analyzed by unpaired t-test. **B**. Tumors from mice described in (A), were excised and cell lysates were prepared. Immunoblot analyses were conducted for the expression levels of AR, HA-tagged SPOP and β-Actin in the tumor lysates. The numbers beneath the bands represent densitometry analysis conducted to quantify the expression of AR and SPOP. **C. Genetic ablation of Spop results in increased**

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AR protein levels in the mouse prostate. Immunoblot analyses were conducted for murine AR, SPOP, and β-Actin in the prostates of nine-month old wild-type and hemizygous SPOP (SPOP+/−) C57/BL6 mice. The numbers beneath the bands represent densitometry analysis to quantify AR and SPOP expression in the prostate lysates.

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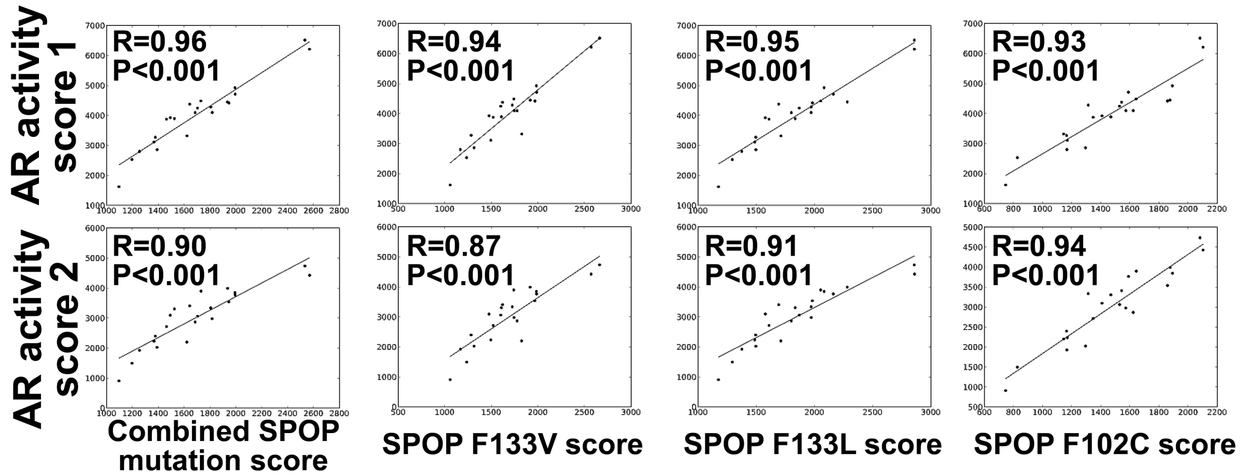

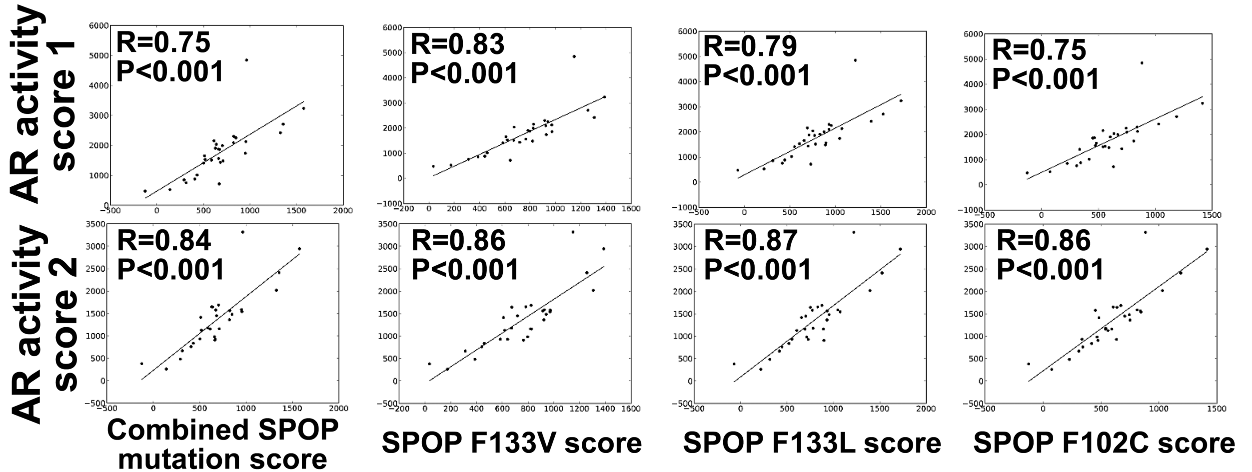

Fig. 7. SPOP gene signature scores in human PC specimens correlate strongly with androgen receptor activity

A–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 *et al* (AR activity score 1) and Nelson *et al* (AR activity score 2)). (A) Relative distribution of SPOP gene signature scores and androgen activity scores over primary PC samples from the Cai *et al* cohort (GSE32269; R range: 0.87–0.94, all comparisons p<0.001). (B) Relative distribution of SPOP mutation scores and androgen activity scores over metastatic PC

samples from the Cai *et al* cohort (GSE32269; R range: 0.75–0.87, all comparisons $p<0.001$).

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