**SPOP Mutation Drives Prostate Tumorigenesis In Vivo through Coordinate Regulation of PI3K/mTOR and AR Signaling**

**Graphical Abstract**

**Highlights**
- Mutations in *SPOP* are driver events resulting in prostate tumorigenesis in the mouse
- SPOP mutation activates PI3K/mTOR signaling through upregulation of SRC3 (NCOA3)
- SPOP mutation maintains AR signaling against PI3K/mTOR-mediated negative feedback
- Mutant SPOP upregulates a network of AR-associated transcription factors

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**In Brief**
Blattner et al. develop a mouse model and use it to demonstrate that human SPOP mutation can drive prostate tumorigenesis through coordinate deregulation of both PI3K/mTOR and AR pathways. The study provides insights to both unique and common features of molecular subtypes of human prostate cancer.

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SPOP Mutation Drives Prostate Tumorigenesis In Vivo through Coordinate Regulation of PI3K/mTOR and AR Signaling

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SUMMARY

Recurrent point mutations in SPOP define a distinct molecular subclass of prostate cancer. Here, we describe a mouse model showing that mutant SPOP drives prostate tumorigenesis in vivo. Conditional expression of mutant SPOP in the prostate dramatically altered phenotypes in the setting of Pten loss, with early neoplastic lesions (high-grade prostatic intraepithelial neoplasia) with striking nuclear atypia and invasive, poorly differentiated carcinoma. In mouse prostate organoids, mutant SPOP drove increased proliferation and a transcriptional signature consistent with human prostate cancer. Using these models and human prostate cancer samples, we show that SPOP mutation activates both PI3K/mTOR and androgen receptor signaling, effectively uncoupling the normal negative feedback between these two pathways.

INTRODUCTION

Recurrent missense mutations in SPOP are the most common point mutations in primary prostate cancer, occurring in about 10% of both clinically localized and metastatic disease (Barbieri et al., 2012; Berger et al., 2011; Blattner et al., 2014; Cancer Genome Atlas Research Network, 2015; Robinson et al., 2015). SPOP mutations define a distinct and key molecular class of prostate cancer, with characteristic genomic alterations, patterns of genomic rearrangements, gene expression profiles, and methylation patterns (Barbieri et al., 2012; Blattner et al., 2014; Boysen et al., 2015; Cancer Genome Atlas Research Network, 2015). SPOP mutations occur early in the natural history of prostate cancer solely as heterozygous missense mutations with dominant-negative and selective loss of function toward the remaining wild-type (WT) allele (Baca et al., 2013; Significance

Recurrent point mutations in SPOP occur early in 10% of prostate cancers, defining a distinct molecular subclass. Our findings show that SPOP mutation drives prostate neoplasia in vivo through coordinate deregulation of both PI3K/mTOR and AR pathways. The discovery that SPOP mutation can activate two of the major pathways in prostate cancer exposes not only the biology of the SPOP mutant subclass, but the central importance of these pathways and their context across the spectrum of prostate cancer. These findings provide insight into both the unique and common features of molecular subtypes of human prostate cancer and highlight potential opportunities for precision therapy.
Boysen et al., 2015; Prandi et al., 2014; Theurillat et al., 2014). To date, no models have established the role of SPOP mutation as a driver of prostate neoplasia in vivo, and studies exploring the downstream effects of SPOP mutations have largely relied on overexpression of mutant SPOP protein in cell lines with alterations outside the genetic context of SPOP mutant prostate cancer (Geng et al., 2013, 2014; González-Billalabeitia et al., 2014; Pierce et al., 2015; Theurillat et al., 2014). Here, we report development of a conditional mouse model to define the role of SPOP mutation as a driver of prostate tumorigenesis in vivo.

RESULTS

SPOP Mutation Drives Prostate Neoplasia In Vivo in Combination with Pten Loss

To determine the impact of SPOP mutation in the prostate, we developed a transgenic mouse with prostate-specific conditional expression of SPOP-F133V, a common missense mutation found in human prostate cancer (Figure 1) (Barbieri et al., 2012; Berger et al., 2011; Cancer Genome Atlas Research Network, 2015). Specifically, we utilized a lox-STOP-lox strategy with the human SPOP-F133V transgene knocked into the Rosa26 locus (hereafter R26F133V) (Figures 1A, S1A, and S1B). These mice were crossed with PbCre mice to express mutant SPOP specifically in the prostate. Immunohistochemistry (IHC) against IRES-linked EGFP confirmed consistent expression of the transgene in the ventral, dorsolateral, and anterior lobes of the prostate with Cre expression (Figures S1C and S1D), and cell lines derived from these prostate confirmed physiologic levels of SPOP-F133V protein expression compared with endogenous Spop protein (Figure S1C). We observed minimal alterations in prostate glandular architecture and histology due to SPOP-F133V expression (Figure 1D), no differences in proliferation as measured by Ki-67 expression, and no differences in overall androgen receptor (AR) expression based on IHC (Figures 1E and S1I). Importantly, the histologic phenotype of HG-PIN in PtenL/L;R26F133V mice was distinct, characterized by a striking degree of nuclear atypia compared with the rarer HG-PIN with minimal atypia in PtenL/L control mice (Figures 1D, 1E, 1H, and S1J). Areas of HG-PIN in SPOP-F133V mice showed high levels of phosphorylated Akt and AR as determined by IHC (Figures 1E, 1G, and S1H), and an increased number of Ki-67-positive cells (Figure 1F). At advanced age (>12 months), areas of invasive cancer were observed in one out of five PtenL/L;R26F133V mice (Figures 1I and S1K). We conclude from these data that in the context of heterozygous Pten loss, SPOP mutation drives a distinct phenotype of HG-PIN with atypia and the potential to transition to invasive carcinoma.

PTEN deletions and mutations, while rare in the early phases of the SPOP mutant subtype of human prostate cancer (Barbieri et al., 2012; Cancer Genome Atlas Research Network, 2015), become more frequent in advanced prostate cancer (Figure S2A) (Grasso et al., 2012; Robinson et al., 2015), suggesting that PTEN deletion may contribute to progression of SPOP mutant prostate cancer (Barbieri et al., 2014; Hoffner et al., 2013). We therefore next examined the impact of SPOP mutation in the setting of conditional homozygous Pten loss. PbCre:PtenL/L mice developed diffuse, highly proliferative HG-PIN without evidence of invasive cancer, but with cystic enlargement of the anterior prostate grossly (Figures 2A–2D). In contrast, by 12 months of age PbCre:PtenL/L;R26F133V mice displayed severely enlarged prostates with areas of solid tumor (Figures 2A and S2B). Histologically, 80% of mice expressing mutant SPOP displayed invasive, poorly differentiated carcinomas (Figures 2C, 2D, and S2C). These tumors were highly proliferative, with transitional sarcomatoid differentiation and maintained expression of AR (Figures 2D and S2D). We conclude from these data that SPOP mutation is able to cooperate with loss of Pten to drive invasive prostate cancer in vivo.

SPOP Mutant Prostate Organoids Show Increased Proliferation without AR Upregulation

Previous reports studying the biology and downstream effects of SPOP mutations in prostate cancer have relied on ectopic overexpression of SPOP proteins in cell lines with multiple pre-existing genetic alterations (An et al., 2014, 2015; Gan et al., 2015; Geng et al., 2014; Theurillat et al., 2014), making the physiologic relevance of these models for normal prostate cell difficult to interpret. SPOP mutation occurs early in the natural history of prostate cancer and defines a distinct genetic subtype not represented by prostate cancer cell lines; therefore, it may be necessary to study its effects in benign prostate cells. Organoid platforms represent an opportunity to study the effect of cancer-associated alterations in genetically normal cells that can recapitulate the cell biology and epithelial architecture of the prostate (Chua et al., 2014; Gao et al., 2014; Karthaus et al., 2014). To establish models to study the underlying biology affected by SPOP mutation in prostate cells, we isolated organoid lines from the prostates of R26F133V mice (Pten WT background) and engineered them to express tamoxifen-inducible Cre (CreERT2) (Figure 3A). After treatment with 4-hydroxytamoxifen or vehicle, cells were sorted for GFP and physiologically relevant levels of mutant SPOP protein expression were confirmed by western blot (Figure 3A). Organoid lines were then grown as 2D monolayer cultures or 3D cultures in Matrigel. As previously described (Chua et al., 2014; Gao et al., 2014; Karthaus et al., 2014), in 3D cultures organoids recapitulated features of
Figure 1. SPOP Mutation Induces a Distinct Phenotype of Early HG-PIN with Nuclear Atypia in Pten<sup>L/+</sup> Mice

(A) Schematic of conditional SPOP-F133V construct in the Rosa26 (R26) locus (top) and of the expressed targeted transgenic transcript after Cre expression driven by probasin (Pb) promoter (bottom).

(B) Left graph: percentage of cells per gland with high AR expression across all lobes. Each point indicates one gland. Right graph: nuclear size of the 20 largest nuclei on H&E section of WT mice (control) (n = 3) and PbCre;R26<sup>F133V</sup> (SPOPmut) mice (n = 4) at 12 months of age. H&E staining shows representative nuclei of SPOPmut and control mice. Scale bar, 50 μm.

(C–H) Control, PbCre;Pten<sup>L/+</sup>, Pten<sup>L/+;</sup>R26<sup>F133V</sup>. (C) Percentage of Pten<sup>L/+</sup> control and Pten<sup>L/+;</sup>R26<sup>F133V</sup> mice, with HG-PIN at 3, 6, and 12 months of age. Number above bar indicates HG-PIN-positive/total number of mice. (D) Percentage of Pten<sup>L/+</sup> control and Pten<sup>L/+;</sup>R26<sup>F133V</sup> mice showing moderate or strong nuclear atypia at 3, 6, and 12 months of age. Number above bar indicates mice with atypia/total number of mice. (E) H&E, p-AKT, and AR IHC of representative glands of 6-month-old CreNeg (WT), Pten<sup>L/+</sup>, and Pten<sup>L/+;</sup>R26<sup>F133V</sup> mice. Scale bar, 50 μm. (F and G) Percentage of cells per gland with positive Ki-67 staining (F) and AR staining (G) divided into glands with histologically normal and HG-PIN phenotype. (H) Examples of HG-PIN in Pten<sup>L/+</sup> and Pten<sup>L/+;</sup>R26<sup>F133V</sup> mice at 6 and 12 months of age. Scale bar, 50 μm.

See also Figure S1.
prostate histology, including growth as multilayered structures with nuclear AR and expression of CK5 and CK8 in basal and luminal cells, respectively (Figure 3B).

Compared with controls, SPOP mutant organoids showed a higher rate of organoid formation capability, and resulting organoids showed more irregular borders with no differences in size (Figures 3B, 3C, and S3A), and increased Ki-67 expression enriched in the basal layer (Figures 3B and 3D). 2D proliferation assays showed increased proliferation of mutant cells (Figure 3E). Increased cell growth was confirmed in multiple SPOP mutant organoid lines, including those with Cre expressed under control of the probasin promoter (Figure S3B).

SPOP mutation also resulted in protein upregulation of previously reported SPOP substrates Dek and Src3 (Figures 3G and S3C) (Geng et al., 2013; Theurillat et al., 2014). We saw no consistent increased expression of AR by either IHC or western blot (Figures 3B, 3F, and 3G), although rare individual biological replicates showed increased AR protein (Figure S3C). We did not observe any alterations in Pten expression, although both AR and PTEN have been reported to be SPOP substrates (Figure 3G) (An et al., 2014; Geng et al., 2014; Li et al., 2014). Taken together, these data support that physiologic levels of mutant SPOP expression alone are sufficient to confer features of transformed cells.

**Transcriptional Profiling of SPOP Mutant Organoids Shows Relevance to Human Prostate Cancer and Nominates PI3K/mTOR Activation**

To provide further insight to the signaling pathways deregulated by SPOP mutation and determine the relevance of these models to human prostate cancer, we performed RNA sequencing (RNA-seq) on SPOP mutant organoids and controls. RNA-seq reads mapped to human and mouse SPOP confirmed appropriate expression of the F133V transgenic transcript without overexpression compared with endogenous mouse Spop (Figures S1C and S4A). We defined differentially expressed genes, with robust clustering according to SPOP status (Figure 4A and Table S1). Interrogating the gene space nominated by SPOP mutant mouse organoids in the transcriptomes of clinically localized human prostate cancer samples (The Cancer Genome Atlas [TCGA]) revealed significant clustering of known prostate cancer genes.
Figure 3. SPOP Mutation Drives Altered Phenotype and Increased Proliferation without AR Upregulation in Mouse Prostate Organoids

(A) Schematic overview of generating inducible SPOP-F133V murine prostate organoids. Sequence from left to right: prostate of 6- to 10-week-old mouse harboring conditionally expressing SPOP-F133V was dissected. Stable lines with tamoxifen-inducible Cre were generated either by infecting the organoids with CreERT2 retrovirus (line A) or crossing SPOP-F133V mice with TMPRSS2-CreERT2 mice (line B). Cells were treated with 4-hydroxytamoxifen or vehicle for 48 hr followed by cell sorting, and epitope-tagged SPOP-F133V expression was confirmed on immunoblot.

(B) Basic characterization of 3D organoid lines with bright-field microscopy, H&E staining, GFP, AR, Ki-67, IHC, and Ck5 and Ck8 IF. Scale bar, 50 μm.

(C) 3D organoid formation assay: Manual count of fully formed organoids within one well 14 days post plating (10 cells per well, 48 wells per line).

(D) Quantification of Ki-67-positive cells per organoid shown as percentage.

(E) 2D growth assay. Shown are the relative changes in DNA content over a period of 72 hr for stable control and SPOPmut cell lines.

(F) Quantification of Ki-67-positive cells per organoid shown as percentage.

(G) Western blot analysis of AR, DEK, SRC3, SPOP_Tag, SPOP, PTEN, and Vinculin expression in control and SPOPmut cell lines. Scale bar, 50 μm.
SPOP mutant tumors \( (p = 2.57 \times 10^{-10}) \), Figure 4B). SPOP mutations at F133 and other amino acids (e.g., Y87, F102, W131) were well represented in the cluster, as were genomic features known to be associated with SPOP mutant prostate cancer (including genomic deletions at 5q21, 6q15, and 2q21) (Barbieri et al., 2012; Boysen et al., 2015), indicating that SPOP-F133V in mouse prostate engaged a transcriptional program consistent with SPOP mutant human prostate cancer (Figure 4B). Differentially
expressed genes in SPOP mutant mouse prostate organoids showed significant overlap with differentially expressed genes in human prostate cancer (Figure 4C). Molecularely, human prostate cancers can be classified into those harboring rearrangements in ETS transcription factors (e.g., TMPRSS2-ERG) and those lacking ETS rearrangements (Rubin et al., 2011). SPOP mutant prostate cancers are exclusively ETS rearrangement negative, and differentially expressed genes in SPOP mutant mouse prostate organoids overlapped specifically with ETS rearrangement-negative human prostate cancer (Figure 4C). Together, these data suggest that expression of SPOP-F133V in mouse prostate epithelium recapitulates transcriptional features of human SPOP mutant prostate cancer.

Having established the relevance of the mutant SPOP organoid model to human SPOP mutant prostate cancer, we next explored the signaling pathways deregulated by SPOP mutation. Unbiased gene set enrichment analysis (GSEA) nominated multiple pathways as deregulated by SPOP mutation (Figure 4D and Table S2). Multiple independently derived organoid lines with induction of SPOP mutation showed strong concordance across prostate gene sets (Figure S4B). We previously reported that SPOP mutation affects genomic stability similarly to BRCA1 inactivation (Boysen et al., 2015) and saw evidence of this in the transcriptional response to SPOP-F133V in mouse organoids (Figure S4-C). Despite previous reports that SPOP mutation drives AR transcriptional output in vitro (Geng et al., 2014) and that SPOP mutant human prostate cancer is associated with increased AR transcriptional output in TCGA data (Cancer Genome Atlas Research Network, 2015), we saw no evidence that gene sets defined by androgen regulation in either mouse or human prostates were enriched in that gene sets defined by androgen regulation in either mouse prostate (Genome Atlas Research Network, 2015), we saw no evidence that SPOP increased AR transcriptional output in TCGA data (Cancer Cell, 2014). We therefore hypothesized that in genetically normal prostate cancer, including deletions and mutations in PTEN, and amplification and mutations in PIK3CA and AKT1 (Figures 5F and S5D), consistent with previous data (Barbieri et al., 2012). Consistent with this, in PtenC4 mice all prostate glands expressing pAKT showed loss of Pten protein expression, while PtenC4 mice showed activation of Akt despite persistent Pten expression (Figures 5G and S5E). Finally, in reverse-phase protein array data from 250 clinically localized human prostate cancer samples (TCGA), SPOP mutant human prostate cancers had significantly higher expression of phospho-T37 and phospho-T70 4eBP1, consistent with PI3K/mTOR activation (Figure S5F). In human prostate cancer samples and mouse organoids, we did not identify gene expression changes in components of PI3K signaling in SPOP mutant tumors (Figure S5G), suggesting that pathway activation was not due to transcriptional upregulation of PI3K components. Taken together, these data suggest that SPOP mutation is associated with activation of PI3K/mTOR signaling in mouse prostate tissue and human prostate cancer samples.

**Activation of PI3K/mTOR Signaling by Mutant SPOP is Mediated by SRC3**

SRC3, encoded by NCOA3, is a bona fide substrate of SPOP and has been shown to be stabilized by prostate cancer-specific SPOP mutants (Geng et al., 2013). We confirmed that SRC3 protein was upregulated by SPOP-F133V in mouse organoids (Figures 3G and S3C) and prostate tissue (Figure 6A). SRC3 has also been reported to activate PI3K signaling through transcriptional upregulation of insulin-like growth factor 1 (IGF-1) (Torres-Arzayas et al., 2004). We confirmed in prostate organoids that mutant SPOP was associated with increased Igf1 mRNA in both Pten WT and Pten-deleted backgrounds (Figure 6B), and knockdown of Src3 with small interfering RNA (siRNA) abrogated the increase in Igf1 expression by mutant SPOP (Figure 6C). Furthermore, inhibition of IGF-1 receptor abrogated the increase in pS6 and pAkt protein levels in SPOP mutant prostate cells (Figure S6A), and stimulation with IGF-1 ligand increased pS6 and pAkt in SPOP WT controls to levels comparable with those in SPOP mutant cells (Figure S6B). We therefore hypothesized that activation of PI3K/mTOR signaling by mutant SPOP could be mediated by SRC3. Knockdown of Src3 in mouse prostate organoids reduced the increase in pS6 protein levels seen with mutant SPOP, in both Pten WT (Figure 6D) and Pten-deleted (Figure 6E) backgrounds. These data support that activation of the control of different promoters (Figures 5C and S5C). Increased activation of mTOR signaling by SPOP-F133V was maintained in prostate tissue from PbCre;PtenC4;R26F133V mice (Figure 5D). Finally, treatment of organoids with Torin1, a potent inhibitor of both mTORC1 (mTOR complex 1) and mTORC2, again revealed increased mTOR output in SPOP mutant organoids (Figure 5E).

To determine whether activation of PI3K signaling by SPOP mutation was supported by genomic events observed in human prostate cancer samples, we examined three publicly available datasets comprising 498 tumors (Baca et al., 2013; Barbieri et al., 2012; Cancer Genome Atlas Research Network, 2015). Analysis showed mutual exclusivity between SPOP mutations and genomic alterations in components of PI3K signaling that are significantly recurrently altered in clinically localized prostate cancer, including deletions and mutations in PTEN, and amplification and mutations in PIK3CA and AKT1 (Figures 5F and S5D).
Figure 5. SPOP Mutation Leads to Activation of PI3K/mTOR Signaling

(A) pS6 staining in representative glands of 1-year-old mice with Pten\^\textsuperscript{+/+} background. SPOP\^F133V mice show focally increased staining over control. Scale bar, 50\,µm.

(B) Left panel: representative pS6 staining of control organoids (left) and SPOP-F133V expressing organoids (right). Scale bar, 50\,µm. Right panel: quantitative comparison of organoids positive for pS6 expression on IHC.

(C) Western blot for pAKT and pS6 in mouse prostate organoids. Control, Pten\^L/L; SPOPmut, Pten\^L/L; R26F133V.

(D) Left: western blot for pAKT, p4EBP1 and pS6, from prostate tissue lysates of 1-year-old WT, PbCre;Pten\^L/L mice (control), and PbCre;Pten\^L/L; R26F133V mice (SPOPmut). Right: relative protein expression across multiple samples from WT, control, and SPOPmut prostate lysates.

(E) Treatment with decreasing concentration of mTOR inhibitor for 1 hr leads to gradual release of mTOR activity (left to right: 100, 50, 10, 5.0 nM Torin1). SPOPmut organoids show higher mTOR activation than control organoids based on p4EBP1 and pS6 protein level.

(F) Mutual exclusivity between mutated SPOP and alterations in PI3K pathway components (PTEN deletions/mutations or PIK3CA and AKT amplification/mutation) across three independent cohorts with a total of 498 samples (SPOP\^\textsuperscript{+} = 440, SPOPmut = 58).

(G) PTEN and pAKT IHC for prostate glands in PbCre;Pten\^\textsuperscript{+/+} (Control) and PbCre;Pten\^\textsuperscript{+/+}; R26F133V (SPOPmut) mice. Scale bar, 50\,µm.

See also Figure S5.
PI3K/mTOR signaling by SPOP mutation is mediated in part through the SPOP substrate SRC3.

**SPOP Mutation Maintains AR Activity against PI3K-Mediated Feedback Inhibition**

PI3K and AR signaling have significant reciprocal negative feedback, with PI3K activation resulting in downregulation of AR signaling (Carver et al., 2011; Lee et al., 2015; Mulholland et al., 2011; Thomas et al., 2013). We therefore hypothesized that in our model systems, previously reported upregulation of AR signaling by mutant SPOP could be masked by the concomitant activation of PI3K/mTOR signaling. We examined the impact of SPOP mutation on AR protein level in mouse organoids with inactivation of PI3K/mTOR signaling. Treatment with Torin1 resulted in a relative increase in AR protein expression with SPOP-F133V (Figure 7A). In addition, modulation of mTOR activity with various doses of Torin1 led to an expected inverse relationship between AR protein levels and mTOR activity (as measured with pS6 and p4eBP1) in control cells (Figure 7A). In contrast, organoids expressing SPOP-F133V maintained AR expression in the face of higher mTOR activity (Figure 7A). We next examined the consequences of SPOP mutation on expression of AR in mouse organoids with activation of PI3K/mTOR signaling. Prostate organoids cultured in low (5 ng/mL) or high (50 ng/mL) epidermal growth factor (EGF) conditions were examined for AR and pS6 protein levels by IHC. As expected, control cells responded to increased EGF with increased pS6 levels, increased Ki-67 staining, and a dramatic decrease in AR protein expression (Figures 7B and S6A). In contrast, SPOP mutant organoids maintained higher AR levels with increased EGF exposure (Figure 7B).

Prostate organoids derived from Pten\(^{L/L}\) mice showed an expected increase in pAkt and downregulation of AR expression (Figure 7C), consistent with previous results (Carver et al., 2011). In contrast, SPOP-F133V in the Pten\(^{L/L}\) background resulted in upregulation of AR protein and increased expression of AR target genes (Figures 7C and 7D). This has been confirmed in prostate tissue from 1-year-old PbCre;Pten\(^{L/L}\) and PbCre;Pten\(^{L/L}\);R26F133V mice compared with age-matched WT mice (Figure S7B). Furthermore, areas of AR and pS6 expression showed clear mutual exclusivity at the individual cell level in Pten\(^{L/L}\) prostate organoids, while combination with SPOP-F133V resulted in co-expression of AR and pS6 (Figure 7E).

Next, we examined the impact of SPOP mutation on AR transcriptional output in human prostate cancer samples, using RNA-seq data from TCGA. When considered in isolation, SPOP mutation had a strong association with higher AR transcriptional score (p = 3.65 \times 10^{-5}, Figure S7C). However, when molecular subtype (ETS and PTEN status) was considered, we observed an attenuation of this phenomenon (Figure S7D). As previously reported, PTEN deletion showed a strong association with decreased AR transcriptional activity (Carver et al., 2011), particularly in cancers harboring ETS rearrangements (p < 0.001, Figure S7D). When compared with tumors of the same PTEN and ETS background, SPOP mutant tumors showed both an increase in PI3K/mTOR activity (as measured by p4EBP1, p = 0.019) and an increase in AR transcriptional activity (p = 0.047, Figure 7F), consistent with the concomitant activation of PI3K and AR signaling in these cancers. Together, these data support that SPOP mutation maintains AR transcriptional output in the setting of PI3K activation, thereby activating two major pathways in the pathogenesis of prostate cancer.
Proteome-wide Analysis Shows Activation of PI3K/mTOR Signaling and Coordinate Upregulation of an AR-Centric Protein Network

To gain further insight into the proteins deregulated by expression of physiologic levels of mutant SPOP, we performed unbiased proteome-wide profiling of control and SPOP mutant mouse prostate cells (Pten WT) using label-free tandem mass spectrometry (MS/MS). We observed significant differential expression in 300 proteins (≥1.5 fold, p < 0.05), with mutant SPOP, resulting in upregulation of 187 and downregulation of 113 proteins.

Figure 7. SPOP Mutation Stabilizes AR and Maintains AR Transcription in the Setting of Activated PI3K/mTOR Signaling

(A) Quantitative western blot analysis. Decreasing Torin1 concentration leads to increased p4eBP1 expression (bars) and either decreased AR expression (blue line, control) or stable AR expression (red line, SPOPmut). AR normalized to 100 nM Torin-Control and pS6 normalized to 0 nM Torin-SPOPmut.

(B) Left: relative AR expression in control organoids (blue) and SPOPmut organoids (red) in 5 or 50 ng/mL EGF culture condition. Right: AR staining of control and SPOPmut organoids in 5 or 50 ng/mL EGF culture condition. Scale bar, 50 μm.

(C–E) Organoids generated from the whole prostate of 1-year-old PbCre;Pten WT, PbCre;Pten F133V, and PbCre;Pten L/L;R26F133V mice. (C) Western blot showing AR, pAKT, SPOP, and PTEN expression. (D) Relative RNA expression measured by qPCR for AR targets FKBP5, PSCA, and NKX3.1. Data are means ±SEM. (E) IHC staining for AR (left) and pS6 (right) in control (top) and SPOPmut expressing organoids. Control organoids show exclusive AR (arrow) or high pS6 (asterisk) staining on a gland-to-gland basis. SPOPmut organoids have co-existing high expression of AR and pS6. Scale bar, 50 μm.

(F) Phospho-4EBP1 levels and AR transcriptional score from human prostate cancer samples (TCGA), divided into SPOP mutant and SPOP WT tumors. Top: distribution of phospho-4EBP1 levels in reverse-phase protein array data from human prostate cancer samples. Bottom: AR transcriptional score in RNA-seq data from human prostate cancer samples. Increasing phospho-4EBP1 and AR score from left to right. Only PTEN WT tumors were considered in this analysis. See also Figure S7.
Figure 8. Proteome-wide Analysis of SPOP-F133V Expression in Mouse Prostate Cells: Activation of PI3K/mTOR Signaling and Upregulation of a Network of AR Transcriptional Partners

(A) Volcano plot of differentially abundant proteins in control versus SPOPmut mouse prostate cells (three independent biological replicates, 

\( Pten^{WT} \)) determined by label-free MS/MS. Log2 (fold change) of SPOP-F133V/control is plotted on x axis; \(-\log_{10}(p\ \text{value})\) is plotted on y axis. Non-axial vertical dashed lines represent ±1.5-fold change; non-axial horizontal dashed line represents \( p = 0.05 \). Each circle denotes a single detected protein; larger circles represent higher confidence in differential abundance based on peptide counts and variability.

(legend continued on next page)
113 proteins (Figure 8A and Table S3). Consistent with hyperactivation of PI3K/mTOR signaling in response to SPOP-F133V, the most altered protein was IRS1 (down 15.9-fold, p = 0.002), known to be degraded in response to mTOR activation (Harrington et al., 2005). Furthermore, Ingenuity pathway analysis of altered proteins highlighted AKT1 as one of the top upstream regulators (Figure 8B and Table S4). These unbiased proteomics data reinforce the conclusion that SPOP mutation results in activation of PI3K/mTOR signaling.

Under these assay conditions and higher level of resolution, several previously established SPOP substrates showed significantly increased expression (Table S3), including TRIM24 (up 3.9-fold, p = 0.002) and AR (up 2.24-fold, p = 0.016). Others demonstrated trends of increased expression but did not meet proteome-wide significance, such as Dek (up 1.36-fold, p = 0.036) and SRC3 (up 2.02-fold, p = 0.12).

We next determined the frequency of the established SPOP binding consensus (SBC) amino acid motif (nonpolar [e]-polar [\text{\text{-S/T-S/T-S/T}}]) in all significant altered proteins. Upregulated proteins were significantly more likely to harbor an SBC than downregulated proteins (Figure 8C and Table S5), consistent with the hypothesis that SPOP-F133V acts as a dominant negative. We next performed de novo motif analysis on the top upregulated proteins to determine whether there were amino acid sequences specifically associated with increased expression in response to SPOP-F133V. This revealed a number of serine-rich motifs distinct from the canonical SBC (Figure S8A). The amino acid sequence S-S-S-x-x-S was the top enriched motif in upregulated proteins, and was present in established SPOP substrates such as Trim24 and AR, AR cofactors such as Rnf14, and other upregulated proteins with potential relevance to transcriptional control and prostate cancer pathogenesis, such as Brd2, Atr, and Setb1 (Figure 8D and Table S6). Interestingly, another highly enriched amino acid sequence contained the L-x-x-L-L motif (Figure S6A and Table S6) known to mediate the interaction of transcriptional co-activators with nuclear hormone receptors (McInerney et al., 1998).

Finally, to gain insight into the associations among proteins deregulated by SPOP-F133V, we performed a network analysis of up- and downregulated proteins using STRING, a database of known and predicted protein-protein interactions (Szklarczyk et al., 2015). Consistent with the hypothesis that mutant SPOP acts as a dominant negative to coordinately upregulate networks of functionally related proteins, we observed a robust network among proteins upregulated by mutant SPOP (Figure 8E); in contrast, there was minimal network structure in downregulated proteins (Figure S8B). In particular, there was a subnetwork of upregulated proteins centered around AR, including the AR-associated transcription factor HOXB13 (Norris et al., 2009), and AR co-activators EP300 (Debes et al., 2002), DNA-PK (Goodwin et al., 2015), SRC3 (Xu et al., 2009), and TRIM24 (Groner et al., 2016). Using immunoblots, we confirmed increased expression of HoxB13 and p300, using both mouse and human prostate cells expressing mutant SPOP (Figures 8F, 8G, and S8D). Taken together, these data suggest that mutant SPOP acts as a dominant negative to coordinately deregulate networks of functionally related proteins, and in particular may effect upregulation of transcriptional complexes consisting of AR and associated transcription factors and co-activators.

**DISCUSSION**

Comprehensive molecular characterization of prostate cancer has revealed numerous molecular alterations with the potential to act as drivers of cancer development and progression. These include several genes previously not implicated in carcinogenesis, making their functional significance unclear. SPOP mutations are the most common point mutations in clinically localized prostate cancer, yet SPOP was only recently implicated as a cancer gene. This study shows that SPOP mutation promotes prostate tumorigenesis in vivo, clearly defining it as a cancer driver (versus passenger) and reinforcing its importance in human prostate cancer.

AR and PI3K signaling represent two critical signaling pathways in the pathogenesis of human prostate cancer. Recent data have exposed reciprocal negative feedback between AR and PI3K signaling (Carver et al., 2011; Lee et al., 2015; Muholand et al., 2007; Norris et al., 2007). Here we show that SPOP mutation disrupts this feedback, allowing coordinate activation of both pathways, supporting prostate carcinogenesis. In human prostate cancer, separate genomic events leading to independent activation of these pathways are common. For instance, ERG rearrangement and PTEN inactivation commonly co-occur in primary prostate cancer (Barbieri et al., 2012; Cancer Genome Atlas Research Network, 2015; Taylor et al., 2010); PTEN loss drives PI3K activation while ERG maintains AR transcription in this setting (Carver et al., 2009; Chen et al., 2013). In human prostate cancer, the SPOP mutant subclass has been associated with increased AR activity (Cancer Genome Atlas Research...
sequences of case with combination with other alterations (Baena et al., 2013; Carver prostate neoplasia in mice, but show neoplastic phenotypes in context of this model: one caveat is that mice of the C57BL/6 in combination with genic pathways, which result in robust neoplastic phenotypes of SPOP-F133V alone may reflect activation of underlying oncogenic abnormalities with prostate-specific conditional expression of SPOP. Unlike other genomic alterations, SPOP mutation is not increased in prevalence in castration-resistant prostate cancer (Robinson et al., 2015) and may even be depleted in these cancers, suggesting preferential response to androgen-deprivation therapy. However, understanding the full extent of mechanisms responsible for the effect of SPOP mutation on these signaling pathways, the interaction between them, and the impact on therapeutic sensitivity requires additional investigation.

Genetically engineered mouse models have played a key role in understanding the biology of prostate cancer, defining the impact of critical alterations observed in human disease, and developing effective treatment strategies. Interestingly, key alterations observed in human prostate cancer (such as overexpression of ERG or ETV1 and deletion of PTEN) are insufficient for prostate neoplasia in mice, but show neoplastic phenotypes in combination with other alterations (Baena et al., 2013; Carver et al., 2009; Chen et al., 2005, 2013). Here, we show this is the case with SPOP mutation as well, at least in the specific genetic context of this model: one caveat is that mice of the C57BL/6 background are often considered relatively tumor resistant (Svensson et al., 2011), and we cannot speculate on the consequences of SPOP mutation in other backgrounds. The cytologic abnormalities with prostate-specific conditional expression of SPOP-F133V alone may reflect activation of underlying oncogenic pathways, which result in robust neoplastic phenotypes in combination with Pten deletion. This spectrum of genetic alterations provides the opportunity to study the role of SPOP mutation as an early event in cancer initiation (Pten+/-), a key driver in cancer progression (Pten-/-), and its role in more advanced disease (Pten-/-).

The study of early alterations in a relatively genetically normal context may be crucial for defining the biological effects on prostate cancer development. SPOP mutation occurs early in the natural history of prostate cancer (Boyesen et al., 2015), and ectopic introduction of SPOP mutations in cell lines with multiple alterations may mask oncogenic effects. We observed that conditional expression of mutant SPOP alone in mouse prostate organoids results in transcriptional changes consistent with human prostate cancer, reinforcing the relevance of our model and the important role of mutated SPOP in prostate cancer pathogenesis. In addition, all SPOP mutations observed in human prostate cancer are heterozygous, with roughly equal expression of the WT and mutant alleles (Barbieri et al., 2012). Our conditional mouse model systems show nearly 1:1 expression of endogenous and mutant SPOP proteins, recapitulating physiologically relevant expression levels in human disease.

SPOP encodes the substrate recognition component of a CUL3-based E3 ubiquitin ligase, and prostate cancer-derived SPOP mutants appear to act as dominant negatives with selective loss of function (Boyesen et al., 2015). Known substrates of SPOP are numerous, and the specific substrates that are de-regulated by SPOP mutations are starting to be defined. These include the chromatin-associated oncogene DEK (Theurillat et al., 2014), the oncogenic co-activator TRIM24 (Groner et al., 2016; Theurillat et al., 2014), and AR itself (An et al., 2014) (Geng et al., 2014). We show that SRC3 (NCOA3), another SPOP substrate stabilized by SPOP mutation (Geng et al., 2013), mediates, at least in part, the activation of PI3K/mTOR signaling by SPOP mutation. Furthermore, an unbiased proteomics approach revealed additional candidate proteins regulated by SPOP mutation. Most importantly, it showed increased expression of not only AR but a network of multiple AR-associated transcription factors and co-activators. Consistent with this, proteins upregulated by mutant SPOP are enriched for the L-x-x-L-L motif, a key mediator of interaction between co-activators and nuclear receptors (Heery et al., 1997; McInerney et al., 1998). This raises the possibility that SPOP mutation can coordinately deregulate multiple components of transcriptional complexes rather than a single substrate, therefore amplifying its impact on prostate cancer pathogenesis. Given the ability of ubiquitin ligases to coordinate networks of signaling events, it is highly likely that no single substrate of SPOP is responsible for all downstream oncogenic phenotypes. Further investigation will continue to define which SPOP substrates, individually or in combination, are critical for prostate tumorigenesis.

The relationship between SPOP mutations and other genomic alterations in the PI3K pathway (such as PTEN deletions) in prostate cancer is likely complex. Here, we show that the histologic phenotypes observed in the prostate when SPOP mutation is combined with Pten loss are distinct from the histology with Pten loss alone, rather than acceleration of the Pten-deleted phenotype. In addition, SPOP mutation primarily affects readouts of mTOR activity, with less effect on Akt activation driven by Pten loss, consistent with parallel signaling rather than truly redundant pathways. SPOP mutations occur early in the natural history of prostate cancer, while PTEN deletions are generally later events. As we and others have shown, SPOP mutations are mutually exclusive with genomic alterations in PI3K pathway components in early, clinically localized disease states, but these alterations co-occur in more advanced cancers, particularly in castration-resistant prostate cancer. Whether the emergence of PTEN alterations in advanced SPOP mutant cancers represents natural biological progression or a mechanism of resistance to therapy will require additional investigation.

In summary, we report that mutation of SPOP, the most common missense mutations in prostate cancer, results in neoplasia in the mouse prostate. Physiologic expression of mutant SPOP activates PI3K/mTOR signaling in vitro and in vivo, and upregulates a network of AR-associated transcription factors and co-activators. SPOP mutation maintains AR transcriptional activity against PI3K/mTOR-mediated negative feedback, effectively activating two pathways critical for the pathogenesis of prostate cancer.
EXPERIMENTAL PROCEDURES

Gene Targeting and Mouse Breeding

All mouse studies are approved by Weill Cornell Medicine (WCM) Institutional Care and Use Committee under protocol 2015-0022. Gene targeting was performed as previously described (Chen et al., 2013). For generation of prostate-specific SPOP-F133V expression, Rosa26SPOP/SPOP mice were crossed with previously described PbCre4;Pten+/− mice (Trotman et al., 2003; Wu et al., 2001). Only male PbCre4-positive mice were used to carry the PbCre4 allele. All described mice are in a C57BL/6 background.

Mouse Prostate Harvest

Prostate tissue was harvested from mice euthanized using CO2, and samples were prepared using liquid nitrogen for fresh-frozen samples or fixed in 4% formalin overnight and embedded in paraffin. Sectioning of the prostate was performed as described by Shappell et al. (2004).

Pathologic Review

All sections were reviewed by two independent board-certified genitourinary pathologists with specific expertise in mouse models of human prostate cancer (B.D.R. and M.I.). All review was performed blinded to genotype.

Protein Analysis

Tissue paraffin embedding, sectioning, and staining with H&E and IHC were performed by the translational research program at WCM Pathology and Laboratory Medicine. A full list of all antibodies used in this study and conditions is provided in Supplemental Experimental Procedures.

Mouse Prostate Organoid Generation and Experiments

Prostate tissue was extracted from euthanized mice and digested as previously described (Lukacs et al., 2019). Cells were plated in Matrigel (Corning, BD 356231) and covered by mouse medium containing 5-50 ng/mL EGF. Medium and culture conditions were as previously described (Karthaus et al., 2014). For inducible organoid systems, mouse prostate cells were virally infected with a CreERT2 construct and selected with puromycin (Line_A) or generated from a mouse containing transgenic TMPRSS2-CreERT2 construct (Line_B [Gao et al., 2016]). CreERT2 was activated by adding 1 µM 4-hydroxytamoxifen (Sigma-Aldrich, T176) into the medium overnight. Either GFP or allele. All described mice are in a C57BL/6 background.

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