B-Raf Activation Cooperates with PTEN Loss to Drive c-Myc Expression in Advanced Prostate Cancer

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Abstract

Both the PI3K → Akt → mTOR and mitogen-activated protein kinase (MAPK) signaling pathways are often deregulated in prostate tumors with poor prognosis. Here we describe a new genetically engineered mouse model of prostate cancer in which PI3K Akt-mTOR signaling is activated by inducible disruption of PTEN, and extracellular signal-regulated kinase 1/2 (ERK1/2) MAPK signaling is activated by inducible expression of a BRAFV600E oncogene. These tissue-specific compound mutant mice develop lethal prostate tumors that are inherently resistant to castration. These tumors bypass cellular senescence and disseminate to lymph nodes, bone marrow, and lungs where they form overt metastases in approximately 30% of the cases. Activation of PI3K → Akt → mTOR and MAPK signaling pathways in these prostate tumors cooperate to upregulate c-Myc. Accordingly, therapeutic treatments with rapamycin and PD0325901 to target these pathways, respectively, attenuate c-Myc levels and reduce tumor and metastatic burden. Together, our findings suggest a generalized therapeutic approach to target c-Myc activation in prostate cancer by combinatorial targeting of the PI3K → Akt → mTOR and ERK1/2 MAPK signaling pathways. Cancer Res; 72(18); 4765–76. ©2012 AACR.

Introduction

Although the majority of prostate cancers can now be treated effectively with surgery or radiation therapy, a subset of highly aggressive tumors progress to lethal metastatic disease (1). Unlike other cancers, it has proven difficult to classify prostate tumors into molecular subtypes that distinguish those that are indolent from those that are aggressive. Nonetheless, certain genes and/or molecular pathways have been associated with disease progression and/or poor prognosis. Among those associated with disease progression, the MYC oncogene is somatically amplified in a subset of advanced prostate tumors (2, 3), whereas nuclear MYC protein is upregulated in cancer progression even in the absence of gene amplification (4), although the underlying mechanisms by which it is activated remain unresolved.

Among the key signaling pathways associated with disease progression and poor outcome, various components of both the PI3-kinase → Akt → mTOR and RAF → MEK → ERK MAPK pathways are frequently coactivated in advanced prostate tumors and associated with poor outcome (5–8). Moreover, studies in cell culture and in vivo have shown the functional significance of coactivation of PI3-kinase → Akt → mTOR and MAPK signaling for prostate cancer tumor progression (9, 10). It is well established that a driving force for activation of PI3-kinase → Akt → mTOR signaling is inactivation of PTEN, which is prevalent in prostate tumors (11, 12). However, despite the prevalence of MAPK activation in a majority of advanced prostate tumors (6), the underlying mechanisms are less clear (13). In particular, the major upstream activators of MAPK signaling, namely RAF kinases, are rarely mutated in prostate tumors (14–16); however, activation of the RAF signaling pathway occurs in more than 90% of prostate tumors (6), and at least one mechanism by which BRAF is activated is through chromosomal rearrangement (17).

In the current study, we have investigated the functional consequences of coactivation of PI3-kinase → Akt → mTOR and MAPK signaling for prostate tumorigenesis and metastasis in vivo by generating a genetically engineered mouse (GEM) model having conditional loss-of-function of Pten combined with activation of oncogenic Braf. These mice develop lethal prostate tumors that metastasize to lymph nodes and lungs in approximately 30% of the cases. Notably, these prostate tumors display robust activation of PI3-kinase → Akt → mTOR and MAPK signaling, coincident with Myc pathway activation.

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Therapeutic treatment with rapamycin and PD0325901, which target PI3-kinase → Akt → mTOR and MAPK signaling, respectively, results in reduced tumor and metastatic burden while suppressing Myc pathway activation. Taken together, these findings suggest that an important consequence of activation of PI3-kinase → Akt → mTOR and MAPK signaling in advanced prostate cancer is Myc pathway activation, which therefore may be a target of therapeutic intervention.

Materials and Methods

**Generation and analyses of GEM**

All experiments using animals were carried out according to protocols approved by the Institutional Animal Care and Use Committee at Columbia University Medical Center. The Nkx3.1<sup>1<sup>CreERT2</sup></sup> allele (18), a conditional allele for Pten (Pten<sup>fl<sup>+/+</sup></sup>, 19) and a lox-stop-lox Braf<sup>fl<sup>+/+</sup></sup> allele expressing an inducible Braf allele (20) have been described. Mice were maintained on a mixed strain C57BL/6 and 129/Sv, and Friend Virus B-Type background and breed to generate the full spectrum of genotypic combinations. Primers for genotyping are listed in Supplementary Table S1. For tamoxifen induction of Cre activity, mice were administered tamoxifen (Sigma Catalog #T5648) in corn oil by intraperitoneal injection (225 mg/kg) or oral gavage (100 mg/kg) once daily for 4 consecutive days, at 2 to 3 months of age as in Wang and colleagues (18). Control mice received only corn oil by the same delivery method. Note that unless otherwise indicated the ages of the mice analyzed refer to time following delivery of tamoxifen. Where indicated, mice were androgen-ablated by surgical castration at 1 month after tumor induction. Following tumor induction, mice were monitored on a daily basis for body condition (i.e., muscle tone and weight) and sacrificed when their body condition score was more than 1.5, as per guidelines of the Institutional Animal Care and Use Committee.

For phenotypic analyses, mice were sacrificed, prostate tissues were collected, photographed, and weights were determined. The prostatic lobes (anterior, dorsolateral, and ventral) were collected individually and fixed in 10% formalin and paraffin-embedded, cryopreserved in optimal cutting temperature (OCT) compound, or snap-frozen in liquid nitrogen. Full necropsy was done on each mouse analyzed and, in addition to prostate, a spectrum of tissues was collected into 10% formalin for evaluation of the metastatic phenotype, which included prostate, a spectrum of tissues was collected into 10% formalin for evaluation of the metastatic phenotype, which included kidneys, liver, lungs, pancreas, small intestine, heart, lumbar lymph node, caudal lymph node, and submandibular lymph nodes. Bone marrow was collected by macrodissection of the femur followed by flushing the marrow with 1 mL of PBS using a 26G needle. DNA was made from the flushed cells and then Platinum Genotype Tsp DNA polymerase (Invitrogen) was used to amplify fragments up to 500 bp therefore avoiding primer dimerization. Primers for genotyping are listed in Supplementary Table S1. Senescence-associated β-galactosidase (SA-β-Gal) staining was done using the Cellular Senescence Assay Kit (Chemicon International) on tissue embedded directly in OCT. Images were photographed using a Nikon Eclipse E800 microscope equipped with a Nikon DXM1200 digital camera. For quantification of immunostaining, a minimum of 3 independent sections from 3 independent mice (i.e., 9 sections) from slides that had been immunostained with Ki67, androgen receptor (AR), the indicated phospho-proteins, cytokertatin 5 or cytokertatin 8, were counted as done previously (22). Statistical analyses were carried out using a 2-tailed t test. GraphPad Prism software (Version 5.0) was used for all statistical analysis and to generate data plots.

For Western blot analyses, total protein extracts were prepared from the dorsolateral prostate or prostate tumors in 1X radioimmunoprecipitation assay buffer (0.1% SDS, 1.0% deoxycholate-sodium salt, 1.0% Triton X-100, 0.15 M NaCl, 10 μmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA) with fresh 1% protease inhibitor (#1697498; Roche Basel) and 1% phosphatase inhibitor (#P2830; Sigma-Aldrich). Protein lysates (10 mg per lane) were resolved by SDS page electrophoresis, immunoblotted with primary antibody followed by the appropriate secondary antibody and visualized using the ECL Plus Western Blotting Detection Kit (#RPN2132; GE Healthcare/Amersham Biosciences). Western blot data were quantified using Imagej (http://rsb.info.nih.gov/ij/; 23). Details of antibodies are provided in Supplementary Table S2.

**Preclinical analyses of GEM**

Rapamycin was purchased from LC labs (Catalog #R-5000, lots #ASW-105 and #ASW-109); PD325901 was a generous gift from Pfizer (Batch U). Rapamycin was delivered via intraperitoneal at 10 mg/kg in 5.2% Tween 80, 5.2% PEG400. PD325901 was delivered via oral gavage at 10 mg/kg in 0.05% hydroxypropyl-methylcellulose. 0.02% Tween 80. Rapamycin and/or PD0325901 (or vehicle) were delivered for 5 consecutive days with 2 days off for a period of 4 weeks. Alternatively, mice were treated with rapamycin and/or PD0325901 (or vehicle) for 4 days and dissected within 6 hours after the last treatment. Mice were weighed daily and observed for signs of distress following dosing. Notably, none of the treatments resulted in appreciable weight loss exceeding 10%.

**Gene expression analysis**

Gene expression profiling analysis was done using total RNA isolated from prostate tissues of Nkx3.1<sup>1<sup>CreERT2</sup></sup>; Pten<sup>fl<sup>+/+</sup></sup> (n = 6), or Nkx3.1<sup>1<sup>CreERT2</sup></sup>; Pten<sup>fl<sup>+/+</sup></sup>, Braf<sup>fl<sup>+/+</sup></sup> mice treated with vehicle (n = 3), or Nkx3.1<sup>1<sup>CreERT2</sup></sup>; Pten<sup>fl<sup>+/+</sup></sup>, Braf<sup>fl<sup>+/+</sup></sup> mice treated with RAP+PD (n = 3). RNA was isolated using the MagMAX-96 Total RNA Isolation Kit (Ambion), which was reverse-transcribed and bionin-labeled using the Illumina TotalPrep RNA Amplification Kit (Ambion). The cRNA (1.5 mg) was hybridized on mouse-6 v2 BeadArrays (Illumina) using an iScan BeadArray scanner (Illumina). Data were loaded and normalized using IlluminaExpressionFileCreator version 2 and Illumina normalizer with collapse mode using the maximum of all the probe values for each gene and without background subtraction. The resulting datasets were preprocessed to remove probesets whose difference between maximum and minimum values...
were less than 100. Data were log transformed for Gene Set Enrichment Analysis (GSEA) analysis. Gene expression microarray data have been deposited in the Gene Expression Omnibus (GEO) under series accession number GSE39190.

The gene signatures of Nkx3.1*CreERT2*, Ptenfl/fl, BrafCA/+ versus Nkx3.1*CreERT2*, Ptenfl/fl, BrafCA/+ mouse prostate tumors and RAP-PD-treated versus vehicle-treated Nkx3.1*CreERT2*, Ptenfl/fl, BrafCA/+ mouse prostate tumors were defined using the Welch t test as a list of genes ranked by their differential expression. To identify pathways commonly deregulated in vehicle- and RAP-PD-treated Nkx3.1*CreERT2*, Ptenfl/fl, BrafCA/+ mouse prostate tumors, enrichment of the differentially expressed gene signatures in human pathways was evaluated using GSEA (24). Using pathways collected in manually drawn, peer-reviewed biologic pathway databases composed of luminal epithelial cells, they displayed an expansion of CK5 positive cells NB or NP single mutants, as had also been observed in the Braf transgenic model (34).

Considering that Braf pathway activation is most prominent in advanced prostate cancer in humans (6), we asked whether the NPB mice expressed AR and were sensitive to androgen depletion. We found that the NPB prostate, as well as the NB and NP single mutants, each express nuclear AR (Fig. 1S–V). Furthermore, following androgen deletion by surgical castration, the NPB mice do not display significant tumor regression at 2 weeks following castration (Supplementary Fig. S2), suggesting that the tumors in the NPB mice are inherently resistant to castration. This contrasts with the NP mouse tumors, which are castration sensitive (37) as are the previously described Braf transgenic model, which is wild type for Pten (34).

Consistent with this observation that the NPB tumors are inherently castration resistant, another notable distinction of the NPB compound mutants relative to the NB and NP single mutants was the virtual absence in the NPB mouse of SA-β-Gal staining, a marker of cellular senescence, which was in striking contrast to the robust SA-β-Gal staining in both single mutants (Fig. 1A–D'). We have shown recently that one of the major differences among otherwise similar mouse prostate tumors is that the castration resistant ones display little or no cellular senescence (37), therefore, this observation further supports the inherent castration resistance of these NPB prostate tumors. As mentioned previously, the prostate tumors from the NPB mice were also much more highly proliferative than the NB or NP single mutants (Fig. 1W–Z, F'), which is also consistent with their limited cellular senescence.

Finally, another striking distinction of the NPB prostate tumors is their propensity to metastasize to lymph nodes and lungs. In particular, NPB mice, but not NB or NP mice, displayed metastases to lungs as well as the lumbar lymph nodes in 30% of the cases (n = 3/10) as evident by analyses of H&E as well as by staining for AR and pan-cytokeratin (Fig. 2; Supplementary Table S3). Notably, the NPB mice also displayed disseminated tumor cells in bone marrow (n = 2/10), as detected by quantitative PCR (qPCR; Supplementary Table S3). In summary, expression of oncogenic BrafV600E combined with Pten inactivation leads to advanced prostate cancer distinguished by its inherent castration resistance, bypass of cellular senescence, and the occurrence of secondary metastases.

Results

Cooperation of BrafV600E activation and Pten inactivation in a mouse model of advanced prostate cancer

To investigate the role of Braf pathway activation in conjunction with Pten loss-of-function, we used a tamoxifen-inducible Cre (CreER(T)) under the control of the Nkx3.1 promoter (18), a prostate-specific homeobox gene whose haploinsufficiency of function leads to prostate intraepithelial neoplasia (PIN; 33). This Nkx3.1*CreERT2* knock-in allele simultaneously inactivates Nkx3.1 and drives tamoxifen-dependent Cre-mediated recombination specifically in the prostate epithelium (Supplementary Fig. S1), including a relevant cell of origin of prostate cancer (18). We crossed the Nkx3.1*CreERT2* allele with a Pten floxed allele (19), or with a conditionally activatable Braf (BrafCA/+), allele, which expresses oncogenic BrafV600E in response to Cre activity (ref. 20; Fig. 1; Supplementary Fig. S1). Notably, our current model, which conditionally expresses activated Braf in prostatic luminal cells, is distinct from a previously reported transgenic model (34).

We found that mice lacking one allele of Nkx3.1, both alleles of Pten, and having an activatable Braf allele [i.e., Nkx3.1*CreERT2* × Ptenfl/fl × BrafCA/+; hereafter denoted Nkx3.1*CreERT2*, Ptenfl/fl, BrafCA/+ (NPB) mice] develop lethal prostate cancer 4 months after tamoxifen induction (i.e., by 6 months of age; Fig. 1A; Supplementary Table S3). In contrast, mice lacking only Pten or having only an activatable Braf allele [denoted Nkx3.1*CreERT2*, BrafCA/+ (NB) or Nkx3.1*CreERT2*, Ptenfl/fl (NP) mice, respectively] rarely develop lethal prostate tumors up to 2 years following tamoxifen induction (Fig. 1A; Supplementary Table S3). Indeed, by 4 months after tamoxifen induction, the prostate tumors from the NPB mice were large in size (~2–3 grams) and highly proliferative, compared with the NB or NP single mutants (Fig. 1B–F, W–Z, F). Notably, cooperation of BrafV600E activation and Pten inactivation has also been observed in an analogous mouse model of melanoma (35, 36).

The progressive phenotype of the NPB mice was further evident by histopathologic analyses, in which these mice displayed PIN by 2 to 4 weeks and invasive adenocarcinoma by 2 to 3 months after tamoxifen induction, which was considerably more aggressive than either the NB or NP single mutants (Fig. 1G–J; Supplementary Table S3). Nonetheless, the prostate tumors from the NPB mice, as well as the NB and NP single mutants, were primarily composed of luminal epithelial cells, as evident by robust staining for cytokeratin 8, with significantly less staining for cytokeratin 5, a marker of basal epithelial cells, and a few scattered neuroendocrine cells (Fig. 1K–R, F). However, although the tumors in the NPB mice were composed of luminal cells, they displayed an expansion of CK5 positive cells NB or NP single mutants, as had also been observed in the Braf transgenic model (34).
Figure 1. Cooperation of Braf activation and Pten loss in prostate cancer. A, survival curve shows the percentage of mice of the indicated genotypes surviving after tumor induction. B, average wet weights of prostate tumors. C–F, whole mount images. G–J, representative H&E images of anterior prostate. K–N, immunofluorescence images show staining for cytokeratin 8 (CK8), which stains luminal epithelial cells, and cytokeratin 5 (CK5), which stains basal epithelial cells. Note that the prostate tumors are primarily luminal as evident by the robust staining for CK8, with more limited staining of CK5. O–R, immunohistochemical staining for synaptophysin. S–V, immunohistochemical staining for AR in the intact mice. Note that the prostates from each of the models express nuclear AR. W–Z, immunohistochemical staining for Ki67. A'–D', SA-β-Gal staining shows prominent senescence in the prostates from the Nkx3.1CE2+/+; BrafCA/+ (NB) and Nkx3.1CE2+/+; Ptenf/f (NP) mice, but not in the Nkx3.1CE2+/+; Ptenf/f; BrafCA/+ (NPB) mice. E', immunofluorescence staining index as measured by the percentage of the indicated CK5 and CK8 positive cells relative to total epithelial cells in the prostates of mice by genotypes as indicated. F', average proliferation assessed by the number of Ki67-positive cells relative to total epithelial cells. Where indicated, P values compare the experimental to the control (Nkx3.1CE2/ prostate) and scale bars represent 100 μm, except in (K–N) where they represent 25 μm.
We further investigated the molecular pathways that distinguish the NPB phenotype from NP phenotype using GSEA to identify biologic processes that are altered in these prostate tumors by comparing the NPB mice with the NP single mutants (Supplementary Table S4, S5A, and S5B). Among the significantly enriched categories identified were those corresponding to cell cycle \( (P < 0.001) \), G1 pathway \( (P = 0.001) \), pathway in cancer \( (P < 0.001) \), and ERK pathway \( (P = 0.004) \); Supplementary Fig. S3; Supplementary Table S5A), consistent with our phenotypic analyses (see Fig. 1). Additional categories that were significantly enriched included focal adhesion \( (P < 0.001) \) and extracellular receptor interaction \( (P < 0.001) \); Supplementary Fig. S3; Supplementary Table S5A).

Considering our observation that Myc is upregulated in the NPB relative to the NP mice (see Fig. 4D), we asked whether the gene signature corresponding to the NPB versus NP prostate was associated with upregulation of the Myc pathway activity (Fig. 5A–C; Supplementary Table S6A). In particular, we compared the mouse prostate signature with 6 independent Myc pathway signatures, which were as follows: (i) genes upregulated and promoters bound by MYC in human B-cells (32); (ii) genes whose promoters contain E-box motifs and whose expression is induced by Myc in neuroblastoma cells (30); (iii) genes identified as Myc target by chromatin immunoprecipitation analyses in human cancer cells (28); (iv) genes differentially expressed in human cancer cells expressing Myc (27); (v) genes upregulated in B cell lymphoma tumors expressing MYC (31); and (vi) genes upregulated in Burkitt's lymphoma cells expressing MYC (29). In comparison with each of these independent Myc signatures, the gene signature corresponding to the NPB versus NP mice was significantly enriched for upregulation of the Myc pathway genes, as evident from the positive enrichment score in each case (Fig. 5A; Supplementary Table S6A). These observations provide further support for our finding that Myc is activated in the NPB mouse prostate tumors.

**Combination targeted therapy reduces tumor and metastatic burden, and alleviates Myc activation**

On the basis of these phenotypic and molecular analyses, we reasoned that the NPB mice would provide an excellent *in vivo* model to study the consequences of therapeutic targeting of the PI3-kinase \( \rightarrow \) Akt \( \rightarrow \) mTOR plus MAPK signaling pathways, and to assess their relevance for Myc pathway activation. Toward this end, we conducted preclinical studies using the combination of rapamycin to target mTOR signaling together with a mitogen-activated protein/extracellular signal–regulated kinase (MEK) inhibitor (PD0325901) to target MAPK signaling. We have previously found that this RAP+PD combination was highly effective for treatment of prostate cancer in a mouse model based on germline inactivation of Nkx3.1 and *Pten* (5). Furthermore, combinatorial targeting of PI3-kinase \( \rightarrow \) Akt \( \rightarrow \) mTOR and MAPK signaling pathways has shown promising results in preclinical studies and clinical trials for several cancers, including prostate as well as lung and breast (5, 44–46), while targeting tumors driven by *Braf* activation combined with *Pten* loss using the same or similar agents has
shown promise in preclinical studies of melanoma and thyroid cancer (35, 47).

Therefore, we evaluated the consequences of RAP and/or PD for therapeutic intervention in the NPB mice in preclinical studies (Fig. 6A; Supplementary Table S7). We found that treatment with both RAP+PD had a significantly impacted on tumor burden as evident by inspection of several independent endpoints. In particular, the NPB mice displayed a significant (P < 0.0001) reduction in tumor weight after treatment (n = 10), as well as histologic evidence of reduced tumor growth (n = 6) and a significant (P < 0.0001) reduction in cellular proliferation (n = 5; Fig. 6B–I, W; Supplementary Table S7). In contrast to the robust efficacy of the RAP+PD combination, the single agents were only modestly effective, as we have observed previously (5), although PD0325901 alone displayed significant efficacy for inhibiting proliferation (Fig. 6F–I, X). Notably, in addition to the effects on tumor burden, the RAP+PD treatment also decreased metastatic burden, as evident by the reduced incidence of lung metastases (n = 1/10) and disseminated tumor cells in the bone marrow (n = 0/10; Fig. 6Y; Supplementary Table S7).

These phenotypic changes in tumor and metastatic burden were accompanied by reduced expression of the relevant targets, namely pAKT(Ser473), pS6(Ser235/236), and pERK (Thr202/Tyr204) (Fig. 6J–V). Furthermore, following just 4 days of RAP and/or PD treatment, we observed a significant reduction in activation of multiple components of the PI3-kinase → Akt → mTOR, MAPK, and Ras signaling pathways (Fig. 7A–C). In particular, PI3-kinase → Akt → mTOR pathway components were reduced following RAP treatment and the
RAP+PD combination, whereas components of the MAPK and Ras pathways were downregulated by treatment with PD as well as with the RAP+PD combination.

Notably, expression of Myc and Cyclin D1 were reduced by treatment with the individual agents and were abolished by the RAP+PD combination (Fig. 7D), suggesting that inhibition of PI3-kinase → Akt → mTOR and MAPK signaling led to Myc downregulation. To confirm this finding, we assessed whether genes associated with Myc activation that were upregulated in the NPB mice (see Fig. 5A) were downregulated in response to RAP+PD treatment. For this purpose, we generated expression profiles from prostate tumors from NPB mice that had been treated with RAP+PD or vehicle (Supplementary Table S8) and then used GSEA analyses to compare this signature to the same Myc gene signatures we had found to be enriched in the NPB versus NP prostate tumors (see Fig. 5A). In contrast to the strong positive enrichment of the Myc pathway signatures identified in the transition from NP to NPB mice, we found an inverse negative enrichment of these Myc pathway signatures when comparing the RAP+PD-treated to the vehicle-treated samples (compare Fig. 5A and B, Supplementary Table S6B). This observation was further confirmed by qPCR validation, focusing on Myc target genes involved in cell cycle progression, such as Cyclin D1, Cyclin D2, Cyclin B1, and glutaredoxin 3 (Fig. 5C). In particular, Myc targets that were upregulated in the NPB prostate tumors were correspondingly downregulated following treatment with RAP+PD (Fig. 5C). In summary, these findings show that combination therapy using rapamycin and PD0325901 to target PI3-kinase → Akt → mTOR signaling and MAPK signaling results in a profound reduction in tumor burden, reduces the occurrence of metastases, and reverts Myc pathway activation.

**Discussion**

Here we describe a new mouse model of advanced prostate cancer based on inducible Pten inactivation combined with inducible expression of oncogenic Braf<sup>V600E</sup>, which leads to dysregulation of both PI3-kinase → Akt → mTOR and MAPK signaling. Our findings suggest that cancer progression that results from activation of Braf signaling together with loss of Pten is the consequence of several important features that distinguish these advanced tumors. In particular, the prostate tumors in the NPB mice are inherently castration resistant and express robust levels of AR, are highly proliferative, display a bypass of cellular senescence, and most notably, metastases to lungs and the lymph nodes. Furthermore, our analyses have revealed that Myc is a major downstream effector of PI3-kinase → Akt → mTOR and MAPK signaling in these NPB prostate tumors, which can be suppressed by therapeutic interventions.
targeting these signaling pathways. Thus, our findings provide a new preclinical model for evaluating agents that target the PI3-kinase → Akt → mTOR and MAPK signaling pathways, as well as novel insights about Myc as a target for therapeutic intervention.

Because upregulation of extracellular signal-regulated kinase 1/2 (ERK1/2) MAPK signaling is prevalent in advanced prostate cancer, the role of RAF kinases, which are the major upstream effectors, has remained controversial. Although point mutation of \(\text{BRAF}\) does not play a major role in prostate tumorigenesis (14–16), activation of RAF → MEK → ERK signaling occurs in a majority of prostate tumors with poor prognosis (6) and at least one mechanism for its activation is via chromosomal translocation of the \(\text{BRAF}\) gene (17). Indeed, the absence of \(\text{BRAF}\) mutations in prostate cancer is perhaps not surprising, because prostate tumors are not typically characterized by mutational activation and are generally more indolent relative to other cancer types. Thus, a major future challenge will be to define how RAF → MEK → ERK signaling is activated in prostate cancer so that the appropriate targets for therapeutic intervention can be identified.

It is interesting to compare the consequences of \(\text{PTEN}\) inactivation combined with oncogenic \(\text{BRAF}^{\text{V600E}}\) expression in melanoma versus prostate cancer. Similar to prostate cancer, combinatorial activation of PI3-kinase → Akt → mTOR signaling and MAPK signaling occurs frequently in melanoma and is associated with poor outcome (14, 48, 49), and GEM models in which \(\text{Pten}\) and \(\text{Braf}\) are perturbed in melanoma have revealed their functional collaboration (35, 36). However, in striking contrast to prostate cancer, \(\text{BRAF}\) mutations are highly prevalent in melanoma (14, 50), and the progression of melanoma is generally much more aggressive than is the case for prostate cancer. Thus, although the molecular consequences of \(\text{BRAF}^{\text{V600E}}\) expression and \(\text{PTEN}\) inactivation (i.e., activation of PI3-kinase → Akt → mTOR signaling and MAPK signaling) may be similar in melanoma and
prostate cancer, the molecular evolution of the tumors is likely to be distinct. Further comparison of the molecular features of prostate cancer and melanoma driven by BRAFV600E expression and PTEN inactivation may provide insights into their common versus tissue-specific molecular mechanisms of cancer progression, which can be facilitated by the availability of complementary mouse models for these tumor sites.

Finally, our current findings have provided new insights into the relationship of Myc upregulation to activation of PI3-kinase → Akt → mTOR and MAPK signaling in prostate cancer. In particular, our findings show that Myc, whose upregulation in prostate cancers is associated with disease progression and outcome (1, 51), is downstream of PI3-kinase → Akt → mTOR and MAPK signaling and can be downregulated by agents that affect these pathways. In the case of melanoma, a mouse model based on Pten loss and Braf activation has revealed the involvement of β-catenin signaling via GSK3 (36), which may also ultimately affect Myc signaling. These observations further underscore the potential value of comparative analyses of molecular pathways of prostate cancer and melanoma through studies of the corresponding mouse models, as well as the

Figure 6. Combination therapy reduces tumor burden. A, design of preclinical studies using combination therapy with rapamycin and PD0325901. Nkx3.1CE2/+;Pten(−/−);BrafCA/+ (NPB) mice were induced with tamoxifen at 2 months of age and drug treatment was initiated 1 month later and continued for 1 month, during which time mice were treated with vehicle or rapamycin and/or PD0325901. Following cessation of treatment, mice were sacrificed (long-term response). For evaluation of immediate response to drug action, a cohort of mice were treated with vehicle or rapamycin and/or PD0325901 for 4 days and sacrificed within 6 hours after the last treatment (short-term response). Unless otherwise indicated the analyses show data for the long-term response. B–E, representative H&E images of mice treated with vehicle, rapamycin, and/or PD0325901 as indicated. F–I, representative Ki67 immunostaining of prostate tissues treated as indicated. J–U, immunostaining for the indicated phospho-proteins. V, immunostaining index as measured by the percentage of the indicated phospho-proteins positive cells relative to total epithelial cells in the prostates of mice treated as indicated. W, average prostate weight for mice treated as indicated. X, proliferation index as measured by the percentage of Ki67 positive cells relative to total epithelial cells in the prostates of mice treated as indicated. Y, the penetrance of lung metastasis and disseminated tumor cells of mice treated as indicated. Where indicated, P values compare the drug- to vehicle-treated groups, and scale bars represent 100 μm. PD, PD0325901; RAP, rapamycin.
significance of Myc as a key downstream target of these signaling pathways.

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

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References

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