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13. SUPPLEMENTARY NOTES

14. ABSTRACT
Prostate cancer is the most common diagnosed and second leading cause of epithelial cancer-related death in men. Small cell neuroendocrine carcinoma (SCNC) accounts for only 1% of diagnosed prostate cancers prior to aggressive therapy. However, after administration of aggressive therapy, tumor resistance is inevitable resulting in the acquisition of SCNC tumors in well over 20% of patients. SCNC tumors are highly aggressive, metastasize readily, and often lead to death of the patient within months after diagnosis. Tyrosine kinases represent an untapped area for therapy in the stratification of SCNC patients. We observed that RET tyrosine kinase is heightened at the mRNA and protein level in SCNC tissues and neuroendocrine cell lines when compared to adenocarcinoma. RET expression and related neuroendocrine markers were upregulated in a model of neuroendocrine differentiation and RET mutations resulted in initiation of prostate cancer. Future work will continue to define the role of RET in prostate cancer.

15. SUBJECT TERMS
Prostate cancer, metastasis, tyrosine kinase, neuroendocrine, RET, small cell, human

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INTRODUCTION

Prostate cancer is the most common diagnosed and second leading cause of epithelial cancer-related death in men[1]. Therefore, much is to be learned about the growth and resistance of epithelial cancer cells to current therapeutics. Small cell neuroendocrine carcinoma (SCNC) accounts for only 1% of diagnosed prostate cancers prior to aggressive therapy[2]. However, after administration of aggressive therapy, tumor resistance is inevitable resulting in the acquisition of SCNC tumors in well over 20% of patients. SCNC tumors are highly aggressive, metastasize readily, and often lead to death of the patient within months after diagnosis. The mechanism for the increase in the SCNC component of prostate cancer after aggressive therapy is unclear and therapies to treat this variant form of the disease are severely lacking. One family of proteins thought to promote cancer growth and resistance to aggressive therapy are the tyrosine kinases. Tyrosine kinase activity has been shown to play a role in prostate cancer, but targeting these same molecules clinically has not been very successful[3]. In this proposal, our objective was to evaluate a novel tyrosine kinase target, RET, in men with SCNC who have become resistant to current treatments. This new target will provide a novel mechanism and treatment strategy that will benefit a growing population of men suffering from metastatic SCNC. Further, the proposed study will help to advance the field of prostate cancer research by providing greater insight into better treatment options and strategies for administration of specific, targeted inhibitors in patients with SCNC.

KEYWORDS

- Prostate cancer
- Small cell neuroendocrine carcinoma
- RET
- Metastasis
- Neuroendocrine differentiation
- Tyrosine kinase

ACCOMPLISHMENTS

What were the major goals of the project?

The major aims of the project are to:
Aim 1. Confirm and establish RET kinase activity in prostatic SCNC tissues.
Aim 2. Evaluate the trans-differentiation capacity of RET kinase *in vitro*.
Aim 3. Determine the functional role of RET kinase in castration resistant prostate cancer.

What was accomplished under these goals?

For Aim 1, we were able to perform immunohistochemistry of RET protein on CRPC tissue microarrays consisting of normal prostate tissue, prostate cancer, and small cell neuroendocrine carcinoma (SCNC) and adenocarcinoma (AdCa). We found that total RET immunostaining was prominent in subsets of patients with both SCNC and AdCa, but lower in normal prostate and prostate cancer tissues (**Figure 1A-D**). The frequency of RET IHC staining was about 50% of the patients analyzed. We also analyzed RET protein via western blot in a large panel of prostate cancer cell lines. Strikingly, RET protein was only observed in the cell lines with a small cell or neuroendocrine phenotype and not cell lines with the adenocarcinoma phenotype (**Figure 1E**). We are not sure why the discrepancy exists between IHC staining and western blot as it pertains to RET kinase levels between SCNC and AdCa. We are still investigating why this is the case. One possibility is that total RET may not be expressed at different levels between SCNC and AdCa suggesting that we need to look at the phosphorylation levels of RET. We have attempted IHC using an antibody that recognizes RET phosphorylation but with little luck to date as the phospho-site antibody does not work very well. Overall, this data suggests that RET protein is indeed present in CRPC and possible enriched in SCNC based on cell line data with the neuroendocrine phenotype. We will need to assess more tissues and eventually generate a phosphosite specific antibody that recognizes RET phosphorylation to measure RET activity.

For Aim 2, we assessed the expression of RET and other neuroendocrine markers at the mRNA level to determine if they are correlated upon androgen withdrawal. Previous studies have shown that LNCaP cells grown in charcoal stripped media (mimics androgen withdrawal) will develop neuroendocrine-like differentiation[4]. We used this model to assess the levels of RET transcript after growing LNCaP cells in charcoal stripped media. By Day 6 and Day 9, we observed noticeable morphology changes of LNCaP cells (**Figure 2A-D**) as well as distinct changes in RET mRNA and the neuroendocrine markers synaptophysin and chromogranin A (**Figure 2E-G**). These preliminary findings suggest that RET kinase increases expression when cells undergo neuroendocrine differentiation after androgen withdrawal. Next, we generated lentiviral vectors against wildtype (WT) RET, and 2 different RET mutants that render the kinase constitutively activated (RET C634W and RET M918T) (**Figure 3A**). All three vectors express RET protein at a high level and were used for both in vitro and in vivo studies (Aim 3). To test the capability of RET kinase to transdifferentiate in vitro, we transduced WT RET and RET mutants into our LNCaP cell line model and monitored the cellular morphology over time (**Figure 3B-G**). Androgen withdrawal did induce the neuroendocrine phenotype (**Figure 3E-G**), but the addition of RET mutants did not exacerbate this phenotype in either normal growth conditions (**Figure 3B-D**) or in androgen depleted conditions (**Figure 3E-G**). Therefore, the potency of androgen withdrawal may override any effects observed by exogenous addition of the RET gene.

For Aim 3, we assessed the capability of WT RET or mutant RET to aid in prostate cancer initiation using the primary human tissue recombination model. Two RET mutants (RET C634W and RET M918T) were chosen that render the kinase constitutively active (**Figure 4A, red arrows**). Primary prostate basal cells were transduced with WT RET, RET C634W, or RET M918T alone or in combination with other oncogenes (**Figure 4B**). WT RET did not produce a tumor phenotype alone or in combination with an activated AKT or N-MYC. Interestingly, both RET mutants synergized with N-MYC to generate a large tumor although this phenotype primarily consisted of squamous differentiation (**Figure 5B, C, bottom panels and Figure 6A**). This tumor expressed RET protein (**Figure 6B**) as well as markers of luminal cells with CK8 expression and low levels of androgen receptor (AR) (**Figure C, F**). In addition, basal cell markers were also present as evidenced by p63 and CK5 (**Figure 6D, E**). In all, the capability of RET mutants to synergize with N-MYC in human prostate cancer models is exciting and worth pursuing further. We will need to determine how best to generate an adenocarcinoma phenotype prior to testing the capability of RET protein to contribute to castration resistance in these models.

What opportunities for training and professional development has the project provided?
Nothing to report.

How were the results disseminated to communities of interest?
Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?
Nothing to report.

IMPACT
What was the impact on the development of the principal discipline(s) of the project?
Therapeutic targeting of tyrosine kinases in late stage prostate cancer are still underdeveloped. We have begun investigation into one particular tyrosine kinase, RET, and observed that RET mRNA and protein are heightened in CRPC and SCNC.

What was the impact on other disciplines?
Nothing to report.

What was the impact on technology transfer?
Nothing to report.

What was the impact on society beyond science and technology?
Nothing to report.

CHANGES/PROBLEMS
Changes in approach and reasons for change.
Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them.
We are still working out conditions to get an antibody to recognize phosphorylated RET kinase at the western blot and IHC level in our models. So far we have not had any luck to get this to stain appropriately and are currently making an antibody to phosphorylated RET for our own use. Any studies proposing to evaluate RET phosphorylation have not been able to be completed because of this technical hurdle.

We did not evaluate the levels of RET mRNA in different populations of primary human cells in Aim 2 because we could not isolate sufficient numbers of neuroendocrine cells for our study. Because of this limitation we determined it would be better to test whether RET and other neuroendocrine markers' mRNA was altered in established cell line models such as LNCaP.

In addition, we have not been able to perform castration experiments in Aim 3 yet because the tumors that develop from RET kinase are primarily squamous, not adenocarcinoma. We are currently investigating how to develop more adenocarcinoma-like tumors prior to castration which will allow us to more accurately assess RET function during castration as well as neuroendocrine differentiation during this process. One approach is to sort out the luminal like cells from the tumors and re-transplant back into mice. This has been down previously in our lab and provides a viable option to test[5]. One explanation for why WT RET did not generate a tumor phenotype is because the endogenous ligand is not present. We are currently in the process of re-evaluating the capability of WT RET to transduce primary human basal cells with the presence of the ligand GDNF. These experiments are underway.

Changes that had a significant impact on expenditures
Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.
Nothing to report.

Significant changes in use or care of human subjects.
Nothing to report.

Significant changes in use or care of vertebrate animals.

Significant changes in use of biohazards and/or select agents.
Nothing to report.

PRODCUTS
Publications, conference papers, and presentations.
Journal publications
Drake JM, Huang J. 2014 PIP5K1α inhibition as a therapeutic strategy for prostate cancer. *PNAS*. (see appendices)

Books or other non-periodical, one time publications.
Nothing to report.

Other publications, conference papers, and presentations.
Poster Presentation at the Keystone Symposium on the Biological Code of Cell Signaling – January 11-16, 2015 Steamboat Springs, CO (see appendices)

Website(s) or other Internet site(s)
Nothing to report.

Technologies or techniques.
Nothing to report.

Inventions, patent applications, and/or licenses.
Nothing to report.

Other products.
Nothing to report.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS
What individuals have worked on the project?
Nothing to report.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to report.

What organizations were involved as partners?
Nothing to report.

SPECIAL REPORTING REQUIREMENTS
None

APPENDICES (see attached)
- Supporting Data (Figures 1-6)
- Publication in *PNAS*
- Keystone Symposium on the Biological Code of Cell Signaling Abstract

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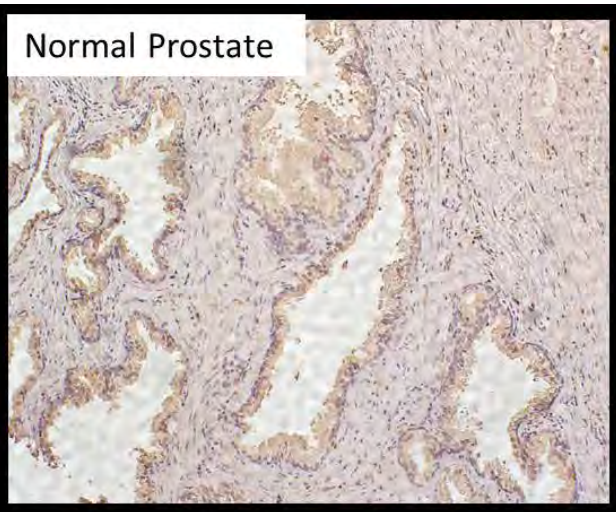
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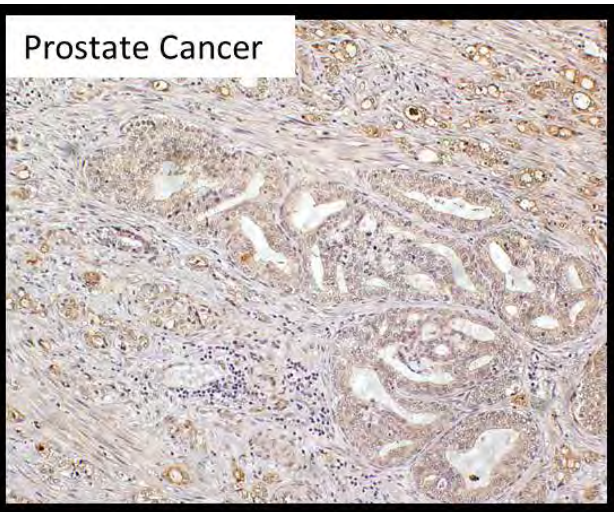
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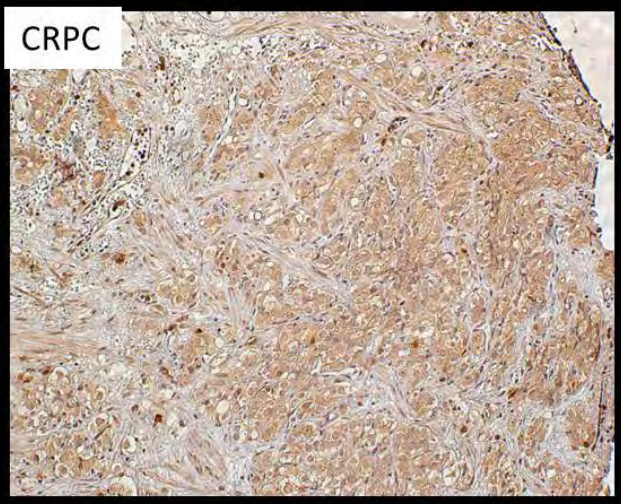
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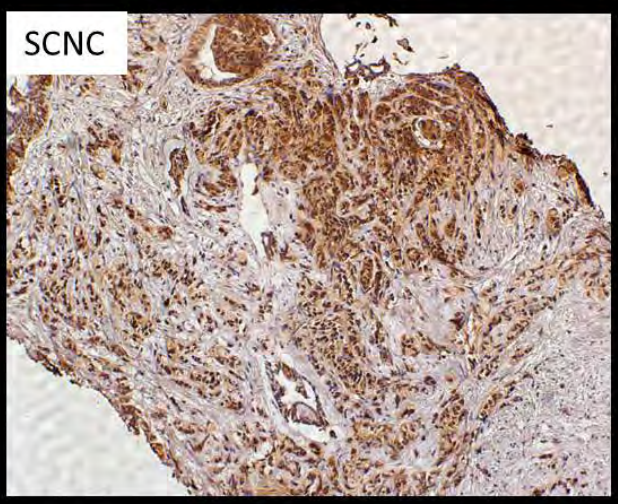
SCNC


AdCa
Small Cell
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LNCaP
VCaP
C4-2
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PC3
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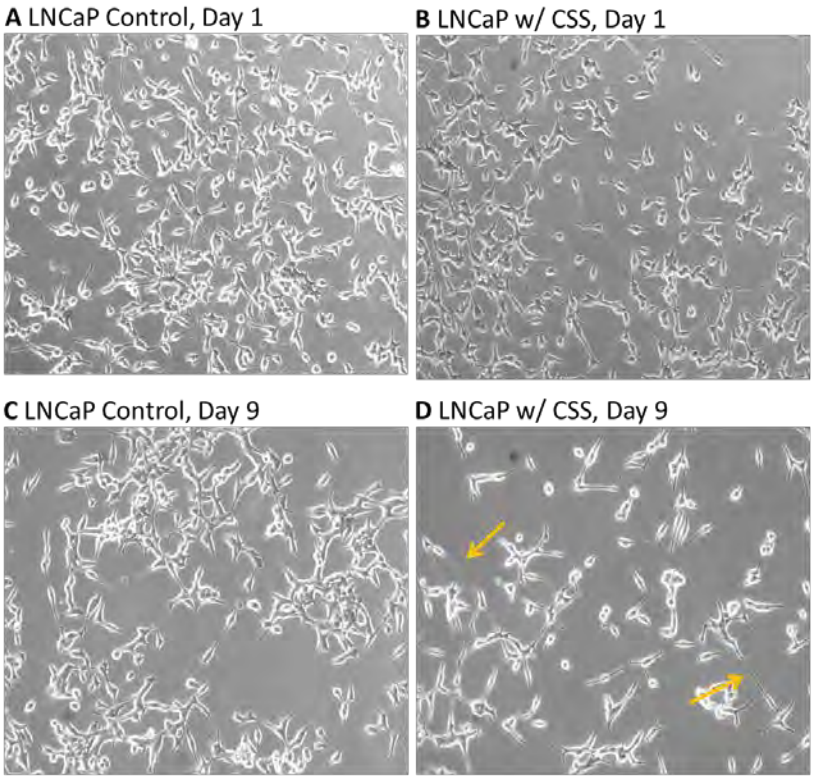
LASCPC1



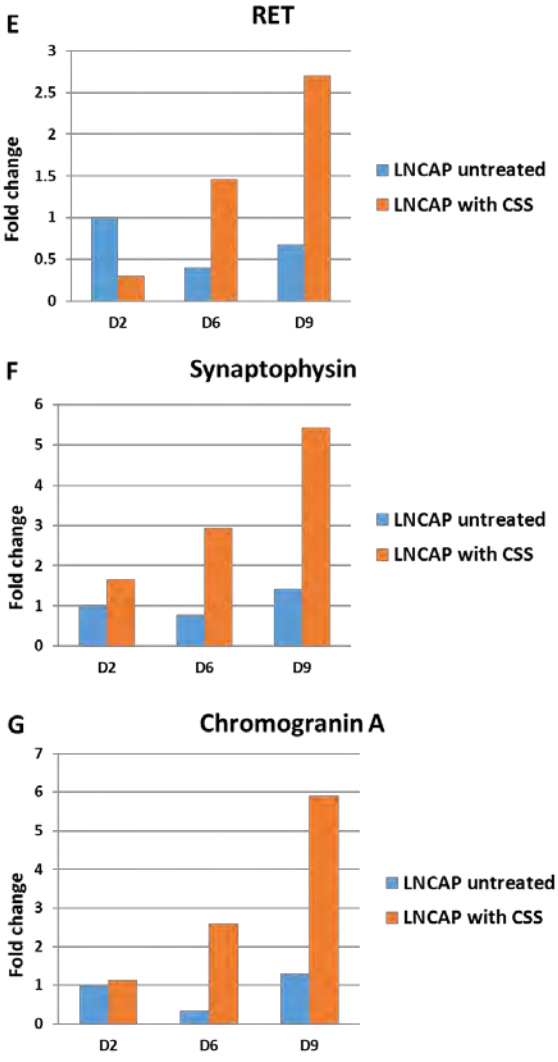


Figure 2. Development of a neuroendocrine phenotype in LNCaP prostate cancer cells. Morphology of LNCaP prostate cancer cells grown I normal growth media (Control) (A,C) or in charcoal stripped medium (CSS) (B,D). By Day 9, noticeable morphological changes are observed that include increased cellular extension that represent neuronal behavior (D, arrows). Analysis of neuroendocrine markers in these cells reveal that by Day 6, RET (E), synaptophysin (F), and chromogranin A (G) mRNA are increased when compared to LNCaP cells grown in normal growth media.

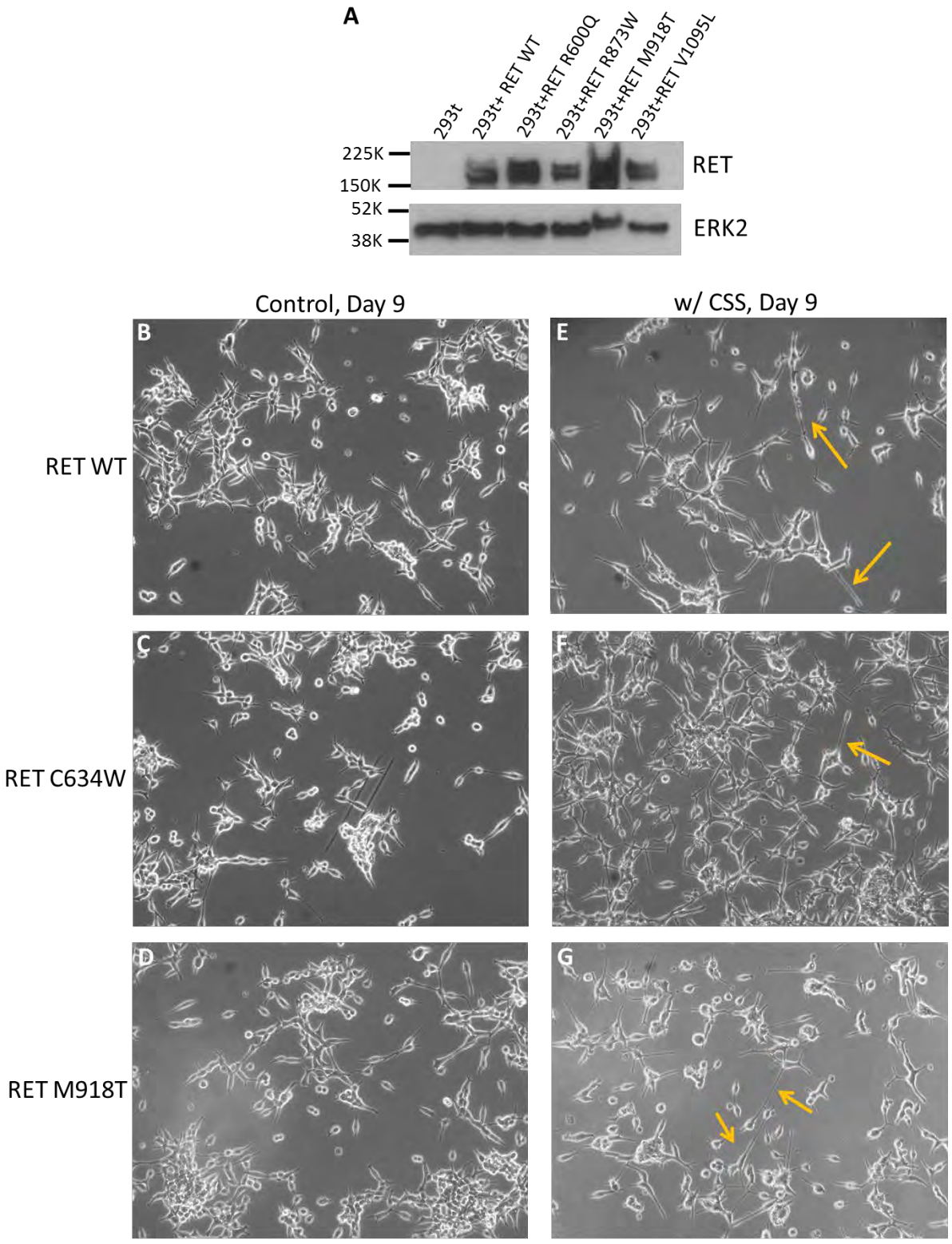


Figure 3. Overexpression of WT RET and RET mutants do not exacerbate the morphology of LNCaP cells. RET WT or activated RET mutants were added via lentiviral transduction to LNCaP cells and assessed for the development of a neuroendocrine phenotype. Total RET expression was measured (A) prior to transduction. LNCaP cells transduced with WT RET or mutant RET protein grown in normal media (Control, B-D) did not display a neuroendocrine phenotype. These same cells grown in charcoal stripped media (CSS, E-G) displayed a neuroendocrine but the addition of RET did not further enhance the morphological phenotype.

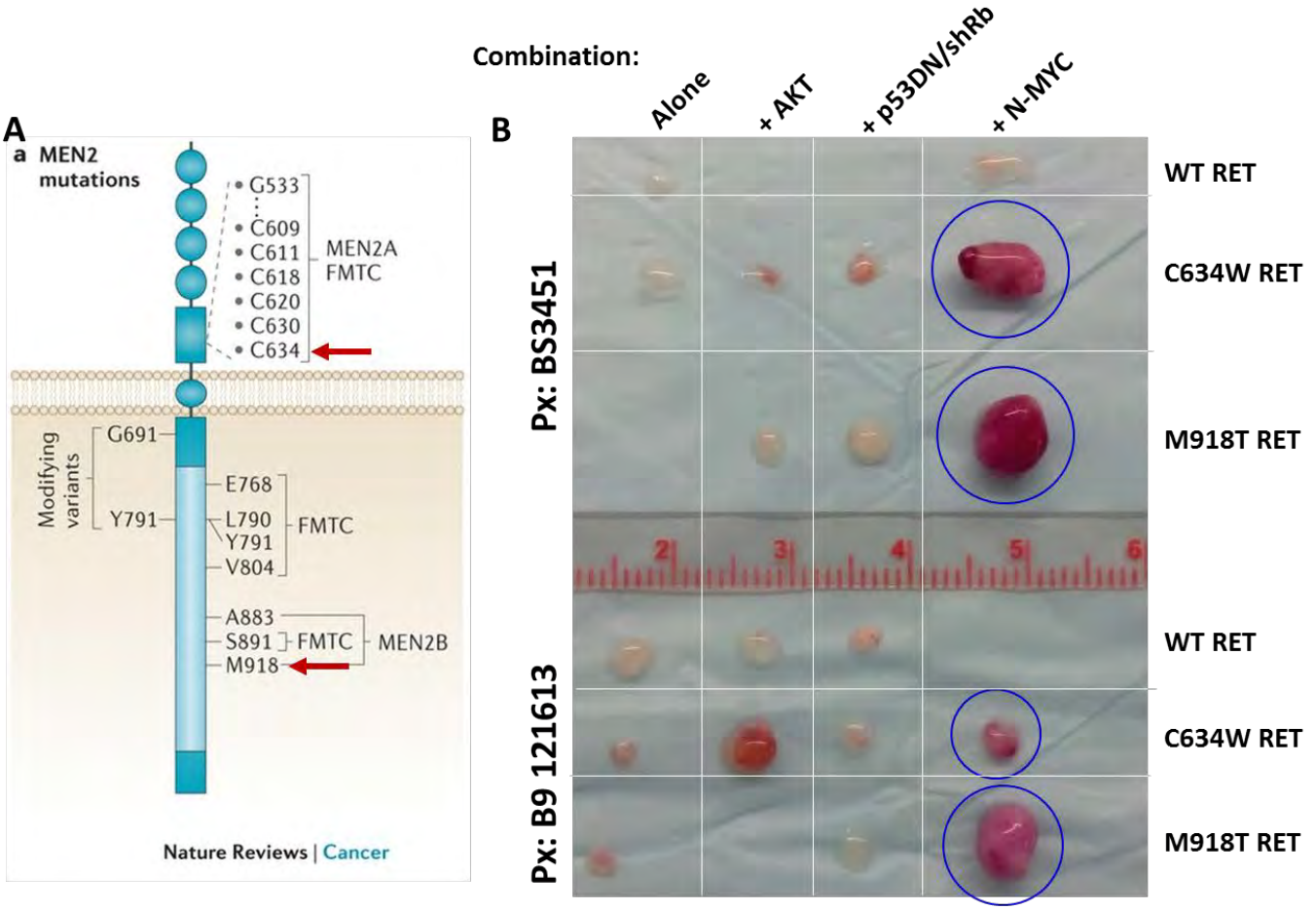


Figure 4. Development of a human tumor after overexpression of RET protein. (A) Diagram of different RET mutations observed in neuroendocrine tumors. Two mutations, RET C634W and RET M918T alters disulfide bonding, increasing dimerization and activates transforming and neuronal differentiation properties without constitutive dimerization, respecively. These mutants are consider constitutive active mutants and were tested initially in the human recombination tumor model. (B) Basal epithelial cells from two different human prostates were transduced with WT RET, RET C634W, or RET M918T alone or in combination with other oncogenic insults. The combination of RET M918T or RET C634W and N-MYC resulted in a robust outgrowth and was assessed for histology (blue circles). Areas with missing grafts indicate that no material was available for recovery.

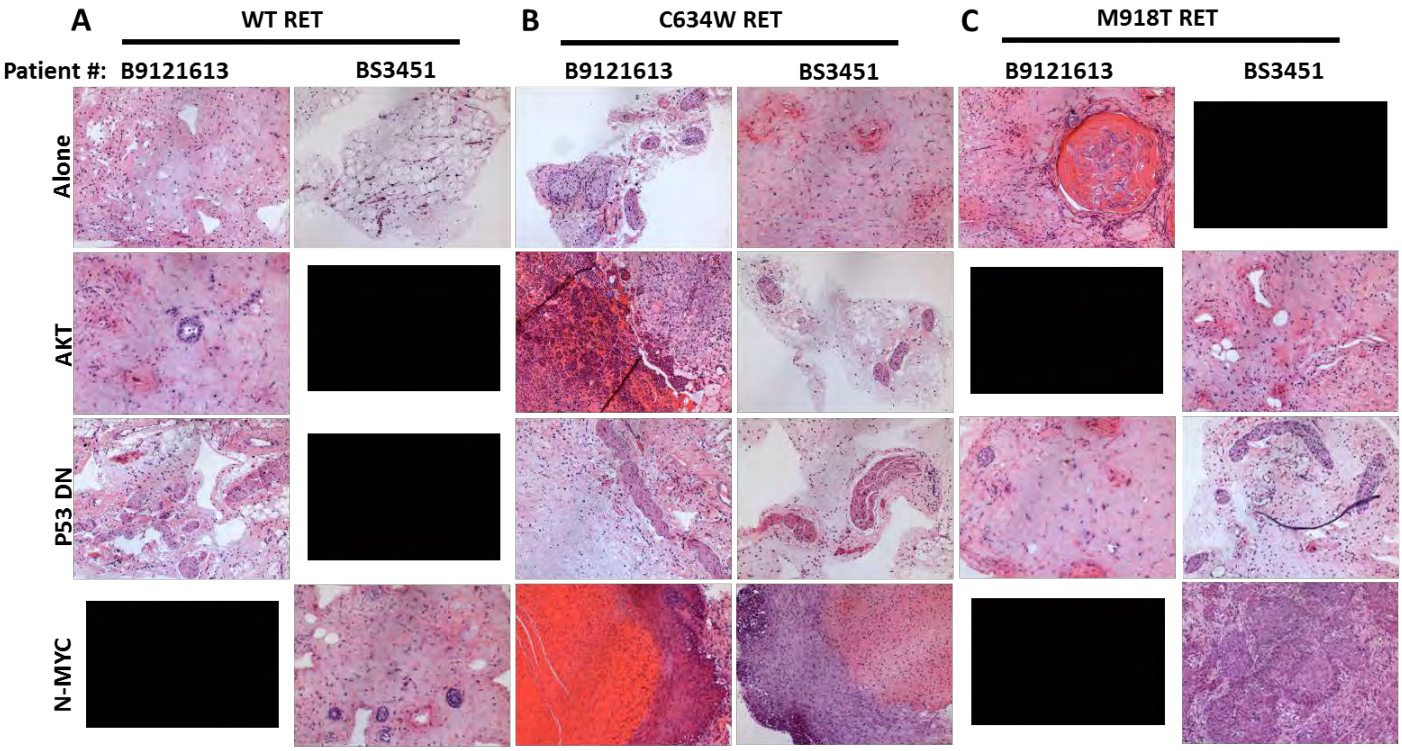


Figure 5. Histological evaluation of RET tumors. (A) WT RET (A), RET C634W (B), and RET M918T (C) alone or in combination with AKT or P53 dominant negative (P53DN) did not produce any appreciable phenotype. WT RET in combination with N-MYC produced small structures containing basal cells. However, RET C634W and RET M918T in combination with N-MYC resulted in much larger structures primarily consisting of squamous cell carcinoma. Very little adenocarcinoma was observed. Black boxes indicate no graft was available for histological evaluation.

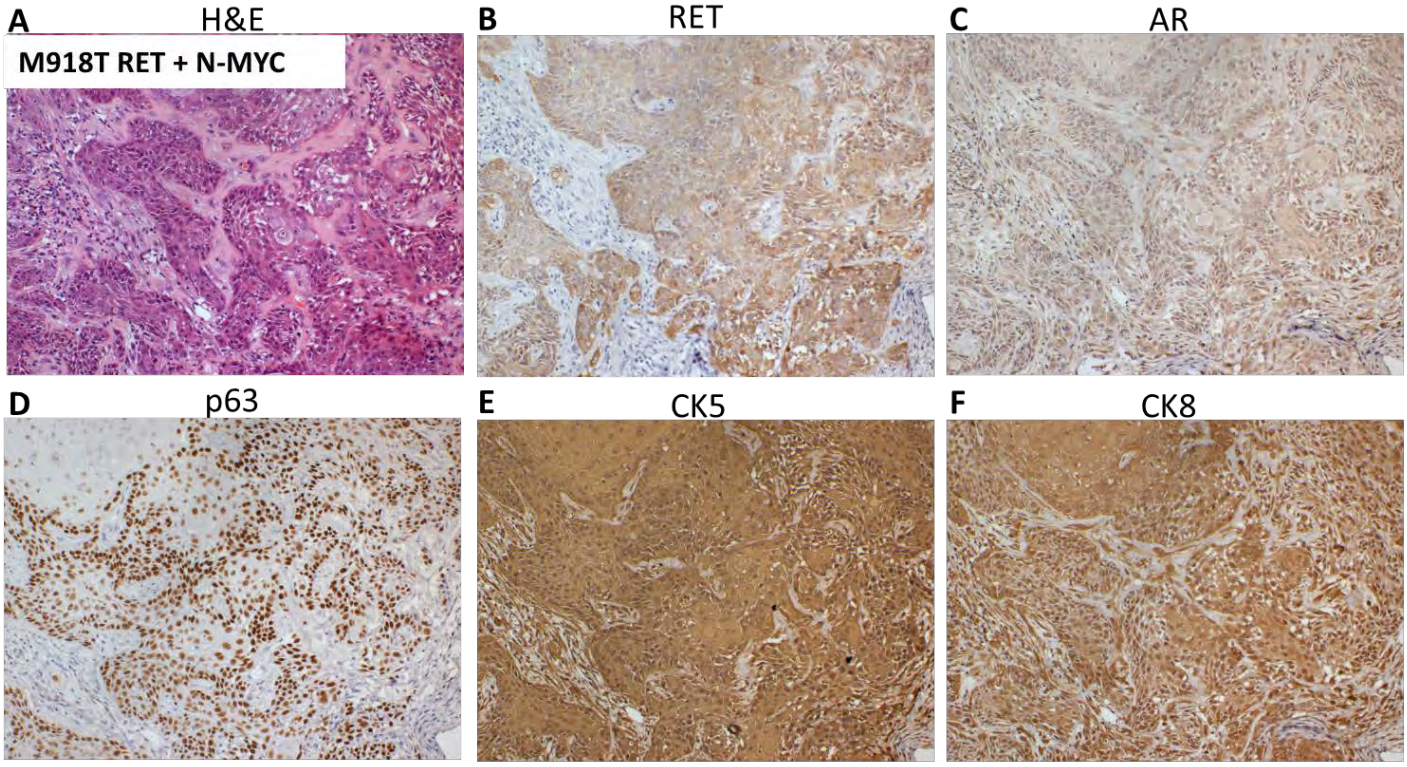


Figure 6. Human RET tumors contain cells of basal origin. RET M918T human tumor (A) was assessed for traditional histological markers of basal and luminal cells. These tumors expressed RET protein (B) and luminal markers with high CK8 (F) and low androgen receptor (AR) (C) as well as markers of basal cells with high p63 (D) and CK5 (E).

PIP5K1α inhibition as a therapeutic strategy for prostate cancer

Justin M. Drake[a,1] **and Jiaoti Huang**[b,c,d,1]

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The phosphatidylinositol 3 kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway is a highly conserved signaling pathway stretching from worms to humans (1). This signaling pathway regulates numerous cellular processes encompassing growth, survival, differentiation, and metabolism and is critical for the survival and growth of cancer (2). Aberrant activation of the PI3K/AKT pathway in cancer can occur through mechanisms such as pathway activating mutations, engagement of receptor tyrosine kinases (RTKs), and loss of tumor suppressors such as phosphatase and tensin homolog (*PTEN*). The many mechanisms by which this pathway is activated in cancer makes PI3K, AKT, and other components of this pathway intriguing targets for therapeutic intervention. In PNAS, Semenas et al. (3) discover a new chemical compound (ISA 2011B) that inhibits the upstream lipid kinase, PI 4 phosphate 5 kinase α (PIP5K1α), extending the arsenal of promising agents targeting the PI3K pathway that may be used therapeutically in advanced prostate cancer.

Prostate cancer results in nearly 30,000 deaths in men in the United States every year. Initially, prostate cancer relies on androgens for growth and will respond to conventional androgen deprivation therapies. However, this response is temporary, and recurrence eventually develops, leading to castration resistant prostate cancer (CRPC) and ultimately the death of the patient. Currently, there are few effective treatment options for CRPC, and many mechanisms have been proposed to explain its occurrence, including persistent androgen receptor (AR) signaling and PI3K pathway activation. Recently, two newly approved drugs, abiraterone acetate and enzalutamide, which inhibit intratumoral androgen synthesis and potently inhibit AR activation, respectively, have resulted in survival benefits for men with CRPC, although lethality in these patients still occurs (4, 5). Therefore, additional therapies must be developed to target alternative pathways in advanced prostate cancer (6, 7).

One of the pathways highly implicated in prostate cancer is the PI3K/AKT pathway. In prostate cancer, the PI3K pathway is activated primarily via the loss of the tumor suppressors *PTEN* or inositol polyphosphate 4 phosphatase II (*INPP4B*) and is aberrantly altered in nearly 100% of metastatic prostate cancer (8). The common signaling molecules studied in this pathway include several downstream players such as PI3K, AKT, and mTOR, whereas little is known about how the most upstream kinase, PIP5K1α, contributes to development, growth, or survival of prostate cancer.

PIP5K1α is part of a family of lipid kinases, and the α isoform predominantly generates PI 4,5 P_2 (PIP2) from PI 4 phosphate (PI4P). PIP2 is phosphorylated by PI3K to generate PI 3,4,5 P_3 (PIP3), which recruits the serine/threonine kinase AKT to the cell membrane, where it becomes activated, initiating signaling cascades. In this study, Semenas et al. (3) find that PIP5K1α is overexpressed in high grade prostate cancer, and its expression correlates with PIP2 and AR expression in these tissues. The authors further demonstrate that PIP5K1α contributed to malignant transformation in prostate cancer cell lines through increased invasion, enhanced stability or expression of AR, and expression of cellular proliferation markers such as cyclin dependent kinase 1 (CDK1). Using a high throughput kinase profiling assay, the authors discovered an inhibitor of PIP5K1α, ISA 2011B. ISA 2011B significantly reversed the protumorigenic effects of PIP5K1α by decreasing prostate cancer cell invasion (via reduced focal adhesion kinase activation), reducing AR and CDK1 expression, inhibiting AKT activation, and increasing apoptosis in vitro. Further, administration of ISA 2011B to mice harboring aggressive PC 3 tumors considerably inhibited growth over the course of 20 d without any measurable toxicity.

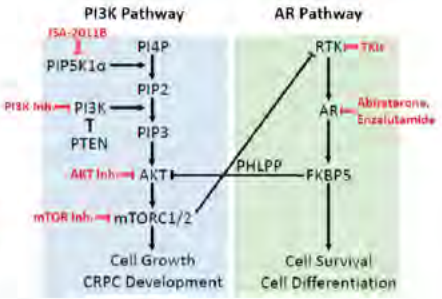

Fig. 1. Cotargeting the PI3K/AKT and AR pathways in CRPC. The administration of AR targeted agents such as abiraterone acetate and enzalutamide is thought to promote the development of castration resistance due to the loss of negative feedback regulation of the PI3K/AKT pathway. The development of the PIP5K1α inhibitor, ISA 2011B, adds another tool for targeting the PI3K pathway, as well as provide the impetus to cotarget with inhibitors of RTK and AR signaling pathways in CRPC. TKI, tyrosine kinase inhibitor.

The development of PI3K pathway inhibitors has been an intense area of focus for researchers and drug companies over the last decade, and the discovery of ISA 2011B may add to the repertoire of existing agents. Early phase clinical trials of pan PI3K and AKT inhibitors, such as perifosine, have shown reasonable toxicity profiles, with hyperglycemia being the most pronounced mechanism based toxicity (9). However, these inhibitors, when applied as single agents to patients with prostate cancer, have not clinically performed well (10). In this study, ISA 2011B displayed no adverse events after administration into mice, and it will be important to determine whether ISA 2011B has a different toxicity profile compared with PI3K or AKT inhibitors. Additionally, although the authors did not report the pharmacokinetics and pharmacodynamics of ISA 2011B in vivo, this information plus the toxicity profile will help to determine the future clinical utility of this compound as a therapeutic agent in cancer.

Author contributions: J.M.D. and J.H. wrote the paper.

The authors declare no conflict of interest.

See companion article 10.1073/pnas.1405801111.

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More recently, focus has turned to inhibition of more downstream effector targets such as mTOR. Due to a wide array of signaling inputs to mTOR, mTOR inhibitors can more broadly be applied to cancers not dependent on the PI3K/AKT pathway, increasing the utility of these inhibitors over PI3K and AKT agents. Allosteric mTOR inhibitors, such as everolimus, have not performed well in clinical trials for prostate cancer (11), but mTOR active site inhibitors, such as MLN0128 (INK128), outperformed everolimus in preclinical models of prostate cancer (12). The increased efficacy of mTOR active site inhibitors is attributed to targeting everolimus resistant substrates such as 4EBP1, as well as completely blocking both mTOR complexes (mTORC1 and C2) and eliminating AKT activation via feedback from S6 kinase (S6K) (13).

Where are we now in the field of PI3K pathway inhibitors for treatment of prostate cancer? There are several PI3K agents available, but to date, the clinical benefit has been limited if these compounds are used as a single agent. The lack of clinical benefit could be due to the activation of alternative signaling pathways such as AR or other kinase pathways. Indeed, development of CRPC may be due to the activation of the PI3K pathway during AR inhibitor therapy, suggesting cross talk between these two pathways (14, 15). As such, combination therapies targeting both the PI3K/AKT pathway and AR are beginning to be tested clinically (16). The development of combination strategies will be imperative to achieve the goal of significantly improving survival in patients suffering from cancer. The central dilemma seems to be in choosing the right combinations. In the PI3K pathway, several combination strategies are currently being or will be tested including PI3K/AKT inhibitors combined with mTOR inhibitors (vertical treatment approach) and PI3K/AKT/mTOR inhibitors with AR inhibitors (horizontal treatment approach) (Fig. 1). Hence, it would be worthwhile to investigate the effects of combination therapies using ISA 2011B with an AR inhibitor, or other pathway inhibitors, in preclinical models of prostate cancer and especially CRPC.

Semenas et al. find that PIP5K1α is overexpressed in high-grade prostate cancer, and its expression correlates with PIP2 and AR expression in these tissues.

Overall, the studies of Semenas et al. uncover the mechanism of an underappreciated lipid kinase in the PI3K pathway, PIP5K1α, and developed a novel inhibitor, ISA 2011B, that targets this kinase. This study will open up new avenues into the investigation of PIP5K1α as a therapeutic target in preclinical models of prostate cancer, in addition to other cancers dependent on the PI3K pathway for growth and survival. It will be important to evaluate this compound further, individually and in combination, in several preclinical models of advanced prostate cancer including genetically engineered mouse models (such as *PTEN*$^{-/-}$) (17), mouse and human tissue recombination cancer models (18 20), and CRPC models (21). If verified, this might be a new promising member in the ever expanding list of PI3K pathway specific inhibitors.

ACKNOWLEDGMENTS. J.M.D. is supported by the Department of Defense Prostate Cancer Research Program Grant W81XWH 14 1 0148. J.H. is supported by the Department of Defense Prostate Cancer Research Program Grants W81XWH 11 1 0227 and W81XWH 12 1 0206, University of California, Los Angeles, Specialized Program in Research Excellence in prostate cancer, National Cancer Institute Grants 1R01CA158627 and 1R01CA172603 01A1, West Coast Prostate Cancer Dream Team supported by Stand Up to Cancer/American Association for Cancer Research/Prostate Cancer Foundation, and Prostate Cancer Foundation Honorable A. David Mazzone Special Challenge Award.

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In Depth Phosphoproteomic Evaluation of Lethal Prostate Cancer Identifies Druggable Kinase Signaling Networks

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Metastatic castration resistant prostate cancer (CRPC) remains incurable due to the lack of effective therapies. The need to identify new actionable targets in metastatic CRPC is crucial as we begin to examine the resistance mechanisms related to androgen withdrawal. Although multiple metastases from the same patient share similar copy number, mutational status, ETS rearrangements, and methylation patterns supporting their clonal origins, it is not known whether actionable targets such as kinases are also similarly expressed and activated in anatomically distinct metastatic lesions of the same patient. We evaluated active kinases using phosphopeptide enrichment and quantitative mass spectrometry to identify therapeutic kinase networks in metastatic CRPC obtained at rapid autopsy. We identified distinct phosphopeptide patterns in metastatic tissues compared to naive primary prostate tissue and prostate cancer cell line-derived xenografts. Evaluation of metastatic CRPC samples for kinase targets revealed SRC, EGFR, RET, ALK, and MAPK1/3 and other activities while exhibiting intrapatient similarity and interpatient heterogeneity. Computational approaches merging metastatic CRPC transcriptomic and phosphoproteomic datasets revealed a highly interconnected network of activated kinases with transcriptional regulators. Interestingly, these kinase activities are not a result of mutation but rather pathway activation within the tumors themselves. In all, this suggests that individualized therapy targeting non-mutated kinases with clinical kinase inhibitors may be an effective strategy in the treatment of metastatic CRPC.