

AWARD NUMBER: W81XWH-19-1-0173

TITLE: Elucidating the Mechanism of RET Kinase Activity in Neuroendocrine Prostate Cancer

PRINCIPAL INVESTIGATOR: Halena VanDeusen

CONTRACTING ORGANIZATION: University of Minnesota

REPORT DATE: AUGUST 2020

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.

1. REPORT DATE AUGUST 2020		2. REPORT TYPE Annual		3. DATES COVERED 1 Aug 2019 – 31 Jul 2020	
4. TITLE AND SUBTITLE Elucidating the Mechanism of RET Kinase Activity in Neuroendocrine Prostate Cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-19-1-0173	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Halena VanDeusen E-Mail: hvandeus@umn.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Minnesota Minneapolis, MN 55455				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Development Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The increased treatment of metastatic castration resistant prostate cancer (mCRPC) with second-generation anti-androgen therapies (ADT) has coincided with a greater incidence of lethal, aggressive variant prostate cancer (AVPC) tumors that have lost dependence on androgen receptor (AR) signaling. These AR independent tumors may also transdifferentiate to express neuroendocrine lineage markers and are termed neuroendocrine prostate cancer (NEPC). Recent evidence generated to achieve the aims of this grant suggests RET kinase signaling is an important driver of NEPC. Genetic knockdown or pharmacological inhibition of RET kinase in multiple mouse and human models of NEPC dramatically reduced tumor growth and decreased cell viability. Our results suggest that targeting RET in NEPC tumors with high RET expression could be an effective treatment option. Identification of aberrantly expressed RET kinase as a driver of tumor growth in multiple models of NEPC provides a significant rationale for further understanding how NEPC tumors gain RET expression, defining the mechanism of RET activation, and validation of RET inhibitors alone or as a component of combination therapies to treat NEPC.					
15. SUBJECT TERMS RET kinase, castration resistant prostate cancer, aggressive variant prostate cancer, kinase inhibitors					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	23	19b. TELEPHONE NUMBER (include area code)

TABLE OF CONTENTS

	<u>Page</u>
1. Introduction	4
2. Keywords	4
3. Accomplishments	4
4. Impact	6
5. Changes/Problems	6
6. Products	7
7. Participants & Other Collaborating Organizations	7
8. Special Reporting Requirements	7
9. Appendices	7

INTRODUCTION

Second-generation ADT, such as abiraterone acetate and enzalutamide, have provided life-extending therapies for recurrent or mCRPC patients. However, the utilization of more effective ADT has coincided with an increase in the development of AVPC. The absence of AR signaling in AVPC renders the existing hormone targeting treatments ineffective and remaining approved therapies, including platinum-based chemotherapy, offer only limited therapeutic benefits. A subset of AVPC tumors are classified as NEPC because they express neuroendocrine genes, which are not typically expressed in prostate adenocarcinoma (AdCa). Receptor tyrosine kinases are an attractive treatment target because these kinases link the extracellular environment to intracellular responses frequently involved in cancer cell proliferation. Recently, RET kinase was identified to be tyrosine phosphorylated in a CRPC patient with small cell neuroendocrine pathology and as an enriched cell surface marker in NEPC. Further, RET knockdown reduced tumor growth of an AR-dependent cell line xenograft, LNCaP, *in vivo*. However, if RET inhibition could be exploited as a therapeutic target in the treatment of neuroendocrine prostate cancer was unknown. The overall objective of this grant is to determine if RET kinase is activated in NEPC and whether it directly contributes to the aggressive phenotype of NEPC. This was divided into two aims: **Aim 1.** Assess the role of RET activity in NEPC through knockdown of RET and identification of activating residues through mutation of activating residues in RET. **Aim 2.** Define bypass kinases that overcome pharmacological RET inhibition using phosphoproteomics and KSEA to identify activated signaling pathways in RET inhibitor resistant xenograft tumors. Currently, there are limited treatment options for patients with aggressive neuroendocrine prostate cancer and none are curative. Identification of aberrantly expressed RET kinase as a driver of tumor growth in multiple models of NEPC provides a significant treatment target and provides rationale for testing the clinical application of RET inhibitors in AVPC patients.

KEYWORDS

RET kinase, castration resistant prostate cancer, aggressive variant prostate cancer, kinase inhibitors

ACCOMPLISHMENTS

What were the major goals of the project?

Within this DOD grant, there were training specific and research specific tasks.

The training specific goal included one major task of:

Major Task 1: Training and educational development in prostate cancer research (Months 1-24, 50% completed)

Milestone 1: Publication of research results and presentation of project national meetings (Expected: Month 24, Actual: first publication in month 10)

The research specific tasks included:

Major Task 1: Generate tools to assess direct role of RET kinase in NEPC (Months 1-12, 50% complete)

Milestone #1: Gain ACURO approval from DOD for animal work (Expected: Month 3, Actual: still in progress)

Milestone #2: Select cell lines for in vivo model tumor growth experiments (Expected: Month 6-12, Actual: 6-12)

Major Task 2: Use NCI-H660 RET knockdown and phosphorylation mutants to define how RET activity influences NEPC (Months 9-12, 50% complete)

Milestone #1: Publish results on requirement of active RET kinase in the ability to sustain NEPC tumor growth (Expected: Month 18, Actual: First manuscript published in month 10)

Major Task 3: Phosphoproteomics of AD80 sensitive and resistant tumors (Months 6-12, 25% complete)

Milestone #3: Identify RET resistance networks for therapeutic targeting (Expected: Month 12, Actual: still in progress)

Major Task 4: Target bypass kinases in dual treatment in vivo experiments (Months 12-24, 0% complete)

Milestone #4: Publish results on mechanisms of resistance to RET inhibitors in NEPC (Expected: Month 24, Actual: still in progress)

What was accomplished under these goals?

Training Major Task 1:

1. Attend quarterly progress meetings with Dr. Drake and Dr. Dehm – Dr. VanDeusen has achieved this goal for the first year of her grant. She discussed her project with Dr. Drake and Dr. Dehm. The

main goal of these meetings was addressing concerns to prepare her RET research for publication, which was accepted into Molecular Cancer Research in early August 2020 (manuscript attached).

2. Present research at the monthly joint lab meetings between Drake and Dehm Labs – Dr. VanDeusen has achieved this goal by attending joint meetings between the two labs. Joint meetings are temporarily suspended due to COVID, but Dr. VanDeusen anticipates presenting her RET research and future directions when the meetings resume.

Research Major Task 1: Generate tools to assess direct role of RET kinase in NEPC

This goal is approximately 50% complete. The tools to identify RET kinase as a critical factor in NEPC cell growth were generated and published in a first author paper in Molecular Cancer Research in August 2020 (Figure 1, manuscript attached). Since the stable knockdown provided a characterizable phenotype, we did not pursue the generation of inducible or CRISPR knockout constructs.

The remaining 50% of tools include generating non-phosphorylatable mutants to identify the key residues required for activation of RET and kinase signaling in NEPC. We identified that RET kinase is active in NCI-H660 cells by immunoprecipitating RET kinase and probing for phosphorylated tyrosine residues (Figure 2A of attached manuscript). Interestingly, these phosphorylated residues are not Y905 or Y1062, which have commercially available phospho-specific antibodies and are well characterized (data not shown). We are shifting our approach to identify the phosphorylated residues to a mass spectrometry-based approach and will validate our findings using non-phosphorylatable mutants. We have purchased custom peptides that mimic trypsin digested RET kinase in the phosphorylated or non-phosphorylated states and use these peptides to generate a mass spectrometry library of fragmentation patterns that we will use to identify the phosphorylated residues of RET in NEPC. We anticipate achieving this goal in the next year of the grant.

Milestone #1: Gain ACURO approval from DOD for animal work – ACURO approval has not yet been granted. Dr. VanDeusen has not yet applied for ACURO approval as preparing the RET studies for publication took priority over initiating the mouse experiments outlined in the grant. We anticipate applying for ACURO approval early in the next year of the grant and anticipate no problems as her mentor, Dr. Justin Drake has achieved ACURO approval for other DOD sponsored grants in the lab.

Milestone #2: Select cell lines for in vivo model tumor growth experiments – PC-3 and NCI-H660 cells were selected for in vitro cell studies. Frozen stocks of these cells have been generated and will be utilized for in vivo tumor model growth experiments in the next year of the grant.

Research Major Task 2: Use NCI-H660 RET knockdown and phosphorylation mutants to define how RET activity influences NEPC

This goal is 50% complete. Using a knockdown approach, we showed that RET kinase is important for growth of NCI-H660 cells. We also utilized three RET inhibitors: AD80, LOXO-292, and BLU-667 to show that RET kinase activity is important for cell growth and nominate RET kinase as an attractive target for treating NEPC (Figure 2C and 2D). These results were published in Molecular Cancer Research in August of 2020.

In the next year of the grant, we will characterize how RET kinase phosphorylation regulates activity in NEPC using the technique outlined in Research Major Task 1.

Milestone #2: Publish results on requirement of active RET kinase in the ability to sustain NEPC tumor growth – This goal was achieved ahead of schedule at month 12 of the grant. The manuscript is attached to the progress report.

Research Major Task 3: Phosphoproteomics of AD80 sensitive and resistant tumors

This goal is 25% complete. We began the process of optimizing mass spectrometry sampling. Unfortunately, due to COVID processing and analysis of mass spectrometry samples at the University of Minnesota core facility has slowed. We anticipate that we will be able to analyze samples and identify resistance pathways in the next year of the grant.

Milestone #3: Identify RET resistance networks for therapeutic targeting – This goal is in progress and has been delayed due to COVID but will be achieved in the next year of this grant.

What opportunities for training and professional development has the project provided? In the first year of this grant, Dr. VanDeusen has had the opportunity to develop her mentoring skills by working with an undergraduate student and providing instruction on cloning and molecular biology techniques. Dr. VanDeusen also attended multiple seminars, both in person (Pharmacology Department and Masonic Cancer Center seminar series) and virtually (Prostate Cancer Foundation), to stay up to date on current prostate cancer research and novel techniques. Unfortunately, due to COVID, conferences that Dr. VanDeusen planned to attend were canceled, but she plans to attend virtual conferences in the next year of her grant. During COVID,

Dr. VanDeusen has also been increasing her knowledge of Python to help her independently analyze mass spectrometry data or other large data sets that she will generate as a part of this grant.

How were the results disseminated to communities of interest?

The results of this research were published in Molecular Cancer Research as a peer reviewed article in August of 2020. The article was featured as a highlight of the issue. Dr. VanDeusen also hopes to present this research at virtual conferences in the next year, including the Society for Basic Urologic Research Annual Meeting.

What do you plan to do during the next reporting period to accomplish the goals?

In the next reporting period, I will focus on completing the second half of Major Tasks 1 and 2 by identifying the phosphorylated residues of RET kinase in NEPC utilizing mass spectrometry and site directed mutagenesis. RET kinase inhibitor resistant tumors are banked in our freezer and will be analyzed by mass spectrometry to identify resistance kinase pathways to achieve Major Task 3. To test whether combination therapies can prevent resistance to RET inhibitors as part of Major Task 4, we will begin in organoid systems. In the first year of this grant, we were able to culture prostate cancer cells as organoids and established it as a technique in the Drake lab. Finally, after acquiring ACURO approval, we will use xenograft tumors to test combination therapies of RET kinase and organoid validated bypass kinase inhibitors.

IMPACT

What was the impact on the development of the principal discipline(s) of the project?

The research supported by this project identified RET kinase as a necessary protein for the growth of neuroendocrine prostate cancer cells (NEPC). By reducing the levels of RET kinase or treating cells with multiple RET kinase inhibitors, proliferation and activation of downstream signaling pathways were reduced. Identification of RET kinase as a driver of tumor growth in multiple models of NEPC provides a significant motivation to test RET inhibitors in clinical applications in NEPC patients.

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

The only change to the approach of this grant is in Major tasks 1 and 2. Instead of generating non-phosphorylatable mutants to identify the activated residues, we are now utilizing a mass spectrometry-based approach. After identifying phosphorylated residues on RET kinase in NCI-H660 cells, we will then use specific non-phosphorylatable mutants expressed in the knockdown cells to determine which residues are required for RET kinase activity. This is a minor change, that we hope will allow us to achieve our goal of identifying the key residues for RET activity directly.

The goal of having a publication by month 18 of the grant was achieved ahead of schedule in month 12. Unfortunately, productivity has slowed due to social distancing and additional safety considerations in place due to COVID. Dr. VanDeusen is approved to continue working in lab under the University of Minnesota's guidelines, but this may cause a delay in mouse and *in vivo* experiments.

Changes that had a significant impact on expenditures

Due to COVID, mass spectrometry and mouse experiments have been delayed. This has caused a significant reduction in the anticipated expenditures. Additionally, due to increased social distancing measures, some funds will be utilized to establish a secondary tissue culture site in the Drake Lab so that Dr. VanDeusen will have a dedicated space to move *in vitro* experiments forward.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report.

PRODUCTS

Publications, conference papers, and presentations

Journal Publications:

Halena R. VanDeusen, Johnny R. Ramroop, Katherine L. Morel, Song Yi Bae, Anjali V. Sheahan, Zoi Sychev, Nathan A. Lau, Larry C. Cheng, Victor M. Tan, Zhen Li, Ashley Petersen, John K. Lee, Jung Wook Park, Rendong Yang, Justin H. Hwang, Ilsa Coleman, Owen N. Witte, Colm Morrissey, Eva Corey, Peter S. Nelson, Leigh Ellis and Justin M. Drake; **Targeting RET Kinase in Neuroendocrine Prostate Cancer**; Molecular Cancer Research; 18: 2020; 1176-1188; (published); (yes)

Books or other non-periodical, one-time publications

Nothing to report.

Other publications, conference papers, and presentations

Nothing to report.

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

Name:	<i>Halena VanDeusen</i>
Project Role:	<i>Principal Investigator</i>
Researcher Identifier (e.g. ORCID ID):	<i>0000-0002-7950-2251</i>
Nearest person month worked:	<i>12</i>
Contribution to Project:	<i>Dr. VanDeusen has been responsible for research and manuscript preparation related to this award</i>
Funding Support:	

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 other organizations were involved as partners?

Nothing to report.

SPECIAL REPORTING REQUIREMENTS

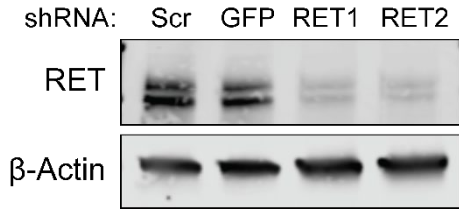
Nothing to report.

APPENDICES

- Figures 1 and 2
- Published Manuscript

Figure 1.

A.



B.

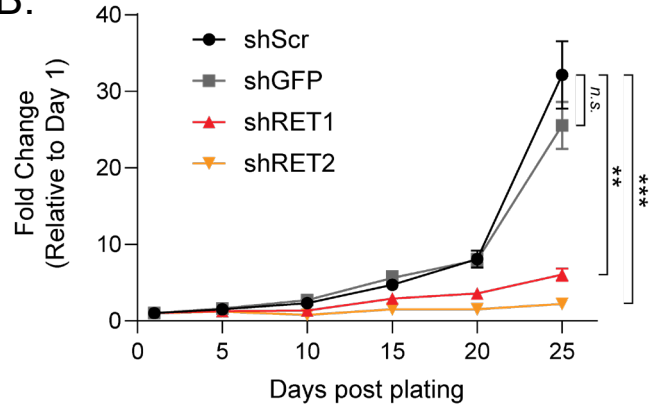


Figure 1. RET expression is important for NEPC cell line growth. A. RET protein expression in NCI-H660 cells stably transduced with scrambled (Scr), anti-GFP or two unique anti-RET shRNA. RET protein levels were reduced in two RET knockdown NCI-H660 cell lines and Actin serves as a loading control. D. RET knockdown reduces cellular proliferation in H660 cell lines. The line graph represents relative cellular proliferation as measured by WST assay of one biological replicate. Cell proliferation was analyzed by linear regression of log transformed data to determine statistical significance and error bars represent the standard deviation of five technical replicates.

Figure 2.

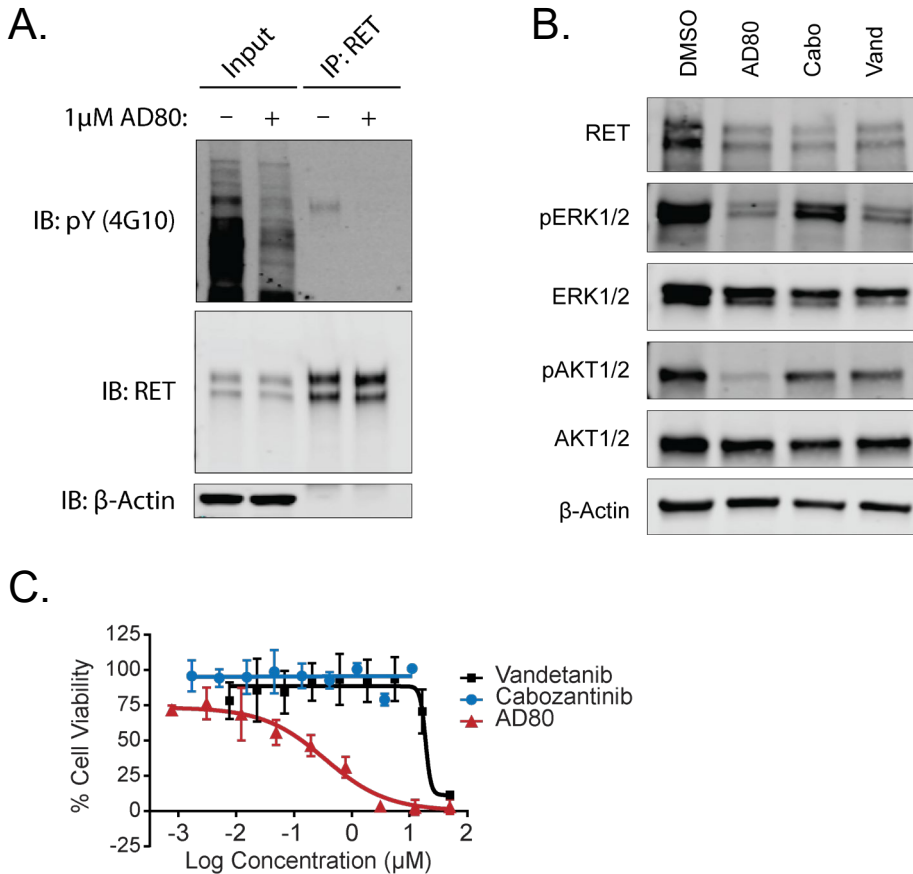


Figure 2. NCI-H660 cells are sensitive to RET inhibition and show greater relative sensitivity to AD80, compared to other RET multi kinase inhibitors.

A. Immunoprecipitation of RET kinase from H660 cells shows that AD80 reduces phosphorylation of tyrosines, as assayed with a total phosphotyrosine antibody 4G10. **B.** NCI-H660 cells treated for 4 hours with 1 μ M of AD80, cabozantinib, or vandetanib, showed reduced levels of RET kinase by western blot relative to DMSO controls. The signaling cascades downstream of RET were analyzed by phosphorylation of ERK1/2 at Try202/Tyr204 and phosphorylation of AKT1/2 at Ser473. The AD80 treatment reduced phosphorylation of both downstream targets to a greater extent than cabozantinib or vandetanib while the total ERK1/2, total AKT1/2 and Actin loading control remained unaffected. **C.** IC₅₀ dose response curves show that NCI-H660 cells are refractory to commonly utilized RET inhibitors vandetanib and cabozantinib but are sensitive to AD80. Error bars represent \pm standard deviation.

Targeting RET Kinase in Neuroendocrine Prostate Cancer



Halena R. VanDeusen¹, Johnny R. Ramroop², Katherine L. Morel³, Song Yi Bae¹, Anjali V. Sheahan³, Zoi Sychev¹, Nathan A. Lau⁴, Larry C. Cheng⁵, Victor M. Tan⁵, Zhen Li⁶, Ashley Petersen⁷, John K. Lee^{4,8}, Jung Wook Park^{9,10}, Rendong Yang¹¹, Justin H. Hwang^{12,13}, Ilsa Coleman⁴, Owen N. Witte¹⁰, Colm Morrissey¹⁴, Eva Corey¹⁴, Peter S. Nelson^{4,8}, Leigh Ellis^{3,13}, and Justin M. Drake^{1,15,16}

ABSTRACT

The increased treatment of metastatic castration-resistant prostate cancer (mCRPC) with second-generation antiandrogen therapies (ADT) has coincided with a greater incidence of lethal, aggressive variant prostate cancer (AVPC) tumors that have lost dependence on androgen receptor (AR) signaling. These AR-independent tumors may also transdifferentiate to express neuroendocrine lineage markers and are termed neuroendocrine prostate cancer (NEPC). Recent evidence suggests kinase signaling may be an important driver of NEPC. To identify targetable kinases in NEPC, we performed global phosphoproteomics comparing several AR-independent to AR-dependent prostate cancer cell lines and identified multiple altered signaling pathways, including enrichment of RET kinase activity in the AR-independent cell lines. Clinical NEPC patient samples and

NEPC patient-derived xenografts displayed upregulated RET transcript and RET pathway activity. Genetic knockdown or pharmacologic inhibition of RET kinase in multiple mouse and human models of NEPC dramatically reduced tumor growth and decreased cell viability. Our results suggest that targeting RET in NEPC tumors with high RET expression could be an effective treatment option. Currently, there are limited treatment options for patients with aggressive neuroendocrine prostate cancer and none are curative.

Implications: Identification of aberrantly expressed RET kinase as a driver of tumor growth in multiple models of NEPC provides a significant rationale for testing the clinical application of RET inhibitors in patients with AVPC.

Introduction

Second-generation ADT, such as abiraterone acetate and enzalutamide, have provided life-extending therapies for patients with

recurrent or mCRPC. However, the utilization of more effective ADT has coincided with an increase in the development of AVPC (1). This subset of mCRPC is characterized by poor prognosis and loss of AR signaling (2). The absence of AR signaling in AVPC renders the existing hormone targeting treatments ineffective and remaining approved therapies, including platinum-based chemotherapy, offer only limited therapeutic benefits (3). A subset of AVPC tumors are classified as NEPC because they express neuroendocrine genes, which are not typically expressed in prostate adenocarcinoma. Recent work has implicated the loss of *RBI* and *TP53* mutations as key alterations in the development of NEPC, and inhibition of kinases such as Aurora A kinase (AURKA), MAPK, or FGFR could provide therapeutic opportunities if selected in the right patient subsets (1, 4–6). Even with these new developments, there still remains a critical need to understand the molecular characteristics and kinase signaling pathways of NEPC tumors to identify and validate effective treatment options.

Receptor tyrosine kinases link the extracellular environment to intracellular responses through multiple signaling cascades. These signaling cascades regulate numerous pathways that are frequently altered in transformed cells, including cell growth, metabolism, proliferation, differentiation, invasion, motility, and cell death (7). RET is a receptor tyrosine kinase that is essential for neural crest development and is frequently mutated or translocated in subsets of endocrine tumors such as multiple endocrine neoplasia 2 (MEN2) and papillary thyroid carcinomas, respectively (8). RET can be therapeutically targeted with some success in these tumor types. Recently, RET kinase was identified to be tyrosine phosphorylated in a CRPC patient with small-cell neuroendocrine pathology (9) and as an enriched cell surface marker in NEPC (10). Furthermore, RET knockdown reduced tumor growth of an AR-dependent cell line xenograft, LNCaP, *in vivo* (11). However, whether RET inhibition could be exploited

¹Department of Pharmacology, University of Minnesota-Twin Cities, Minneapolis, Minnesota. ²Departments of Cancer Biology and Genetics, Comprehensive Cancer Center, The Ohio State University, Columbus, Ohio. ³Department of Oncologic Pathology, Dana-Farber Cancer Institute, Boston, Massachusetts. ⁴Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle, Washington. ⁵Graduate Program in Quantitative Biomedicine, School of Graduate Studies, Rutgers University, New Brunswick, New Jersey. ⁶Cancer Metabolism and Growth Program, Rutgers Cancer Institute of New Jersey, New Brunswick, New Jersey. ⁷Division of Biostatistics, School of Public Health, University of Minnesota-Twin Cities, Minneapolis, Minnesota. ⁸Department of Medicine, University of Washington, Seattle, Washington. ⁹Department of Pathology, Duke School of Medicine, Duke University, Durham, North Carolina. ¹⁰Department of Microbiology, Immunology, and Molecular Genetics, University of California-Los Angeles, Los Angeles, California. ¹¹The Hormel Institute, University of Minnesota, Austin, Minnesota. ¹²Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts. ¹³Broad Institute of Harvard and MIT, Cambridge, Massachusetts. ¹⁴Department of Urology, University of Washington, Seattle, Washington. ¹⁵Department of Urology, University of Minnesota-Twin Cities, Minneapolis, Minnesota. ¹⁶Masonic Cancer Center, University of Minnesota-Twin Cities, Minneapolis, Minnesota.

Note: Supplementary data for this article are available at Molecular Cancer Research Online (<http://mcr.aacrjournals.org/>).

H.R. VanDeusen and J.R. Ramroop contributed equally to this article.

Corresponding Author: Justin M. Drake, University of Minnesota Medical School, 6-120 Jackson Hall, 321 Church St SE, Minneapolis, MN 55455. Phone: 612-624-7151; E-mail: jdrake@umn.edu

Mol Cancer Res 2020;18:1176–88

doi: 10.1158/1541-7786.MCR-19-1245

©2020 American Association for Cancer Research.

as a therapeutic target in the treatment of neuroendocrine prostate cancer is unknown.

Here, we evaluated the phosphoproteome of multiple AR-independent and AR-dependent prostate cancer cell lines to identify altered kinase signaling pathways that are unique to AR-independent prostate cancers. Several downstream signaling networks of RET kinase, and RET kinase itself, were enriched and activated in the AR-independent cell lines when compared with AR-dependent cell lines. In addition, RET kinase was overexpressed in NEPC tumors in multiple clinical datasets. We found that the NEPC cell line, NCI-H660, was dependent on RET expression for proliferation and that targeted RET pathway inhibitors, AD80, and two other inhibitors currently being evaluated in the clinic, LOXO-292 and BLU-667 (12, 13), potentially induced cell death more effectively than currently approved RET inhibitor therapies, cabozantinib and vandetanib (14, 15). Finally, we found that AD80, LOXO-292, and BLU-667, were effective in inducing cell death in NEPC organoid models and AD80 was able to reduce tumor growth of NEPC xenograft tumor models. These results indicate that RET kinase is required for tumor growth of several models of NEPC, and that inhibiting RET induces cell death in neuroendocrine prostate cancer cells that are resistant to current ADT therapies. These results ultimately nominate RET as a key candidate to test further in the development and effective treatment of NEPC.

Materials and Methods

Phosphoproteomics of prostate cancer cell lines

Cultured prostate cancer cells were scraped, pelleted, and snap frozen. Phosphopeptide enrichment and trypsin digestion were performed as described previously (16). Briefly, cells were lysed in 6 mol/L guanidium hydrochloride buffer (6 mol/L guanidinium chloride, 100 mmol/L Tris pH 8.5, 10 mmol/L Tris (2-carboxyethyl) phosphine, 40 mmol/L 2-chloroacetamide, 2 mmol/L Vanadate, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L Beta-glycerophosphate, 10 mg/mL N-octyl-glycoside), sonicated, and cleared. Five milligrams of total protein was digested with trypsin and a 4G10 antibody-based immunoprecipitation (IP) was used to enrich phosphotyrosine peptides. The IP supernatant containing the phosphoserine/threonine (pS/T) peptides (2.5 mg) were desalted on C18 columns and separated via strong cation exchange chromatography. In separate, parallel reactions the pY and pS/T peptides were enriched from nonphosphorylated peptides using titanium dioxide columns. Finally, the pY and pS/T peptides were each desalted with C18 tips prior to mass spectrometer analysis (LC/MS-MS with a dual pump nanoRSLC system (Dionex) interfaced with a Q Exactive HF (Thermo Fisher Scientific; ref. 17). Technical duplicates were run for all samples and data were analyzed using MaxQuant Andromeda version 1.5.3.30 (parameter settings in ref. 18) against the Uniprot human reference proteome database with canonical and isoform sequences (downloaded September 2016 from <http://uniprot.org>). Datasets are accessible through dataset identifiers PXD012970 and PXD012971 (19) through the ProteomeXchange Consortium via the PRIDE partner repository.

Phosphoproteome MS data analysis was performed as described previously (20). For supervised clustering, pY and pS/T data were filtered using a 4-fold change cutoff comparing NEPC versus adenocarcinoma from the original excel tables (See Supplementary Tables S2 and S3). We expanded upon our previously published mCRPC dataset (PXD002286) by decreasing the phosphosite localization probability cutoff from 0.99 to 0.75 (16). This increased our identifications nearly 50% and have now reported those extra identifications in this manuscript as Supplementary Table S6. Hierarchical clustering was per-

formed on mass spectrometry and gene expression data using Cluster (Version 3.0) with the Pearson correlation and pairwise complete linkage analysis (21). Java TreeView version 1.1.6r4 was used to visualize clustering results (22).

Kinase substrate enrichment analysis

KSEA was performed as described previously (23). Briefly, phosphopeptides were rank-ordered by average fold change between AR-independent (AVPC) versus AR-dependent (adenocarcinoma) prostate cancer cell lines. An enrichment score was calculated using the Kolmogorov–Smirnov statistic and statistical significance was calculated via permutation analysis. The normalized enrichment score (NES) was calculated by taking the enrichment score and dividing by the mean of the absolute values of all enrichment scores from the permutation analysis. The Benjamini–Hochberg procedure was utilized to calculate false discovery rate for each kinase. For pY analyses, cutoffs of FDR<0.05, hits>4, and NES>1.3 were used. For pS/T analyses, cutoffs of FDR<0.02, hits>5, and NES>2 were used.

Tissue culture

Human prostate cancer cell lines LNCaP, VCaP, C4-2, 22Rv1, DU-145, PC3, and NCI-H660 cells were obtained from the ATCC. Cell lines were validated annually by Promega PowerPlex16HS Assay at the University of Arizona Genetics core and cells were tested for Mycoplasma contamination by PCR amplification every three months (24). Cells were not used beyond fifteen passages without reauthentication. LNCaP, VCaP, C4-2, 22Rv1, DU145, and PC3 cells were grown in appropriate media as recommended by ATCC (Life Technologies) supplemented with 10% FBS (Sigma-Aldrich) and 1% penicillin–streptomycin (Life Technologies). NCI-H660 cells were grown in Advanced DMEM/F12 (Gibco), with 1× B27 Supplement (Gibco), 10 ng/mL EGF (PeproTech), 10 ng/mL bFGF (PeproTech), and 1% penicillin–streptomycin 1× Glutamax (Life Technologies). LASCPC-01, cMyc/myrAKT, PARCB-1-3, and -5, and EF1 cell lines were obtained from Dr. Owen Witte at UCLA and cultured as described previously (10, 25–27). H660 organoids were cultured as described in ref. 28. Mouse organoids were established by enzymatic digestion of GEMM primary prostate tumor tissue in 5 mg/mL Collagenase type II (Gibco) in DMEM/F12 (Gibco) media with 10 μmol/L Y-27632 dihydrochloride (Tocris Bioscience). Digested cells were seeded into 100% Matrigel and cultured as described by Drost and colleagues 2016 (ref. 28). NCI-H660 organoids were seeded into Prostate 18 QGel 3D Matrix (QGel) according to manufacturer's instructions and cultured in RPMI-HITES media with B27 supplement (Gibco), 1.25 mmol/L N-acetylcysteine (Sigma), 5 ng/mL EGF (PeproTech), 500 nmol/L A83-01 (Tocris Bioscience), 5 ng/mL FGF2 (PeproTech), 10 ng/mL FGF10 (PeproTech), 10 mmol/L Nicotinamide (Sigma), and 1 μmol/L Prostaglandin E2 (Tocris Bioscience). Culture media was replenished every 4 days and organoids were passaged by sequential digestion in 1 mg/mL Dispase II (Gibco) followed by TrypLE Express (Gibco) and mechanical disruption through a needle to dissociate to single cells before resuspension as a 3D culture. RET immunofluorescence in SKO and DKO organoids was followed standard staining procedures using the RET antibody (Cell Signaling Technology E1N8X, 1:100). All cells were grown and maintained in a humidified incubator at 37°C and 5% CO₂.

Dependency analysis

Gene dependency data is based on pooled genome-scale shRNA screens from DEMETER-adjusted (29) Project Achilles 2.201 data (30). DEMETER scores for RET was ranked for all cell lines was ranked and

VanDeusen et al.

plotted across 503 cell lines. Of the same data, to statistically compare the patterns of RET dependency in 8 prostate cancer cell lines to other genes, we ranked the DEMETER score of 11,280 genes in eight prostate cell lines and computed the spearman correlation coefficient for each gene dependency relative to RET dependency.

Generating stable RET knockdown cell lines and cell growth assay

pLKO.1 scramble shRNA and shGFP plasmids were a gift from David Sabatini (Addgene plasmid #1864 and #30323) and two pLKO.1-shRET plasmids (RET1: CCACCCACATGTCATCAAATT, RET2: GGGCGACCGTACATGACTATA) used to generate the Project Achilles dataset were kindly provided by laboratory of Dr. William C. Hahn (Dana-Farber Cancer Institute, Boston, MA) from the RNAi Consortium at the Broad Institute. Lentiviral particles were generated by transfecting 293T cells with 13 μ g pMDL, 5 μ g pRev, 7 μ g pVSVg, and 20 μ g pLKO.1 shRNA plasmid using calcium phosphate. NCI-H660 or PC3 cells were transduced with lentivirus in the presence of polybrene (10 μ g/mL). After 72 hours of infection, stable cells were selected by puromycin (0.5 μ g/mL for NCI-H660 cells; 1 μ g/mL for PC3 cells).

Stable cells were seeded into 96-well plates at cell density of 1,000 cells/well ($n = 5$) for NCI-H660-derived cell lines and 200 cells/well ($n = 3$) for PC3-derived cell lines. Then, cells were cultured for indicated length of days. Cell culture media of NCI-H660 and PC3-derived stable cell lines was replenished every 5 days or 3 days, respectively. Cell proliferation was measured every 5 days for NCI-H660-derived stable cell lines and every 2 to 3 days for PC3-derived stable cell lines using WST reagent (Takara).

Immunoprecipitation and Western blots

Cells for Western blot analysis were lysed with 1% SDS/2% β -mercaptoethanol (β -ME) and boiled for 10 minutes following a freeze thaw after lysis. The protein concentration was determined using Bio-Rad Quick Start Bradford Protein Assay Kit following manufacturer's protocol. Twenty micrograms of protein per lane was loaded into GenScript SurePage 4% to 12% gel, transferred to a nitrocellulose membrane, blocked in 5% BSA in 1xTBST for one hour at room temperature before incubating in primary antibodies (diluted in 1% BSA in TBST) overnight at 4°C. Membranes were washed with 1x TBST before incubating in LI-COR IR-conjugated secondary antibodies (diluted 1:5,000) for 1 hour at room temperature, washed again and imaged using the LI-COR Odyssey System and adjusted with the LI-COR Image Studio Lite software (v5.2). The following antibodies were used for Western blot analysis at 1:1,000 fold dilutions unless otherwise indicated: Total RET (Cell Signaling Technology E1N8X), phospho-ERK1/2 T202/Y204 (Cell Signaling Technology D13.14.4E), total ERK1/2 (Cell Signaling Technology 137F5), pAKT1/2 S473 (Cell Signaling Technology D9E), AKT (Cell Signaling Technology, C67E7), phospho-tyrosine (Millipore Sigma 4G10, 1:500), AR (Santa Cruz Biotechnology, sc-7305, 1:500), α -Tubulin (Santa Cruz Biotechnology, sc32233), and β -actin (Cell Signaling Technology 13E5, 1:5,000).

For immunoprecipitation analysis, cells were lysed with cell lysis buffer containing 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L Na₂EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L beta-glycerophosphate, 1 mmol/L Na₃VO₄, 1 μ g/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride. The protein concentration was determined by bicinchoninic acid protein assay (Pierce) according to the manufacturer's instructions. The immunoprecipitation was performed using Dynabeads Protein A (Life Technologies) following the manufacturer's protocol with modifica-

tion. Briefly, total RET (Cell Signaling Technology C31B4, 1:50) antibody was preincubated with the beads overnight at 4°C. Then, equal amount of each cell lysate was incubated with the RET antibody-conjugated beads overnight at 4°C. After washing the bead-RET antibody-antigen complex four times with cell lysis buffer, the antigen was eluted with 2x Laemmli Sample Buffer (Bio-Rad)/5% β -ME by heating at 95°C for 5 minutes before analysis by Western blot analysis.

LD₅₀ value measurement

AD80, BLU-667, cabozantinib, and vandetanib were all obtained from Selleckchem and LOXO-292 was obtained from MedChemExpress. All drugs were resuspended in DMSO. Cells were treated with drug for 72 hours prior to the WST assay and viability was measured using the WST reagent (Takara) following manufacturer's protocol. Each concentration data point was conducted in triplicate. Each compound was tested at a minimum of ten dose levels, separated by 4-fold dilution concentration intervals. LD₅₀ values were calculated using GraphPad Prism 7. Reported values were calculated from a single WST assay, but were confirmed by repeating the entire assay in duplicate.

Organoid dose response

For assays, organoids were seeded as single cells in 40 μ L of 33% Matrigel (mouse organoids) or Prostate 18 QGel 3D Matrix (NCI-H660 organoids) in 96-well tissue culture plates and cultured for 2 days at 37°C to allow organoid formation. Once formed, organoids were treated with AD80 (at concentrations of ranging from 0.1 μ mol/L to 30 μ mol/L), LOXO-292 or BLU-667 (at concentrations ranging from 0.01 μ mol/L to 100 μ mol/L), or 10 μ mol/L enzalutamide (Medchem-Express) for 72 hours. After treatment, cells were stained with 10 μ L ReadyProbes Cell Viability Imaging Kit Blue/Red (Invitrogen) per well for 30 minutes at room temperature and z-stack images of stained cells were taken using an EVOS FL Auto 2 Cell Imaging System (Invitrogen). The percentage of cell death was calculated by identifying the percentage of PI-positive cells per organoid in at least 10 organoids for each treatment condition and the LD₅₀ was calculated in GraphPad Prism 7.

In vivo studies

Experiments were carried out on 8-week-old male NOD-SCID mice in accordance with Institutional Animal Care and Use Committee (IACUC)-approved protocols at Rutgers University (New Brunswick, NJ). Xenografts were generated via subcutaneous injection of 1×10^6 NCI-H660 cells per animal mixed at a 1:1 ratio with Corning Matrigel Matrix into the right flank. Tumors were allowed to grow to approximately 100–200 mm³ before mice were randomly allocated into vehicle (5% DMSO) or AD80 (10 mg/kg/day in the first experiment or 20 mg/kg/day in the second experiment) treatment groups. Dosing proceeded once daily, 5 days a week for 22 days by oral gavage. Tumor volume and animal weight were measured every two days. Tumors volume was measured by caliper and expressed in mm³ (tumor volume = $0.5 a \times b^2$, where a and b represents long and short diameter, respectively) and maximal tumor volume permitted by Rutgers University IACUC was never exceeded.

IHC

Xenograft tumors were formalin-fixed paraffin-embedded and sectioned following standard procedure. To stain, sections were deparaffinized by baking at 65°C for 1 hour and hydrated with sequential washes in xylenes, 100% ethanol, 95% ethanol, 70% ethanol, and 1x PBS, prior to citrate buffer pH 6.0 antigen retrieval. To stain,

tissues were washed with 0.1% TBST, blocked with 2.5% normal horse serum for 1 hour at room temperature before incubating in primary antibody (RET: Cell Signaling Technology E1N8X, 1:500 and Ki67: Cell Signaling Technology 8D5, 1:400) overnight at 4°C in a humidified slide box. Slides were washed with 0.1% TBST and incubated in HRP-conjugated secondary antibody (Vector Laboratories, MP-7500-15) for 1 hour at room temperature and developed using a DAB peroxidase substrate kit (Vector Laboratories, NC9567138). Reaction was stopped with water before proceeding to counterstaining with hematoxylin for 1 minute. Slides were destained in tap water, dehydrated with ethanol and xylenes, and mounted. Tumor sections were imaged on a Zeiss Axiovert A2. Average RET or Ki67 staining was determined by color deconvolution followed by measurement of the mean gray value in the DAB channel in Fiji (31). Mean gray value was converted to optical density with the following equation: $OD = \text{Log}(\text{Max gray value}/\text{Mean gray value})$. Values for images from five distinct fields of view were averaged to create a single data point for each tumor in each treatment group.

Terminal deoxynucleotidyl transferase dUTP nick end labeling assay

The Click-iT Plus TUNEL Assay for In Situ Apoptosis Detection, Alexa Fluor 488 Kit was used according to the manufacturer's protocol (Invitrogen). Nuclei were counterstained with Hoechst 33342 (Thermo Fisher Scientific). A DNase-treated positive control section was incubated in 1 U of DNase I diluted into 1× DNase I Reaction Buffer (20 mmol/L Tris-HCl, pH 8.4, 2 mmol/L MgCl₂, 50 mmol/L KCl) for 30 minutes at room temperature (Invitrogen). The Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells in tissue sample slides were identified by comparing with the DNase-treated positive control and the no-TdT enzyme negative control. Percent TUNEL-positive area was determined by using Fiji to measure the TUNEL-positive area divided by total tumor area × 100 for each tumor. Adjacent tissue sections were stained with hematoxylin and eosin by the University of Minnesota Clinical and Translational Science Institute Histology & Research Laboratory.

Statistical analysis

For xenograft tumor volume experiments, means and confidence intervals (CI) were calculated on the log scale due to skew and reported in terms of geometric means after exponentiation. Tumor growth rates were fit with a linear mixed effect model in R. All other statistical analyses and Pearson correlations were performed using GraphPad Prism 7 with the tests indicated in the figure legends. $P < 0.05$ was considered to indicate a statistically significant difference. P values were determined with significance indicated as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$.

Results

AR-independent cell lines have altered phospho-tyrosine and phospho-serine/threonine kinase signaling pathways

To identify the unique kinase signaling pathways required for growth and proliferation of AR-independent prostate cancer, we performed phospho-proteomic profiling. We compared AR-dependent cell lines (LNCaP, VCaP, C4-2, and 22Rv1), to AR-independent cell lines that are resistant to ADT and harbor mutations commonly found in NEPC tumor samples [DU145, PC3, NCI-H660, cMyc/myrAKT, LASCPC-01 (26), EF-1 (10), and PARCB-1,-2,-3, and -5 (27); Supplemental Fig. 1 and Supplemental Table S1]. Supervised hierarchical clustering between the AR-dependent and AR-independent groups revealed

distinct patterns in phospho-serine/threonine (pS/T) and phospho-tyrosine (pY) peptides (Fig. 1A and B, respectively; Supplementary Tables S2 and S3). Kinase substrate enrichment analysis (KSEA) identified AURKA as the most highly enriched pS/T kinase (Fig. 1C) and this kinase has been previously reported to be significantly upregulated in NEPC (4). Interestingly, among the tyrosine kinases, RET kinase was also significantly enriched (Fig. 1D), suggesting that RET kinase is activated in AR-independent cell lines (full pS/T and pY KSEA results are in Supplementary Tables S4 and S5, respectively). We confirmed that the RET protein is highly upregulated in the NEPC subset of AR-independent cell lines (Supplemental Fig. S1). Further investigation into the RET pathway via our cell line-derived and previously published mCRPC rapid autopsy phosphoproteomic datasets (9) (expanded phosphoproteome dataset in Supplementary Table S6, see Materials and Methods) identified hyperphosphorylation and, in some cases, activation, of several RET pathway targets including MAPK, AKT, and STAT3 (Fig. 1E and F), further confirming RET pathway activity in AVPC cell lines and tumors.

RET kinase gene expression is upregulated in patients with neuroendocrine prostate cancer

We took advantage of several clinical prostate cancer gene expression datasets to determine whether RET kinase was overexpressed along with known markers of NEPC. Analysis of the University of Washington rapid autopsy dataset (32) which contains multiple metastatic tumors from patients with CRPC revealed that the NEPC (AR-negative, NE-positive) subset had enrichment of RET kinase expression concomitant with increased neuronal lineage genes *ASCL1* and *chromogranin A (CHGA)* and decreased luminal epithelial lineage genes *AR*, *NKX3-1*, and *KLK3* expression (Fig. 2A). Among all patient samples included in the dataset, there was a strong correlation between levels of RET and neuronal lineage markers, while there was a negative correlation with RET expression and the AR-responsive genes (Fig. 2B). Overall, the NE-positive patient population had increased RET expression compared with the AR-positive population (Fig. 2C). An additional patient-derived xenograft (PDX) transcript dataset comparing metastatic NEPC to metastatic adenocarcinoma showed a similar correlation and upregulation of expression of RET and neuronal lineage markers in the NEPC population (Fig. 2D-F; ref. 33). This trend was also observed in additional prostate cancer datasets (Supplemental Fig. 2; refs. 6, 34–36). Overall, these independent datasets demonstrate that RET kinase is frequently overexpressed in clinical NEPC tumors and supports our cell line phosphoproteomic and KSEA analyses, suggesting enhanced RET activity drives NEPC proliferation and survival and therefore nominates RET as a candidate therapeutic target for NEPC tumors.

RET expression correlates with neuroendocrine transcription factors in prostate cancer cell lines and is necessary for NEPC proliferation

The robust levels of RET gene expression in NEPC patient samples suggests it is a potential target in NEPC. To validate the correlation of RET gene expression in prostate cancer cell lines, we examined relative RET dependency in the publicly available pooled genome-scale RNAi screen of 503 cancer cell lines, which includes seven prostate cancer cell lines and one basal prostate cell line (30). We compared the patterns of RET dependency relative to 11,280 genes in the eight prostate cell lines (Supplemental Table S7). As shown in Fig. 3A, strong correlations were observed between the dependencies of RET and NEPC driver genes (*POU3F2*, *SOX2*, *ONECUT2*, and *ASCL1*). In contrast, a negative correlation was seen between the dependencies of RET and

VanDeusen et al.

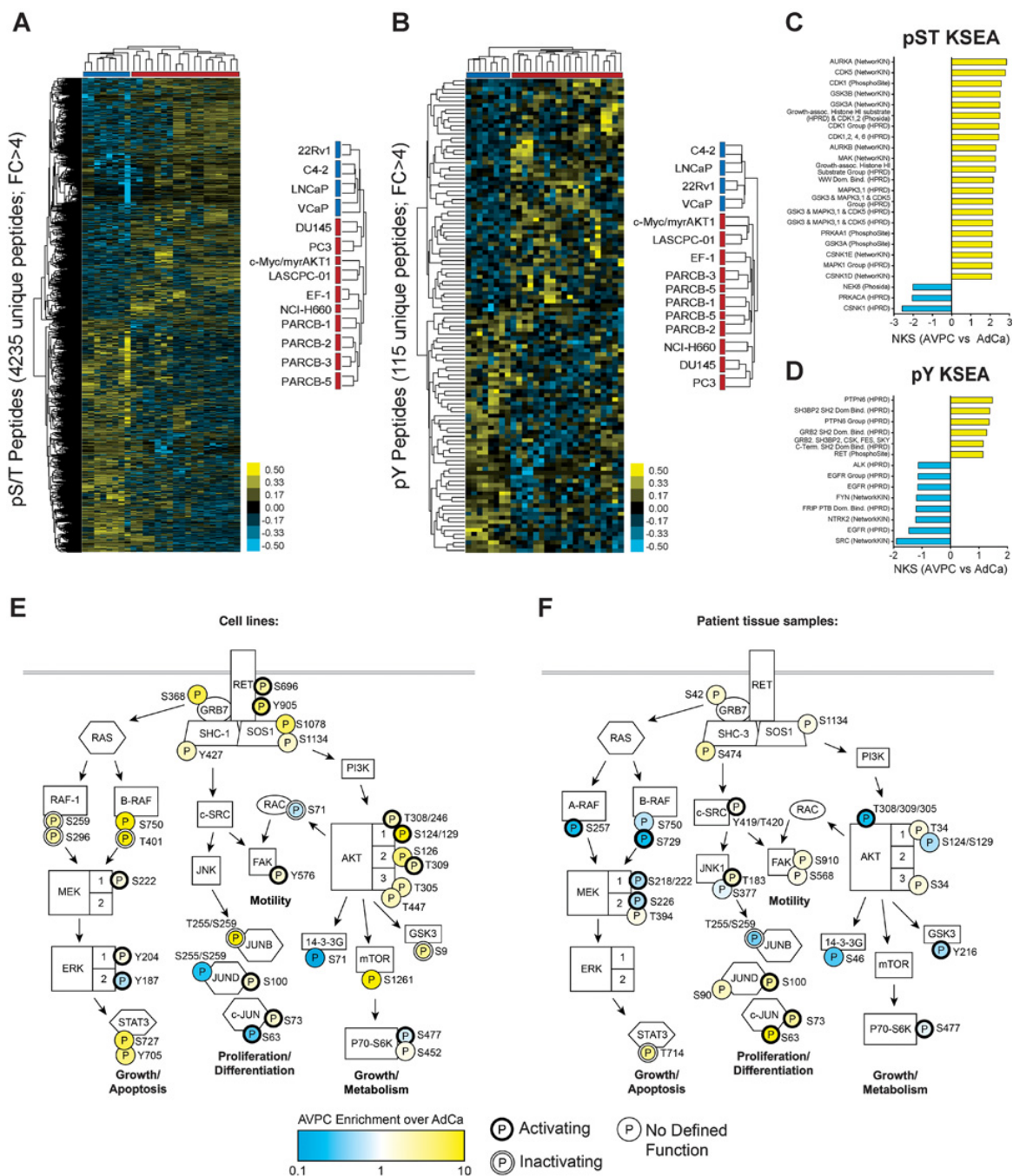


Figure 1.

Global phosphorylation and kinase signaling pathways are differentially regulated in AVPC cell lines compared with adenocarcinoma cell lines. Supervised hierarchical clustering heatmap of 4,235 unique phosphoserine/threonine (pS/T) enriched peptides (A) and 115 unique phosphotyrosine (pY) enriched peptides (B) from adenocarcinoma cell lines (blue: C4-2, 22Rv1, LNCaP, and VCaP) and AVPC cell lines (red: cMyc/myrAKT, LASCPC-01, EF-1, PARCB-1, PARCB-2, PARCB-3, PARCB-5, NCI-H660, DU145, and PC3). Yellow, hyperphosphorylation; blue, hypophosphorylation. C and D, Kinase substrate enrichment analysis (KSEA) performed on the 10 AVPC and 4 adenocarcinoma cell lines in A and B, showed multiple predicted alterations to kinase signaling. C, KSEA for pS/T analysis used a false discovery rate (FDR) <0.05, substrate hits > 5, and normalized K score >2.0. D, KSEA for pY analysis used an FDR < 0.1, substrate hits > 4, and normalized K score >1.1. E, Phosphorylated residues identified in the global phosphoproteomics from A and B or F, human phosphoproteome data (23) were mapped onto signaling pathways downstream of RET kinase. Yellow, enriched in AVPC relative to adenocarcinoma; blue, reduced in AVPC relative to adenocarcinoma. Thick black outline, activating phosphorylation; white outline, inactivating phosphorylation; thin outline, no defined function.

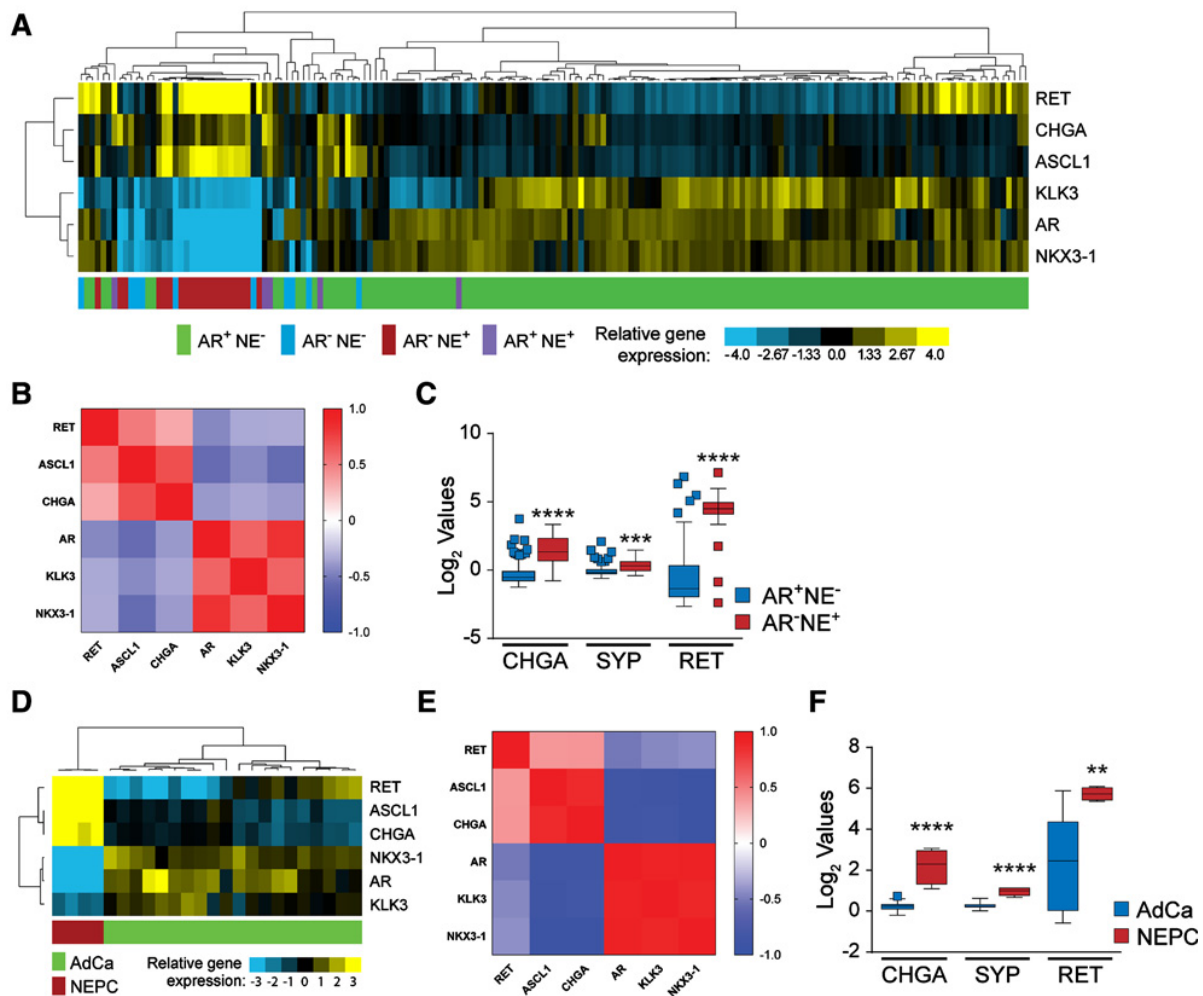


Figure 2.

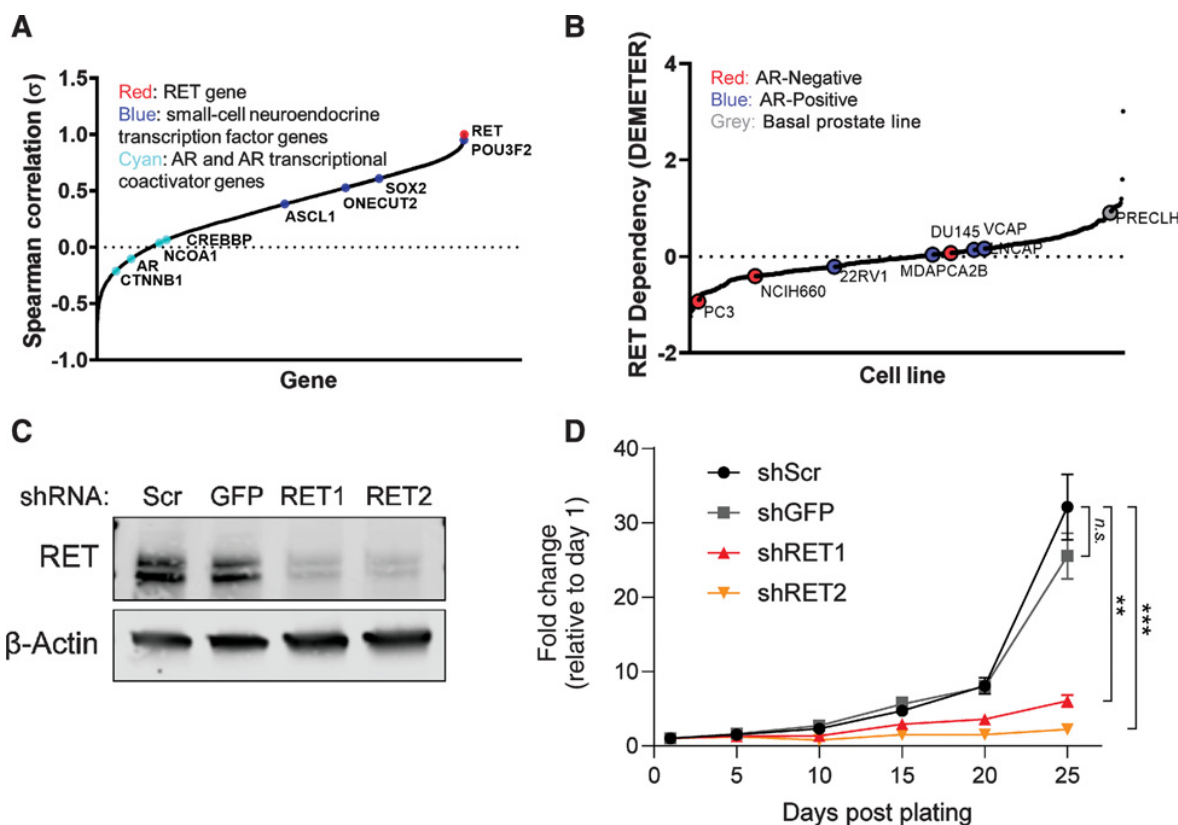
RET kinase along with other neuroendocrine transcripts are upregulated in NEPC relative to adenocarcinoma patient samples. **A**, Microarray data from the University of Washington rapid autopsy data of metastatic prostate cancer biopsies (32) were clustered on the basis of gene expression of RET, neuroendocrine markers: CHGA and ASCL1, as well as androgen-regulated genes: KLK3, AR, and NKX3-1. Upregulation of expression is represented by yellow, while downregulated genes are represented by blue. Patient samples were classified by AR and NE markers as AR+NE- (green, $n = 134$), AR-NE- (blue, $n = 10$), AR-NE+ (red, $n = 20$), and AR+NE+ (purple, $n = 7$). **B**, Pearson correlation matrix of gene expression from **A** showing a correlation of RET gene expression with neuroendocrine markers and negative correlation with AR-responsive markers. **C**, Box and whisker plot of average transcript measurements of CHGA, SYP, or RET in adenocarcinoma (AR+NE-) versus the NEPC (AR-NE+) patients. The data is represented in Tukey plots and expression values were analyzed by Student *t* test. **D**, Agilent oligo array expression analysis of four neuroendocrine AR-negative LuCaP patient derived xenografts (PDX) and 20 LuCaP adenocarcinoma PDX published by Zhang and colleagues (33) were clustered as in **A**. **E**, Pearson correlation matrix of expression data represented in **D**. **F**, Box and whisker plot shows an upregulation in CHGA, SYP, and RET kinase in NEPC versus adenocarcinoma PDX samples. Data is represented as in **C**.

AR. AR expectedly showed strong correlation with AR regulators (CTNNB1, NCOA1 and CREBBP). To determine whether RET expression was required for cellular proliferation or viability, we compared the Project Achilles DEMETER scores of RET, highlighting the prostate cancer cell lines (Fig. 3B; ref. 29). The DEMETER score indicates how gene suppression affects cell viability compared with all other cell lines upon suppression of the same target gene. Among the seven prostate cancer cell lines, two of the AR independent AVPC cells, PC3 and NCI-H660, exhibited greater relative dependency on RET compared the 501 other cell lines (ranked 10th and 76th; Supplementary Table S8). This indicates RET kinase is required for the growth

some AR-independent prostate cancer cell lines and not in the AR-dependent lines.

We validated the findings from the high-throughput short hairpin RNA (shRNA) screening by generating stable RET knockdown cell lines. The two most RET dependent cell lines from the large-scale screen, PC3 and NCI-H660, were stably transduced with two unique anti-RET shRNA constructs and the downregulation of RET protein or mRNA expression were confirmed (Fig. 3C; Supplementary Fig. S3A). RET knockdown strongly suppressed the growth of NCI-H660 cells, and to a lesser extent the PC3 cells. Interestingly, this correlates with the relative level of RET protein expression by Western blot analysis,

VanDeusen et al.

**Figure 3.**

RET expression correlates with NE markers in prostate cancer cell lines and is important for NEPC cell line growth. **A**, RET expression dependency profiling for 11,280 genes across eight prostate cell lines (PRECLH, LNCaP, VCaP, DU145, MDA PCa 2b, 22Rv1, NCI-H660, and PC3). RET expression was positively correlated with NEPC driver genes (blue) and negatively correlated with AR and AR regulators (cyan). **B**, Relative RET dependency scores reflect the ability of 503 cancer cell lines to maintain proliferation after RET knockdown (taken from the Project Achilles 2.201). Among the eight prostate cancer cell lines, PC3 and NCI-H660 cells showed the greatest dependency on RET. **C**, RET protein expression in NCI-H660 cells stably transduced with scrambled (Scr), anti-GFP, or two unique anti-RET shRNA. RET protein levels were reduced in two RET knockdown NCI-H660 cell lines and β -actin serves as a loading control. **D**, RET knockdown reduces cellular proliferation in H660 cell lines. The line graph represents relative cellular proliferation as measured by WST assay of one biological replicate. Cell proliferation was analyzed by linear regression of log-transformed data to determine statistical significance and error bars represent the SD of five technical replicates.

which is readily detectable in NCI-H660 cells and much lower in the PC3 cells (Supplementary Fig. S1). After 25 days of incubation, the total number of NCI-H660 cells decreased by 81% (shRET1, $P = 0.00013$) and 93% (shRET2, $P = 8.82 \times 10^{-5}$) compared with the scrambled shRNA (shScr) cells (Fig. 3D). The number of stable RET knockdown PC3 cells was 45% (shRET1, $P = 0.0021$) and 50% (shRET2, $P = 0.0021$) lower compared with shScr cells at day 8 (Supplementary Fig. S3B). Taken together, this suggests that RET kinase plays a role in enhancing NEPC cell growth and can be an effective therapeutic target for NEPC treatment.

RET kinase inhibitors block RET signaling in NEPC cells

AD80 is a novel, more selective inhibitor of the RET pathway than previous multityrosine kinase inhibitors such as cabozantinib or vandetanib (14, 15). However, AD80 still targets multiple other cellular kinases such as p70S6K, SRC, and VEGF receptors (15). We also utilized the newer RET inhibitors, LOXO-292 and BLU-667, which are currently undergoing clinical trials in RET fusion driven solid tumors and are considered specific RET inhibitors with few adverse effects (12, 13). To determine whether prostate cancer cells are

sensitive to RET inhibition, we determined the IC_{50} of AD80, LOXO-292, BLU-667 in our panel of prostate cancer cell lines (Supplementary Fig. S4A). AD80 was consistently among the most effective at reducing viability, and the NCI-H660 cells were the most sensitive to AD80 of the RET inhibitors tested (Supplementary Figs. S4A–S4C). To confirm that RET kinase is active and can be inhibited by these drugs, we treated NCI-H660 cells with AD80, LOXO-292, BLU-667, or DMSO for 4 hours and evaluated the tyrosine phosphorylation of RET (37). RET kinase immunoprecipitated from RET inhibitor-treated NCI-H660 cells showed a reduction in total tyrosine phosphorylation, indicating that AD80, LOXO-292, and BLU-667 all inhibit the activation of RET in NCI-H660 cells (Fig. 4A). Next, we measured the downstream targets of RET by looking at phosphorylation of ERK1/2 and AKT1/2. Interestingly, all three drugs reduced ERK1/2 phosphorylation of residues Tyr202/Try204 in a dose-dependent manner, but AD80 was the most effective in reducing phosphorylation of both ERK1/2 and AKT1/2, while having no effect on the total protein levels (Fig. 4B). Cabozantinib and vandetanib also decreased the levels of phospho ERK1/2 and AKT1/2 in NCI-H660 cells (Supplementary Fig. S4D). Finally, we treated the RET

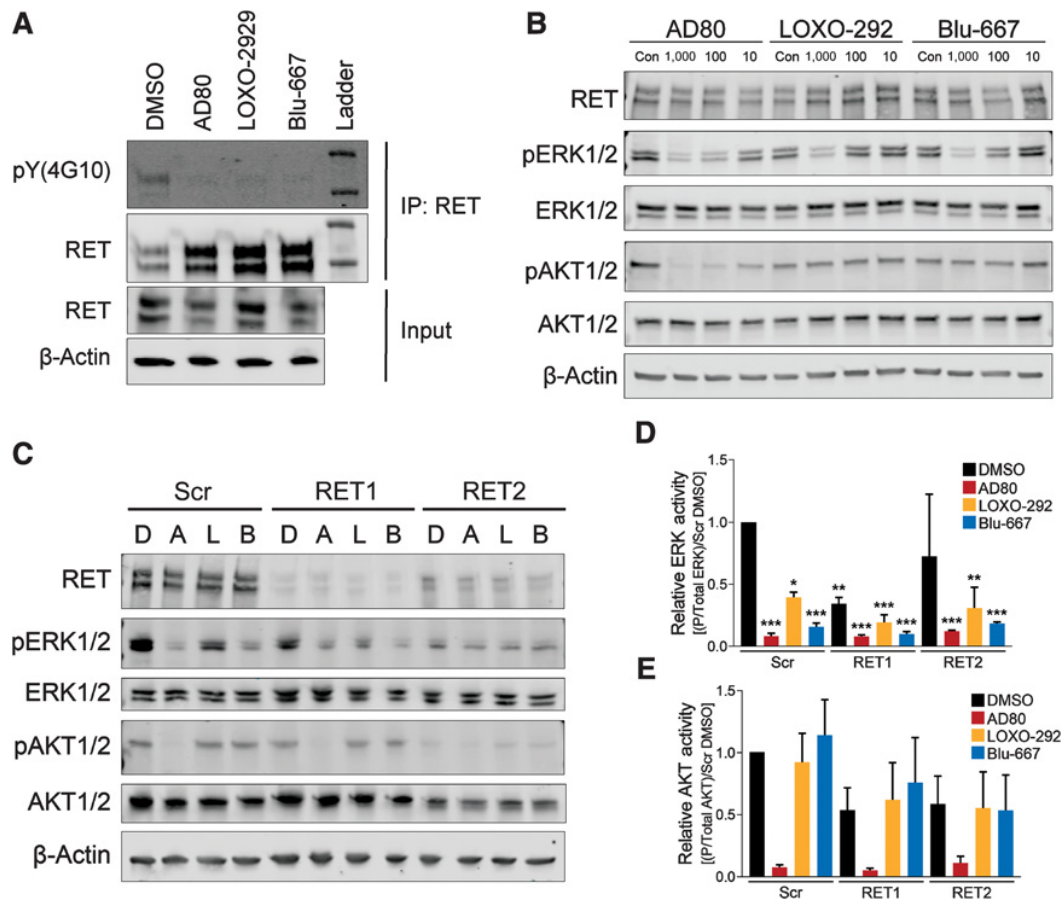


Figure 4.

NCI-H660 cells are sensitive to RET inhibition and show sensitivity to RET inhibitors. **A**, Immunoprecipitation of RET kinase from H660 cells shows that 4-hour treatment with 1 $\mu\text{mol/L}$ AD80, LOXO-292, or BLU-667 reduces RET tyrosine phosphorylation, as assayed with a total phosphotyrosine antibody 4G10. **B**, NCI-H660 cells treated for 4 hours with DMSO (Con) the indicated concentrations (nmol/L) of AD80, LOXO-292, or BLU-667, showed reduced activity of the MAPK and AKT signaling cascades downstream of RET. Activity was analyzed by Western blot analysis for phosphorylation of ERK1/2 at Tyr202/Tyr204 and phosphorylation of AKT1/2 at Ser473. The AD80 treatment reduced phosphorylation of both downstream targets, while LOXO-292 and BLU-667 reduced the activity of ERK1/2. In all treatments the total ERK1/2, total AKT1/2, and actin loading control remained unaffected. **C**, The activity of pERK1/2 (Tyr202/Tyr204) and pAKT1/2 (Ser 473) in NCI-H660 scrambled control and RET knockdown cells was assayed after a 4-hour treatment with DMSO (D), or 1 $\mu\text{mol/L}$ of AD80 (A), LOXO-292 (L), or BLU-667 (B). **D**, The relative ERK1/2 activity was measured by comparing pERK1/2 (Tyr202/Tyr204) to total ERK1/2 protein and normalized to the scrambled DMSO-treated sample. The ERK1/2 activity is reduced by both RET knockdown and after treatment with RET inhibitors. The bars represent the average values from three experiments and the error bars are SD. **E**, Quantification of AKT1/2 activity (pAKT1/2 S473 relative to total AKT protein and normalized DMSO-treated Scr cells) shows AD80 potently inhibits AKT1/2 activity while knockdown may reduce activity slightly. Bars represent the mean from three experiments and the error bars are SD.

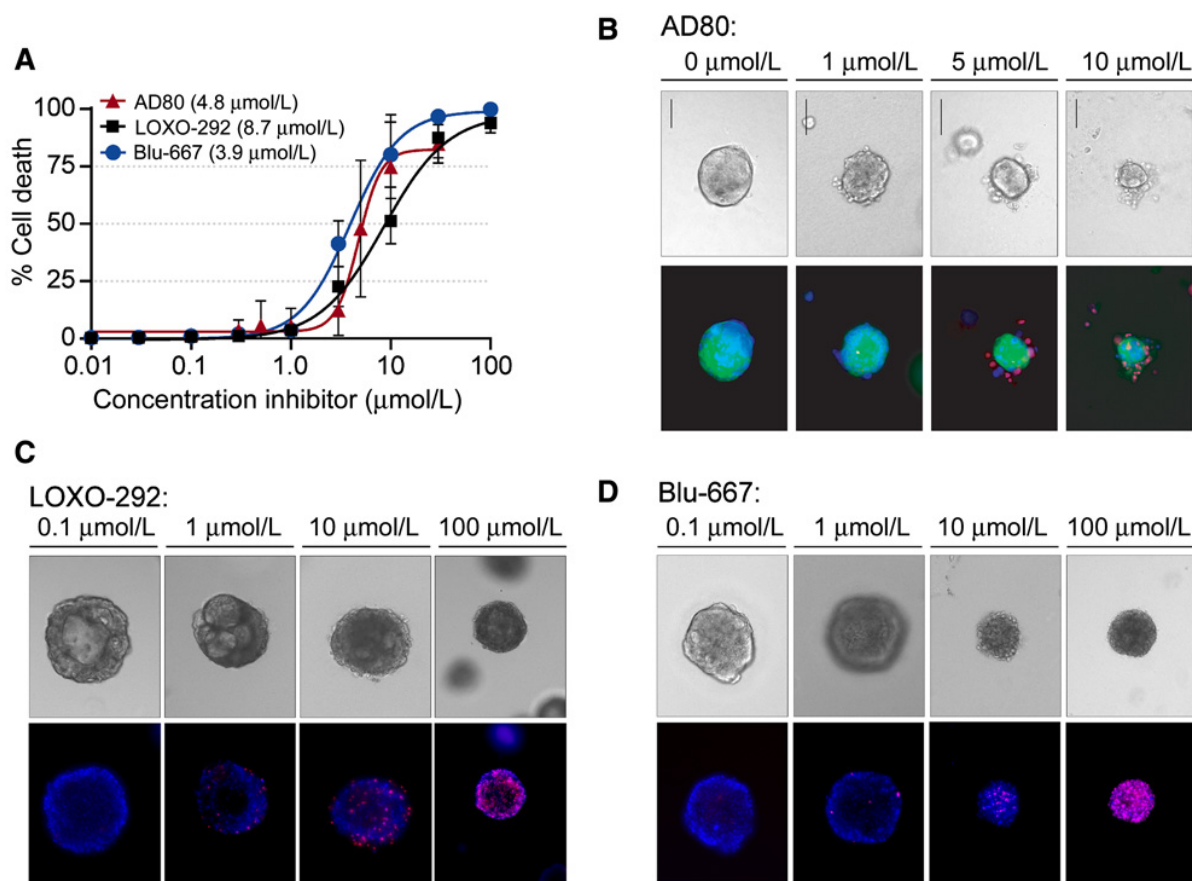
knockdown cells with AD80, LOXO-292, or BLU-667 (Fig. 4C). We found that RET knockdown reduced the levels of ERK1/2 phosphorylation and to a lesser extent AKT1/2 (Fig. 4D and E). The effect of AD80, LOXO-292, and BLU-667 was reduced in the RET knockdown cells, suggesting that RET is required for full activation of ERK1/2 and AKT1/2 (Fig. 4D and E). The ability of these RET inhibitors to reduce viability (IC_{50}) combined with genetic knockdown of RET in NCI-H660 cells (Fig. 3) suggests that RET kinase is critical to the growth and survival of NEPC cells with high RET expression and can be pharmacologically inhibited.

RET inhibition induces cell death in NEPC 3D culture models

We cultured NCI-H660 cells as 3D spheroids and tested the ability of AD80 to induce cell death (Supplementary Fig. S4E). The

calculated LD_{50} for the NCI-H660 organoids was 1.4 $\mu\text{mol/L}$, slightly higher than cells in 2D culture. The organoids clearly displayed an increase in dead cells at higher doses of AD80 (Supplementary Fig. S4F). We extended our RET inhibitor treatment studies to a second organoid model of NEPC (5). Tumors derived from the prostate epithelium of *Pten*^{-/-}*Rb*^{-/-} (DKO) mice express higher levels of RET mRNA than *Pten*^{-/-} (SKO) or wild-type (WT) animals (Supplementary Fig. S5A; ref. 5). Immunofluorescence staining also confirmed an increase of RET kinase protein in the DKO organoids and low to absent RET kinase in the SKO organoids (Supplementary Fig. S5B). The DKO organoids were also resistant to enzalutamide treatment, mimicking the ADT-resistant characteristic of NEPC prostate cancer cells that express high levels of RET (Supplementary Fig. S5C). Treating the

VanDeusen et al.

**Figure 5.**

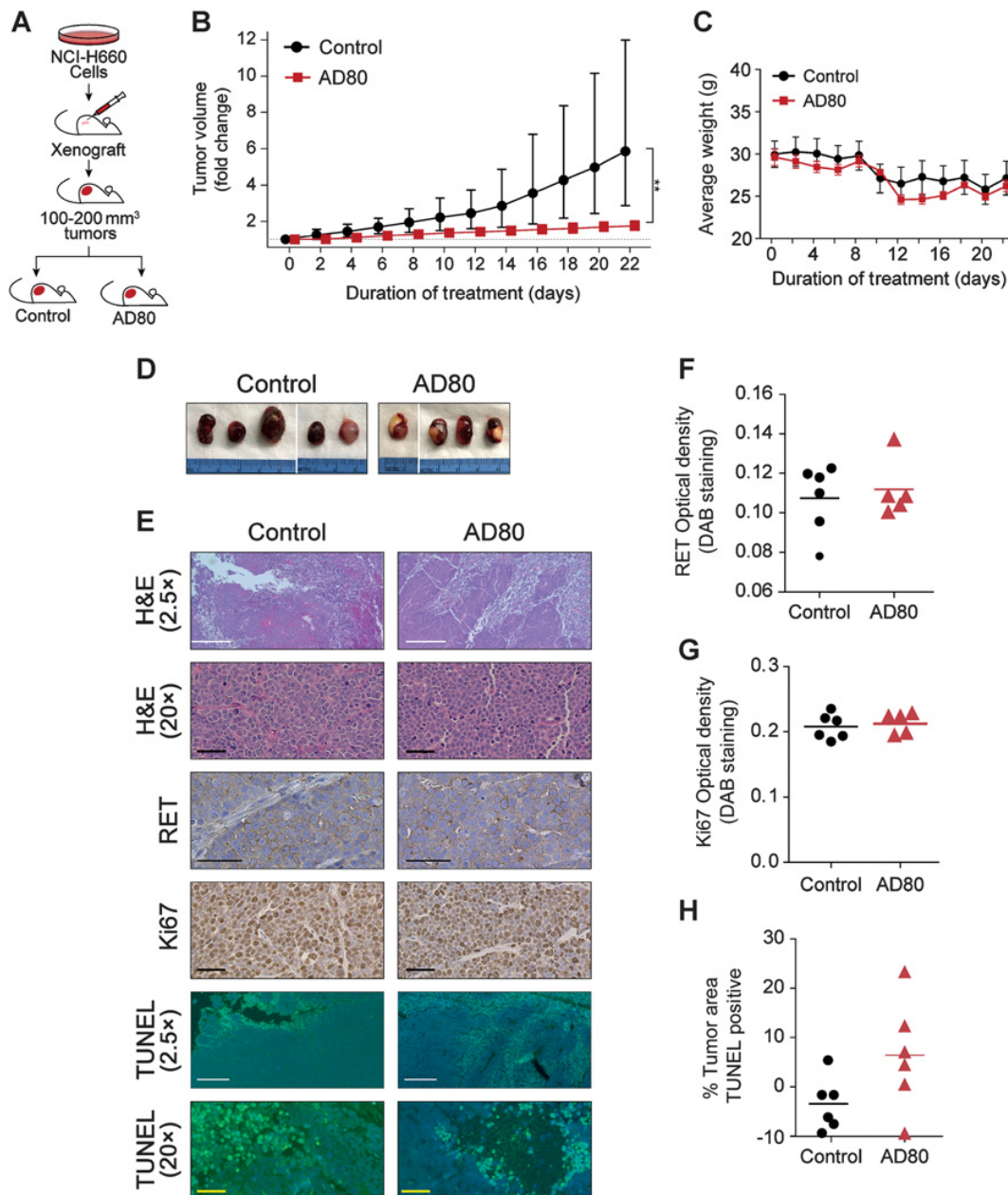
Organoid NEPC models are sensitive to treatment with multiple RET inhibitors. **A**, A dose-response curve of *Pten*^{-/-} and *Rb*^{-/-} prostate specific double knockout (DKO) organoids treated with increasing concentrations of AD80, LOXO-292, and BLU-667. Viability was measured by staining for dead cells. Circles represent mean and error bars \pm SD. **B**, Bright field images and corresponding fluorescence images of GFP-labeled DKO organoids treated with the indicated concentrations of AD80. Blue, DAPI staining of nuclei; red, propidium iodide staining of dead cells. Scale bar, 100 μm . Representative brightfield and fluorescent images of LOXO-292 (**C**) and BLU-667 (**D**) DKO organoids treated with the indicated concentrations of drugs stained as described with the GFP channel omitted.

DKO organoids with increasing concentrations of AD80, LOXO-292, or BLU-667 induced a dose-dependent increase in cell death, as assayed by live-dead PI staining of the organoids (**Fig. 5A–D**). All three drugs displayed a similar LD₅₀, suggesting that RET inhibition is effective in preventing tumor growth in a second model of NEPC.

AD80 reduces growth of NEPC xenograft tumors *in vivo* by increasing cell death

To test the efficacy of AD80 in an *in vivo* model system of NEPC, we generated NCI-H660 xenograft tumors in NOD-SCID mice. Once tumors reached 100–200 mm³, mice were randomized and placed into one of two treatment groups: Control (DMSO) or 10 mg/kg AD80 (**Fig. 6A**). Over the course of the 22-day treatment, AD80-treated tumors showed a significant reduction in overall tumor volume (**Fig. 6B**) without a significant effect on animal weight (**Fig. 6C**). This experiment was repeated in a second cohort of mice with 24 days of treatment and higher dose of AD80 (20 mg/kg; Supplementary Fig. S6). The higher dose of AD80 was associated with increased toxicity, but showed similar inhibition of

tumor growth throughout the 24-day treatment (Supplementary Fig. S6A–S6C). To interrogate the molecular characteristics of AD80 treatment, the tumors (**Fig. 6D**) were fixed and sectioned for staining. Sections stained with hematoxylin & eosin (H&E) showed similar tumor morphology (**Fig. 6E**; Supplementary Fig. S6D). IHC staining for RET showed similar expression and localization among the treatment groups (**Fig. 6E** and **F**; Supplementary Figs. S6D and S6E). There was also no difference in tumor proliferation as assayed by Ki67 staining among the treatment groups in either cohort of mice (**Fig. 6E** and **G**; Supplementary Figs. S6D and S6F). However, TUNEL staining showed large regions of positive staining and the percentage of total tumor area that stained positive trended higher in the AD80-treated groups (**Fig. 6E** and **H**; Supplementary Fig. S6D and S6G). Taken together, these results suggest that AD80 treatment is effective in limiting tumor growth by inducing cell death in neuroendocrine cells with high RET expression and that the specific population of patients that have high RET expression, are refractory to ADT, and have few remaining therapeutic options may benefit from RET kinase inhibitor therapies.

**Figure 6.**

AD80 reduces NCI-H660 xenograft tumor growth. **A**, Schematic of *in vivo* study in which NCI-H660 cells were injected subcutaneously into the right flank of NOD-SCID mice and tumors were allowed to grow to approximately 100 to 200 mm³ before being randomly assigned into two treatment groups: control (DMSO alone, $n = 6$) or AD80 (10 mg/kg/day, $n = 6$). **B**, The fold change in tumor volume by treatment group was plotted as a function of the number of days of treatment. Means and CIs were calculated on the log scale and reported in terms of geometric means after exponentiation with error bars \pm 95% CI. There was evidence of an overall treatment effect on tumor growth rate ($P = 0.006$) with a significantly lower tumor volume at day 22 ($P = 0.006$). **C**, Average animal weights were measured at the same time as tumor volumes and no differences in average animal weight between treatment groups was observed over the duration of the study. Symbols represent means with error bars \pm SE. **D**, Following the termination of the xenograft tumor experiment, tumors were excised from animals that survived to the end of the study and photographed with a centimeter scale ruler. Separate images from the same group are divided by a white line. **E**, Representative images of H&E (2.5x and 20x), RET IHC (20x), Ki67 IHC (20x), and TUNEL (2.5x and 20x) stained sections of tumors from each group. White scale bars, 500 μ m. Yellow and black scale bars, 50 μ m. Average optical density of RET staining (**F**) and Ki67 staining (**G**) from five distinct fields of view in each tumor are represented by symbols with a horizontal bar representing the mean. Quantification was analyzed by one way ANOVA. **H**, Quantification of the average TUNEL positive area (2.5x) was analyzed with the Kruskal-Wallis test ($P = 0.1727$). Symbols represent averages for individual tumors with a horizontal line representing the mean. Bars represent the mean with error bars represent \pm SE.

Discussion

Increasing evidence points to the activation of kinase pathways as possible key mechanisms that bypass AR-targeted therapies and allow the tumors to continue to survive such a harsh therapeutic environment (1, 4, 20, 23). Utilizing phosphoproteomics, we showed that AR-independent cell lines have altered kinase signaling pathways compared with AR-driven adenocarcinomas, which includes activation of RET kinase. Multiple proteins downstream of the RET kinase pathway were phosphorylated on activating residues in both the cell line and in mCRPC autopsy patient samples. RET mutations or activating rearrangements are drivers of tumor development and growth in MEN2, medullary thyroid cancer, and small-cell and non-small cell lung cancers, and drugs targeting RET can extend survival of these patients (38–40). Cabozantinib, which inhibits RET kinase and other receptor tyrosine kinases including VEGR1/2, has extended survival in certain cancers with activating RET mutations (41, 42). In prostate cancer, cabozantinib showed promise in phase II clinical trials but failed to meet the endpoint criteria in phase III trials (NCT01605227) (43, 44). However, this was tested in a nonstratified patient population and did not focus on NEPC or patients with high RET expression (45). A retrospective evaluation of postdocetaxel patients with CRPC in the COMET-1 and COMET-2 phase III clinical trials where cabozantinib was compared with prednisone and prednisone plus mitoxantrone suggest a subpopulation exists that may benefit from cabozantinib treatment, highlighting the importance of molecular stratification of patients for individualized treatments (43, 44, 46). Recently, RET knockdown in a prostate AdCa cell line, LNCaP, was reported to restrict tumor growth, but it remains unclear if and how RET contributes to tumor progression in NEPC (11).

We found that overall RET expression in prostate cancer patient samples is highly variable, but that RET kinase expression correlated very strongly with NEPC. In the datasets we analyzed, there were examples of metastatic and treatment induced NEPC tumors (based on molecular and pathologic features) that lacked RET gene expression. Inversely, there were also patients classified as AR-positive adenocarcinomas that displayed high levels of RET gene expression but lacked expression of other neuroendocrine lineage markers (Fig. 2; Supplementary Fig. S2). It is important to note that the transition from adenocarcinoma to NEPC may be dynamic (5) and RET expression in AR-positive tumors may suggest that these tumors are either a heterogeneous phenotype or are transitioning from adenocarcinoma to NEPC. Currently, little is known about the regulation of RET gene expression in prostate cancer. Several key epigenetic regulators (such as CBX2, EZH2, BRN2, and SOX2) have been identified as possible modulators that can switch tumors between an adenocarcinoma and NEPC state (5, 47–49). We found that RET kinase dependency correlated with several of these transcription factors (Fig. 3). Alterations in DNA methylation or transcriptional regulation resulting from the loss of proteins such as RB1 may further alter RET expression and activity. Therefore, it remains to be determined how robust RET expression is gained during the transition from mCRPC to a NEPC phenotype. In small-cell lung cancer, ASCL1 was shown to induce RET gene expression and this mechanism of regulation may hold true in NEPC, but has not been validated (50).

Regardless of the dynamics of RET expression in disease progression, we showed that multiple RET kinase pathway inhibitors effectively restricted growth in the *Rb/Pten* knockout organoids and AD80 reduced the growth of the NCI-H660 cell line and spheroids *in vitro*, as well as NCI-H660 tumors *in vivo*. We validated our inhibitor studies by

knocking down RET in NCI-H660 and PC3 cell lines and saw a similar reduction in overall growth. The pharmacologic and genetic inhibition of RET kinase suggests that RET kinase signaling is important for NEPC tumor progression. To identify patients that could benefit from treatment including RET inhibition, it will be important to generate assays or validate markers of RET activity in NEPC. Pathology, loss of AR signaling, or expression of neuroendocrine genes are not sufficient alone to identify all patients with high levels of RET expression that may benefit from RET-targeted therapies. Moving forward, it will be important to identify the subset of patients that would benefit from inhibition of RET kinase. Development of biomarkers for transcriptional activators, RET protein, or markers of RET activity will enable pre-selection of individuals who would benefit from RET inhibitors. Understanding the regulation of RET gene expression, correlation of RET expression and activity and disease progression, as well as the contribution of RET kinase to mCRPC tumor progression could inform better treatment strategies.

Disclosure of Potential Conflicts of Interest

O.N. Witte reports personal fees and other from Allogene Therapeutics, other from Kronos Bio, other from Trethera, other from Sofie Biosciences, other from Breakthrough Properties, other from Nammi Therapeutics, and personal fees and other from Vida Ventures during the conduct of the study. P.S. Nelson reports personal fees from Astellas, personal fees from Janssen, and personal fees from Bristol Myers Squibb outside the submitted work. No potential conflicts of interest were disclosed by the other authors.

Disclaimer

The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Authors' Contributions

H.R. VanDeusen: Conceptualization, data curation, formal analysis, validation, investigation, methodology, writing-original draft, writing-review and editing. **J.R. Ramroop:** Conceptualization, data curation, formal analysis, validation, investigation, methodology, writing-original draft, writing-review and editing. **K.L. Morel:** Data curation, formal analysis, investigation, writing-review and editing. **S.Y. Bae:** Data curation, formal analysis, investigation, methodology, writing-review and editing. **A.V. Sheahan:** Data curation. **Z. Sychev:** Formal analysis, methodology. **N.A. Lau:** Data curation, investigation. **L.C. Cheng:** Data curation, formal analysis, investigation. **V.M. Tan:** Data curation, investigation. **Z. Li:** Data curation, formal analysis, investigation, methodology. **A. Petersen:** Software, formal analysis, methodology, writing-review and editing. **J.K. Lee:** Data curation, supervision, investigation, writing-review and editing. **J.W. Park:** Resources, resources. **R. Yang:** Resources, formal analysis. **J.H. Hwang:** Formal analysis, investigation, visualization, methodology, writing-review and editing. **I. Coleman:** Resources. **O.N. Witte:** Resources, supervision, investigation, writing-review and editing. **C. Morrissey:** Resources, data curation, supervision, writing-review and editing. **E. Corey:** Resources, data curation, supervision, writing-review and editing. **P.S. Nelson:** Resources, data curation, supervision, writing-review and editing. **L. Ellis:** Conceptualization, data curation, formal analysis, supervision, funding acquisition, investigation, visualization, methodology, writing-review and editing. **J.M. Drake:** Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, writing-original draft, project administration, writing-review and editing.

Acknowledgments

We thank members of the Drake lab for providing advice and input on the manuscript. We also thank the members of the Biological Mass Spectrometry Facility of Robert Wood Johnson Medical School and Rutgers, The State University of New Jersey, for providing advice and performing mass spectrometry on our samples. We thank Ryder Clifford from QGel for providing reagents. We thank the patients and their families, Celestia Higano, Evan Yu, Elaha Mostaghel, Heather Cheng, Bruce Montgomery, Mike Schweizer, Andrew Hsieh, Daniel Lin, Funda Vakar-Lopez, Lawrence True and the rapid autopsy teams for their contributions to the University of Washington Medical Center Prostate Cancer Donor Rapid Autopsy Program and

the Development of the LuCaP PDX models. H.R. VanDeusen is supported by Department of Defense Prostate Cancer Research Program W81XWH-19-1-0173. This work was also supported by the Department of Defense grant (W81XWH-17-1-0414; W81XWH-17-1-0415; W81XWH-18-1-0347), the Prostate Cancer Biorepository Network (PCBN; W81XWH-14-2-0183), the Pacific Northwest Prostate Cancer SPORE (P50CA97186), the PO1 NIH grant (PO1 CA163227), the Richard M. LUCAS Foundation, and the Institute for Prostate Cancer Research (IPCR). L.C. Cheng and V.M. Tan are supported by the National Institute of General Medical Sciences of the NIH under award number T32 GM008339. J.W. Park is supported by the NIH/NCI grant K99/R00 Pathway to Independence award K99CA218731/R00CA218731. R. Yang is supported by Department of Defense Prostate Cancer Research Program W81XWH-19-1-0161. J.H. Hwang is supported by American Cancer Society-AstraZeneca (PF-16-142-01-TBE). O.N. Witte is supported by the Zimmerman Family, the Concern Foundation, and by a Prostate Cancer Foundation Challenge Award. O.N. Witte is also supported by the West Coast Prostate Cancer Dream Team supported by a Stand Up to Cancer-Prostate Cancer Foundation-Prostate Cancer Dream Team Translational Cancer Research Grant (SU2C-AACR-DT0812; co-principal investigator: O.N. Witte). This research grant is made possible

by the generous support of the Movember Foundation. Stand Up To Cancer is a division of the Entertainment Industry Foundation. The research grant is administered by the American Association for Cancer Research. L. Ellis is supported by the Department of Defense Prostate Cancer Research Program W81XWH-18-1-0541. J. M. Drake is supported by the Department of Defense Prostate Cancer Research Program W81XWH-14-1-0148, W81XWH-15-1-0236 and W81XWH-18-1-0542, Prostate Cancer Foundation Young Investigator Award, and the New Jersey Health Foundation. Research reported in this publication was also supported by the National Center for Advancing Translational Sciences of the National Institutes of Health Award Number UL1-TR002494.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 23, 2019; revised April 1, 2020; accepted May 19, 2020; published first May 27, 2020.

References

- Bluenn EG, Coleman IM, Lucas JM, Coleman RT, Hernandez-Lopez S, Tharakan R, et al. Androgen receptor pathway-independent prostate cancer is sustained through FGF signaling. *Cancer Cell* 2017;32:474–89.
- Beltran H, Tomlins S, Aparicio A, Arora V, Rickman D, Ayala G, et al. Aggressive variants of castration-resistant prostate cancer. *Clin Cancer Res* 2014;20:2846–50.
- Aparicio AM, Harzstark AL, Corn PG, Wen S, Araujo JC, Tu SM, et al. Platinum-based chemotherapy for variant castrate-resistant prostate cancer. *Clin Cancer Res* 2013;19:3621–30.
- Beltran H, Oromendia C, Danila DC, Montgomery B, Hoimes C, Szmulewitz RZ, et al. A phase II trial of the aurora kinase inhibitor alisertib for patients with castration-resistant and neuroendocrine prostate cancer: efficacy and biomarkers. *Clin Cancer Res* 2019;25:43–51.
- Ku SY, Rosario S, Wang Y, Mu P, Seshadri M, Goodrich ZW, et al. Rb1 and Trp53 cooperate to suppress prostate cancer lineage plasticity, metastasis, and anti-androgen resistance. *Science* 2017;355:78–83.
- Beltran H, Rickman DS, Park K, Chae SS, Sboner A, MacDonald TY, et al. Molecular characterization of neuroendocrine prostate cancer and identification of new drug targets. *Cancer Discov* 2011;1:487–95.
- Blume-Jensen P, Hunter T. Oncogenic kinase signalling. *Nature* 2001;411:355–65.
- Arighi E, Borrello MG, Sariola H. RET tyrosine kinase signaling in development and cancer. *Cytokine Growth Factor Rev* 2005;16:441–67.
- Drake JM, Graham NA, Lee JK, Stoyanova T, Faltermeier CM, Sud S, et al. Metastatic castration-resistant prostate cancer reveals intrapatient similarity and interpatient heterogeneity of therapeutic kinase targets. *Proc Natl Acad Sci U S A* 2013;110:E4762–9.
- Lee JK, Bangayan NJ, Chai T, Smith BA, Pariva TE, Yun S, et al. Systemic surfaceome profiling identifies target antigens for immune-based therapy in subtypes of advanced prostate cancer. *Proc Natl Acad Sci U S A* 2018;115: E4473–82.
- Ban K, Feng S, Shao L, Ittmann M. RET signaling in prostate cancer. *Clin Cancer Res* 2017;23:4885–96.
- Subbiah V, Gainor JF, Rahal R, Brubaker JD, Kim JL, Maynard M, et al. Precision targeted therapy with BLU-667 for RET-driven cancers. *Cancer Discov* 2018;8: 836–49.
- Subbiah V, Velcheti V, Tuch BB, Ebata K, Busaidy NL, Cabanillas ME, et al. Selective RET kinase inhibition for patients with RET-altered cancers. *Ann Oncol* 2018;29:1869–76.
- Plenker D, Riedel M, Bragelmann J, Dammert MA, Chauhan R, Knowles PP, et al. Drugging the catalytically inactive state of RET kinase in RET-rearranged tumors. *Sci Transl Med* 2017;9:eaah6144.
- Dar AC, Das TK, Shokat KM, Cagan RL. Chemical genetic discovery of targets and anti-targets for cancer polypharmacology. *Nature* 2012;486:80–4.
- Cheng LC, Li Z, Graeber TG, Graham NA, Drake JM. Phosphopeptide enrichment coupled with label-free quantitative mass spectrometry to investigate the phosphoproteome in prostate cancer. *J Vis Exp* 2018;138:e57996.
- Scheltema RA, Hauschild JP, Lange O, Hornburg D, Denisov E, Damoc E, et al. The Q exactive HF, a Benchtop mass spectrometer with a pre-filter, high-performance quadrupole and an ultra-high-field Orbitrap analyzer. *Mol Cell Proteomics* 2014;13:3698–708.
- Cox J, Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol* 2008;26:1367–72.
- Vizcaino JA, Cote RG, Csordas A, Dianas JA, Fabregat A, Foster JM, et al. The PRoteomics IDentifications (PRIDE) database and associated tools: status in 2013. *Nucleic Acids Res* 2013;41:D1063–9.
- Drake JM, Paull EO, Graham NA, Lee JK, Smith BA, Titz B, et al. Phosphoproteome integration reveals patient-specific networks in prostate cancer. *Cell* 2016;166:1041–54.
- Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci U S A* 1998;95:14863–8.
- Saldanha AJ. Java Treeview—extensible visualization of microarray data. *Bioinformatics* 2004;20:3246–8.
- Drake JM, Graham NA, Stoyanova T, Sedghi A, Goldstein AS, Cai H, et al. Oncogene-specific activation of tyrosine kinase networks during prostate cancer progression. *Proc Natl Acad Sci U S A* 2012;109:1643–8.
- Uphoff CC, Drexler HG. Detecting mycoplasma contamination in cell cultures by polymerase chain reaction. *Methods Mol Biol* 2011;731:93–103.
- Stoyanova T, Cooper AR, Drake JM, Liu X, Armstrong AJ, Pienta KJ, et al. Prostate cancer originating in basal cells progresses to adenocarcinoma propagated by luminal-like cells. *Proc Natl Acad Sci U S A* 2013;110:20111–6.
- Lee JK, Phillips JW, Smith BA, Park JW, Stoyanova T, McCaffrey EF, et al. N-Myc drives neuroendocrine prostate cancer initiated from human prostate epithelial cells. *Cancer Cell* 2016;29:536–47.
- Park JW, Lee JK, Sheu KM, Wang L, Balanis NG, Nguyen K, et al. Reprogramming normal human epithelial tissues to a common, lethal neuroendocrine cancer lineage. *Science* 2018;362:91–5.
- Drost J, Karthaus WR, Gao D, Driehuis E, Sawyers CL, Chen Y, et al. Organoid culture systems for prostate epithelial and cancer tissue. *Nat Protoc* 2016;11:347–58.
- Tsherniak A, Vazquez F, Montgomery PG, Weir BA, Kryukov G, Cowley GS, et al. Defining a cancer dependency map. *Cell* 2017;170:564–76.
- Cowley GS, Weir BA, Vazquez F, Tamayo P, Scott JA, Rusin S, et al. Parallel genome-scale loss of function screens in 216 cancer cell lines for the identification of context-specific genetic dependencies. *Sci Data* 2014;1:140035.
- Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods* 2012;9: 676–82.
- Kumar A, Coleman I, Morrissey C, Zhang X, True LD, Gulati R, et al. Substantial interindividual and limited intraindividual genomic diversity among tumors from men with metastatic prostate cancer. *Nat Med* 2016;22:369–78.
- Zhang X, Coleman IM, Brown LG, True LD, Kollath L, Lucas JM, et al. SRRM4 expression and the loss of REST activity may promote the emergence of the neuroendocrine phenotype in castration-resistant prostate cancer. *Clin Cancer Res* 2015;21:4698–708.
- Abida W, Cyrta J, Heller G, Prandi D, Armenia J, Coleman I, et al. Genomic correlates of clinical outcome in advanced prostate cancer. *Proc Natl Acad Sci U S A* 2019;116:11428–36.

VanDeusen et al.

35. Aggarwal R, Huang J, Alumkal JJ, Zhang L, Feng FY, Thomas GV, et al. Clinical and genomic characterization of treatment-emergent small-cell neuroendocrine prostate cancer: a multi-institutional prospective study. *J Clin Oncol* 2018;36:2492–503.
36. Beltran H, Prandi D, Mosquera JM, Benelli M, Puca L, Cyrta J, et al. Divergent clonal evolution of castration-resistant neuroendocrine prostate cancer. *Nat Med* 2016;22:298–305.
37. Mulligan LM. RET revisited: expanding the oncogenic portfolio. *Nat Rev Cancer* 2014;14:173–86.
38. Phay JE, Shah MH. Targeting RET receptor tyrosine kinase activation in cancer. *Clin Cancer Res* 2010;16:5936–41.
39. Donis-Keller H, Dou S, Chi D, Carlson KM, Toshima K, Lairmore TC, et al. Mutations in the RET proto-oncogene are associated with MEN 2A and FMTC. *Hum Mol Genet* 1993;2:851–6.
40. Dabir S, Babakoochi S, Kluge A, Morrow JJ, Kresak A, Yang M, et al. RET mutation and expression in small-cell lung cancer. *J Thorac Oncol* 2014;9:1316–23.
41. Ernani V, Kumar M, Chen AY, Owonikoko TK. Systemic treatment and management approaches for medullary thyroid cancer. *Cancer Treat Rev* 2016;50:89–98.
42. Gautschi O, Milia J, Filleron T, Wolf J, Carbone DP, Owen D, et al. Targeting RET in patients with RET-rearranged lung cancers: results from the global, multi-center RET registry. *J Clin Oncol* 2017;35:1403–10.
43. Smith M, De Bono J, Sternberg C, Le Moulec S, Oudard S, De Giorgi U, et al. Phase III study of cabozantinib in previously treated metastatic castration-resistant prostate cancer: COMET-1. *J Clin Oncol* 2016;34:3005–13.
44. Sonpavde GP, Pond GR, Fizazi K, de Bono JS, Basch EM, Scher HI, et al. Cabozantinib for progressive metastatic castration-resistant prostate cancer following docetaxel: combined analysis of two phase 3 trials. *Eur Urol Oncol* 2018 Nov 30 [Epub ahead of print].
45. Smith DC, Smith MR, Sweeney C, Elfiky AA, Logothetis C, Corn PG, et al. Cabozantinib in patients with advanced prostate cancer: results of a phase II randomized discontinuation trial. *J Clin Oncol* 2013;31:412–9.
46. Basch EM, Scholz M, de Bono JS, Vogelzang N, de Souza P, Marx G, et al. Cabozantinib versus mitoxantrone-prednisone in symptomatic metastatic castration-resistant prostate cancer: a randomized phase 3 trial with a primary pain endpoint. *Eur Urol* 2019;75:929–37.
47. Clermont PL, Lin D, Crea F, Wu R, Xue H, Wang Y, et al. Polycomb-mediated silencing in neuroendocrine prostate cancer. *Clin Epigenetics* 2015;7:40.
48. Bishop JL, Thaper D, Vahid S, Davies A, Ketola K, Kuruma H, et al. The master neural transcription factor BRN2 is an androgen receptor-suppressed driver of neuroendocrine differentiation in prostate cancer. *Cancer Discov* 2017;7:54–71.
49. Mu P, Zhang Z, Benelli M, Karthaus WR, Hoover E, Chen CC, et al. SOX2 promotes lineage plasticity and antiandrogen resistance in TP53- and RB1-deficient prostate cancer. *Science* 2017;355:84–8.
50. Kosari F, Ida CM, Aubry MC, Yang L, Kovtun IV, Klein JL, et al. ASCL1 and RET expression defines a clinically relevant subgroup of lung adenocarcinoma characterized by neuroendocrine differentiation. *Oncogene* 2014;33:3776–83.

Molecular Cancer Research

Targeting RET Kinase in Neuroendocrine Prostate Cancer

Halena R. VanDeusen, Johnny R. Ramroop, Katherine L. Morel, et al.

Mol Cancer Res 2020;18:1176-1188. Published OnlineFirst May 27, 2020.

Updated version Access the most recent version of this article at:
doi:[10.1158/1541-7786.MCR-19-1245](https://doi.org/10.1158/1541-7786.MCR-19-1245)

Supplementary Material Access the most recent supplemental material at:
<http://mcr.aacrjournals.org/content/suppl/2020/05/23/1541-7786.MCR-19-1245.DC1>

Cited articles This article cites 48 articles, 22 of which you can access for free at:
<http://mcr.aacrjournals.org/content/18/8/1176.full#ref-list-1>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link
<http://mcr.aacrjournals.org/content/18/8/1176>.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.