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14. ABSTRACT
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 androgen receptor (AR) signaling. AVPC tumors may also express neuroendocrine markers, 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 AR-negative to AR-positive prostate cancer cell lines and identified multiple altered signaling pathways, including enrichment of RET kinase activity in the AR-negative cell lines. Clinical NEPC and NEPC patient derived xenografts displayed upregulated RET transcript and RET pathway activity. Pharmacologically inhibiting RET kinase in NEPC models dramatically reduced tumor growth and cell viability in mouse and human NEPC models. Our results suggest that targeting RET in NEPC tumors with high RET expression and may be a novel treatment option.

15. SUBJECT TERMS
RET kinase, castration resistant prostate cancer, aggressive variant prostate cancer, kinase inhibitors, mouse models, organoids

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INTRODUCTION

Prostate cancer (PCa) is the most common male cancer in the United States. Blocking androgen synthesis or signaling through the androgen receptor (AR) is the first line treatment. However, prostate cancer inevitably develops castration resistant prostate cancer (CRPC) and an emerging subset (35%) of CRPC patients develop a highly aggressive tumor phenotype designated aggressive variant prostate cancer (AVPC) and treatment provides modest 1-2 years survival rates. AVPC is characterized by low to absent AR levels and expression of neuronal, reprogramming and stem related gene signatures. Previously, we observed tyrosine phosphorylation of RET, suggesting activity of this kinase in a patient with prostate AVPC. Our objectives are to 1.) Functionally assess the role of RET kinase in the transition to AVPC, 2.) Determine if RET kinase collaborates with EZH2 to drive AVPC, 3.) Determine RET dependent kinase signaling driving lineage plasticity, and 4.) Conduct preclinical trials to evaluate co-inhibition of RET kinase and EZH2 for treatment of AVPC, and re-sensitizing AVPC to enzalutamide. These all are important and unanswered questions involving the most lethal PCa phenotype observed clinically. Our success will drive the PCa field forward, and significantly alter clinical management of patients with AVPC.

KEYWORDS

Prostate Cancer; ADT; Castration Resistance; Enzalutamide; Lineage Plasticity, AVPC; NEPC; RET; Tyrosine Kinase, EZH2; Methylation; Epigenetics, Tyrosine Kinase Inhibitor, Organoids.

ACCOMPLISHMENTS

What were the major goals of the project?

Within the first year of this DOD application, the major goals for the Drake and Ellis lab were to:

Major Task #1:

1. Gain IACUC and ACURO approval for both laboratories as stated in Milestone #1.
2. Generate genetically engineered mice to stand as our breeding colony for generation of our planned experimental genotypes.

Major Task #2:

3. Assess the function of RET knockdown in cell line models.

What was accomplished under these goals?

Major Task #1:

1. Gain IACUC and ACURO approval – This milestone was achieved as both IACUC and ACURO animal protocols were approved in both laboratories.
2. Generate genetically engineered mice to stand as our breeding colony for generation of our planned experimental genotypes. We had achieved our goal of generating 2x breeding colonies to generate experimental mice.

   Breeder Set 1: (Subtask 1) male mice with the genotype of PbCre:Pten$^{fl/fl}$:Rb1$^{fl/fl}$:Ret$^{fl/+}$ and female mice with the genotype Pten$^{fl/fl}$:Rb1$^{fl/fl}$:Ret$^{fl/fl}$.

   Breeder Set 2: (Subtask 2) male mice with the genotype of PbCre:Pten$^{fl/fl}$:Rb1$^{fl/fl}$:Ezh2$^{fl/+}$:Ret$^{fl/+}$ and female mice with the genotype Pten$^{fl/fl}$:Rb1$^{fl/fl}$:Ezh2$^{fl/fl}$:Ret$^{fl/fl}$.

Major Task #2:

3. Assess the function of RET knockdown in cell line models. The robust RET expression in NEPC suggests it is a potential target in NEPC. In additional cancer cell line models, we sought to broadly examine if RET suppression regulated viability of NEPC or other cancer models. To do so, we examined relative RET dependency in 503 cancer cell lines in publicly available pooled genome-scale RNAi screens[1], which includes 7 prostate cancer cell lines and 1 basal prostate cell line. In each cell line, the DEMETER score for each gene indicates how gene suppression impacts cell viability compared to all other cell lines upon suppression of the same target gene. We specifically examined RET dependency based on DEMETER scores in Project Achilles data[2]. Among the 7 prostate cancer cell lines, two of the AR-negative AVPC cells, PC3 and NCI-H660, exhibited greater relative dependency on RET compared the 501 other cell lines

(ranked 10th and 76th) including a normal prostate epithelial PRECLH cell line (**Fig. 1A**). We also compared the patterns of RET dependency relative to 11280 genes in 8 prostate cell lines. As shown in **Fig. 1B**, 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 AR. AR expectedly showed strong correlation with AR regulators (CTNNB1, NCOA1 and CREBBP). This indicates RET kinase suppression has the greatest efficacy in cells that exhibit NEPC features but may not be efficacious in AR-driven models. In addition, we have created RET shRNA constructs and transduced them into several prostate cancer cell lines. We noticed that 3 of the 5 shRNA RET constructs were able to knockdown RET in NCI-H660 cells (**Fig. 2A**) and corresponding downstream RET signaling such as MAPK1/3 (**Fig. 2A**). We have begun assessing the functional consequences of RET knockdown in both NCI-H660 and PC-3 cell lines (another NE cell line). We now have data showing that RET knockdown in PC-3 cells resulted in reduced cell proliferation, suggesting a functional role for RET kinase in NEPC (**Fig. 2B**). We are now testing the role of RET knockdown in NCI-H660 cells and also evaluating whether RET knockdown alters NE gene expression as well as EZH2 activity (subtask #2).

Major Task #3:

4. We have assessed the phosphoproteome of AR+ and AR- (AVPC) prostate cancer cell lines (**Fig. 3**) and found RET activity to be high in the AR- lines (**Fig. 3D**). Part of our analyses has also been to assess whether RET signaling is indeed activated in these AR- cell lines. We mapped out the RET pathway and found several components of the RET pathway activated based on our phosphoproteomic screen (**Fig. 4)**. Once the mice have been bred (Major Task #1), we will also assess the role of RET signaling in these mouse models of RET knockout using phosphoproteomics.

Major Task #5:

5. We have begun to evaluate several different RET inhibitors including vandetanib, cabozantinib, and AD80 on a compendia of prostate cancer cell lines (Subtask 1). We have found that AD80, a more potent RET inhibitor, reduce the IC50 concentration and was more effective at cell killing than vandetanib and cabozantinib in NEPC models such as NCI-H660 cells (**Fig. 5**).
6. We have also tested AD80 on mouse organoid models of NEPC. Working with Dr. Ellis and using his double knockout (DKO) mouse model of $PTEN^{-/-}$ and $RB^{-/-}$, mimicking NEPC[3], we were able to show increased RET mRNA (**Fig. 6A**), protein (**Fig. 6B**) and the organoids grown from these DKO tumors showed a dose dependent effect to a RET inhibitor AD80 (**Fig. 6C, D**), and were not responsive to high doses of enzalutamide (**Fig. 6E7**). We also and found that this compound can synergize with enzalutamide (**Fig. 7A-C**), suggesting that RET inhibition may re-sensitize AR- tumors to enzalutamide (Subtask 2). We are investigating the mechanism as to how this may occur.

What opportunities for training and professional development has the project provided? Within year 1, the trainee, Dr. Yuzhen Zhou has successfully completed her official animal training with the Animal Resource Facility at Dana-Farber Cancer Institute. Dr. Zhou has also been efficiently trained within the Ellis lab to successfully maintain mouse breeding colonies and genotype mice. Dr. Halena VanDeusen in the Drake laboratory was able to attend the Society for Basic Urologic Research Annual Meeting and present her work on this project.

How were the results disseminated to communities of interest? To date, we have presented some aspects of our work to conferences including the Prostate Cancer Foundation Retreat and Society for Basic Urologic Research Annual Meeting in 2018. We have also published this work to the BioRxiv and submitting the manuscript to the journal *Cancer Research* in November 2019. Currently, the data presented includes RET kinase inhibition on cell lines and xenograft tumors. No genetically engineered mouse data has been generated and matured so that is ready for dissemination to the community.

What do you plan to do during the next reporting period to accomplish the goals? We will continue our breeding of experimental mice (GEMMs) so that we can age and begin to collect samples for post-mortem analysis including histopathology and phosphoproteomics, RNA- and ChIP-seq analysis (Completion of

Milestone #2, Major Task #3, and provide necessary information for Milestone #3). We will also complete *in vitro* drug response assays (Subtasks 1 and 2 in Major Task 5) with Dr. Drake's lab.

IMPACT

What was the impact on the development of the principal discipline(s) of the project?
Nothing to report.

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

1. In **major task 1** and listed about as we had proposed to use males that carried homozygous floxed alleles of Pten (PbCre:Pten$^{fl/fl}$:Rb1$^{fl/fl}$:Ret$^{fl/}$). We have found this to be insufficient to produce our required experimental mice in a timely manner. We have been successful at only being able to produce one litter from each male with this genotype. This is believed to be because of accelerated disease kinetics and because of the disease burden in male mice their reproducibility is significantly diminished.

2. **Change in post-doc** – originally another post-doc (Dr. Zach Richards) was employed by Dr. Ellis to work on this award. Dr. Richards decided to leave academic research in pursuit of a job with industry. With this, Dr. Ellis hired Dr. Yuzhen Zhou to perform the research outlined.

   Both changes have resulted in significant delay from accomplishing our intended goals, but we have regained momentum and will be caught up very soon.

3. In **major task 2** we have moved away from the LNCaP model grown in charcoal stripped serum as the results are too variable. We have since moved to use other prostate cancer cell line models that represent AVPC such as NCI-H660 and PC-3. We have now created shRNA RET knockdown lines from these cell lines and assessing functionally the role of RET and also creating RET overexpressed cell lines in 22Rv1 and LNCaP cells. This has delayed our progress a bit but we are catching up quickly.

Actual or anticipated problems or delays and actions or plans to resolve them

1. To rectify this, we have now started to produce and use male mice that are heterozygous for floxed alleles of Pten (PbCre:Pten$^{fl/+}$:Rb1$^{fl/fl}$:Ret$^{fl/}$). Since using these male mice as breeders, we have observed the ability to produce approximately 3-4 litters until disease progression becomes an issue towards reproducibility.
2. Dr. Ellis hired a postdoctoral fellow, Dr. Yuzhen Zhou to perform the research outlined within the SOW.

Changes that had a significant impact on expenditures
None of the changes described above will result in >25% change in budget allocation.

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
Nothing to report.

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

Other publications, conference papers, and presentations
National Presentations:
Masonic Cancer Center Seminar Series, University of Minnesota, Minneapolis, MN, "RET tyrosine kinase in neuroendocrine prostate cancer."
GU Seminar Series, Dana Farber Cancer Institute, Boston, MA, "Targeting RET tyrosine kinase in neuroendocrine prostate cancer."

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 AND OTHER COLLABORATING ORGANIZATIONS
What individuals have worked on the project?

Name:	Dr. Leigh Ellis
Project Role:	Co-Principal Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0003-4739-5049
Nearest person month worked:	
Contribution to Project:	Co-PI
Funding Support:	NCI, DOD

Name:	Dr. Yuzhen Zhou
Project Role:	Post-doc
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	Post-doc researcher
Funding Support:	N/A

Name:	Dr. Justin M. Drake
Project Role:	Co-Principal Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	
Contribution to Project:	Co-PI
Funding Support:	DOD

Name:	Dr. Halena VanDeusen
Project Role:	Post-doc
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12
Contribution to Project:	Post-doc researcher
Funding Support:	

Name:	Dr. Song Yi Bae
Project Role:	Post-doc
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	4
Contribution to Project:	Post-doc researcher
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
None

APPENDICES

- Figure 1-7
- References

Figure 1

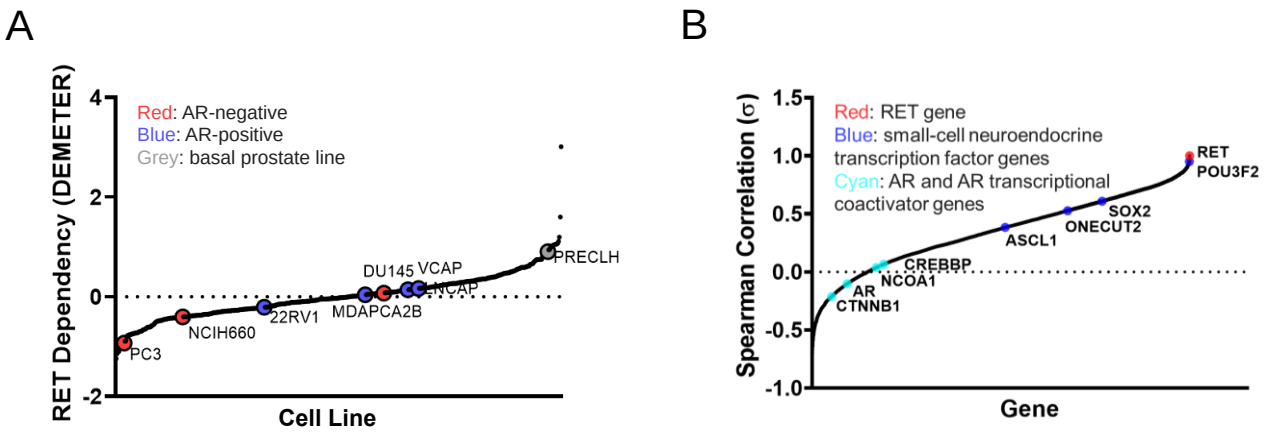


Figure 1. RET dependency in prostate cancer cell lines. A. Relative dependency scores for RET across 503 cancer cell lines. Among the 8 prostate cancer cell lines, PC3 and NCI-H660 cells showed the greatest dependency on RET. **B.** RET dependency profiling for 11280 genes across 8 prostate cancer cell lines. RET dependency was positively correlated with NEPC drivers genes (blue) and negatively correlated with AR and AR regulators (cyan).

Figure 2

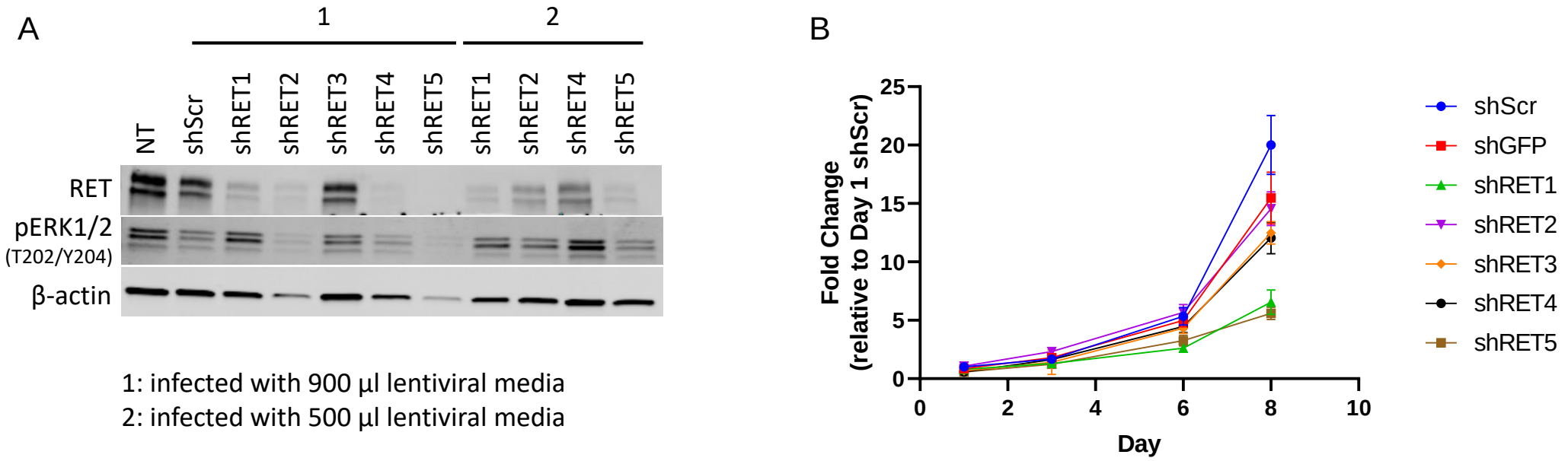


Figure 2. Knockdown of RET kinase reduced cell proliferation. A. 5 different RET shRNA constructs were transduced into NCI-H660 cells. RET knockdown and downstream MAPK signaling was assessed via western blot. **B.** Cell proliferation assay in PC-3 cells after RET knockdown. PC-3 cells were plated at a density of 200 cells/well in a 96-well plate and measurements taken on Days 1, 3, 6, 8 using the WST colorimetric assay. Values are plotted relative to the scrambled control. shRET1 and 5 showed the greatest effect on reducing cell proliferation, which coincides with the knockdown of RET by these constructs in A.

Figure 3

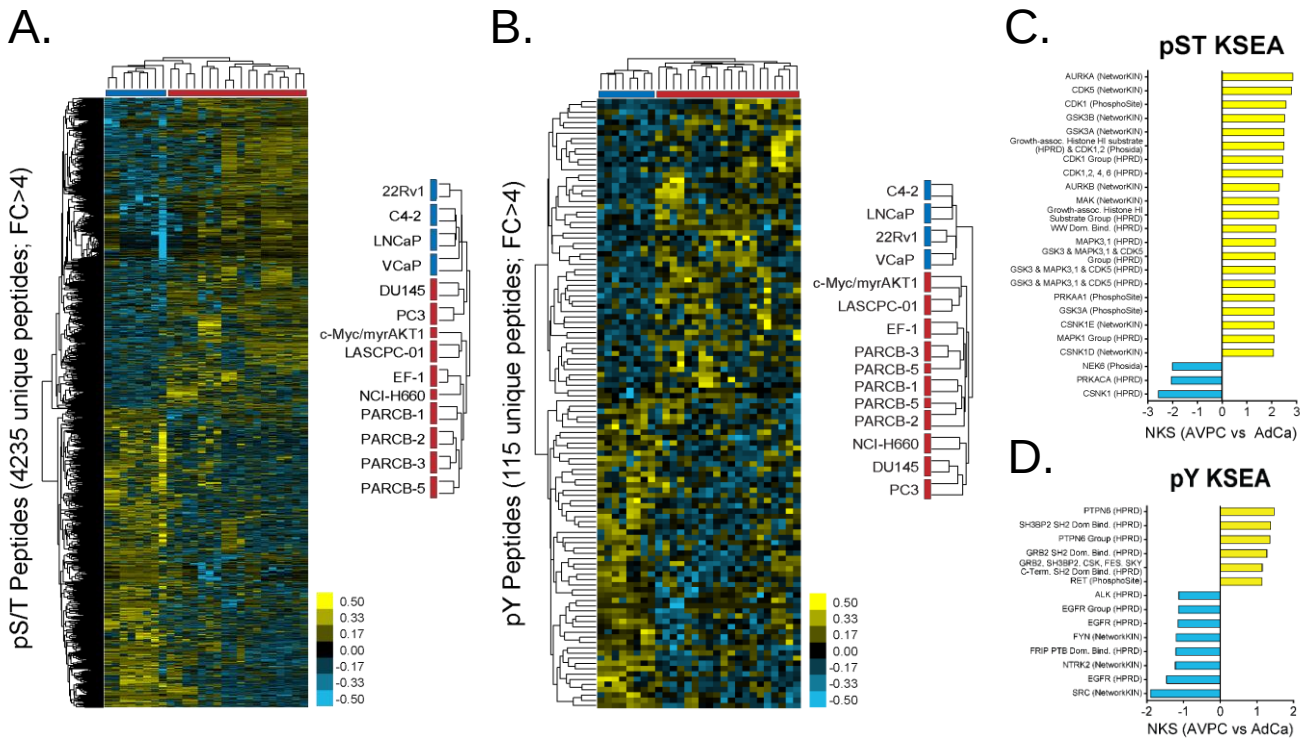


Figure 3. Global phosphorylation and kinase signaling pathways are differentially regulated in AVPC cell lines compared to AdCa cell lines. A and B. Supervised hierarchical clustering heatmap of 4,235 unique phosphoserine/threonine (pS/T) enriched peptides (**A**) and 115 unique phosphotyrosine (pY) enriched peptides (**B**) from AdCa 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 AdCa cell lines in A and B, showed multiple 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.

Figure 4

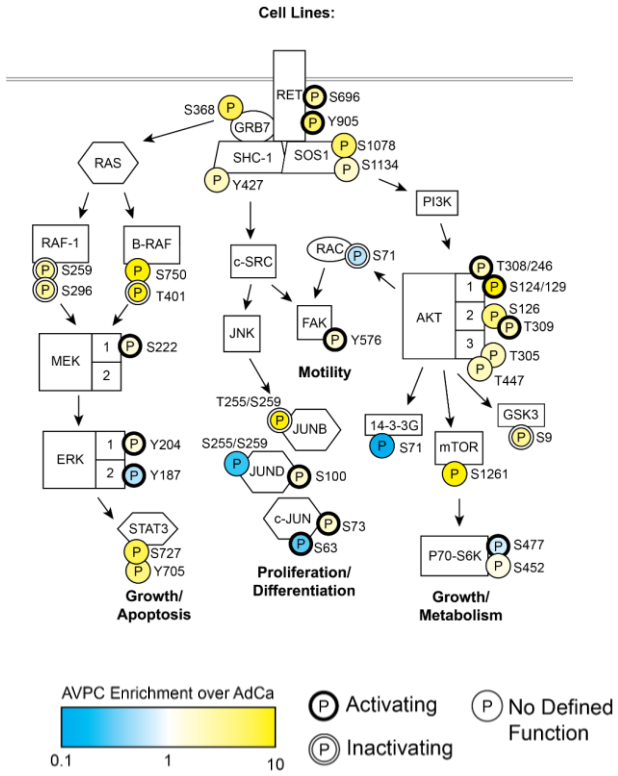
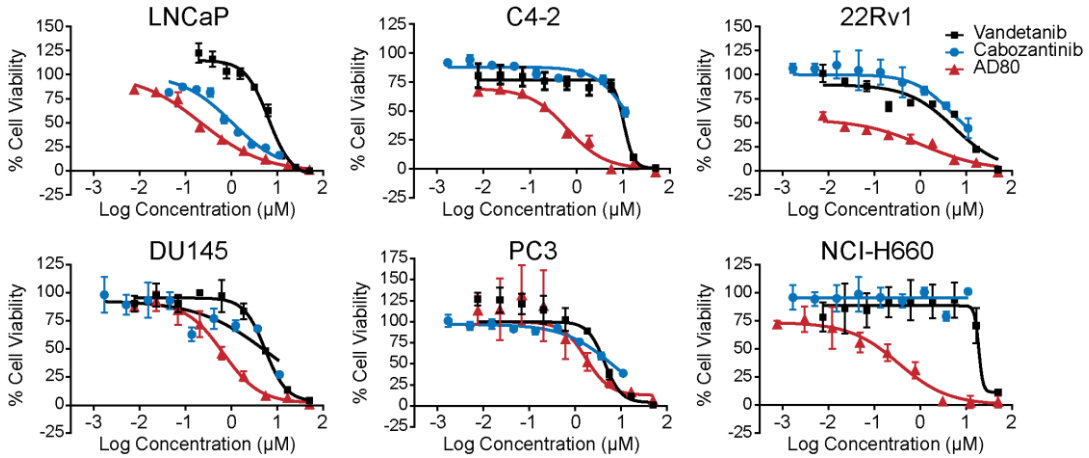


Figure 4. Global phosphorylation and kinase signaling pathways are differentially regulated in AVPC cell lines compared to AdCa cell lines. Phosphorylated residues identified in the global phosphoproteomics (from **Fig. 3A and B**) were mapped onto signaling pathways downstream of RET kinase. Yellow = Enriched in AVPC relative to AdCa; Blue = Reduced in AVPC relative to AdCa. Thick black outline = activating phosphorylation; white outline = inactivating phosphorylation; thin outline = no defined function.

Figure 5

A.



B.

IC50 (μM)	LNCaP	C4-2	22Rv1	DU145	PC3	NCI-H660
Vandetanib	7.3	10.4	5.5	5.4	4.3	19.0
Cabozantinib	0.83	14.2	4.5	6.3	6.7	ND
AD80	0.26	0.63	1.1	0.65	1.7	0.37

A. IC_{50} dose response curves of AdCa (LNCaP, C4-2, and 22Rv1), AVPC (DU145 and PC3), and NEPC (NCI-H660) cell lines treated with varying concentrations of multi-tyrosine kinase inhibitors vandetanib, cabozantinib, AD80. Error bars represent ± SD. **B.** Table of the calculated IC_{50} values (μM) for each of the cell lines and three drugs. ND is not determined.

Figure 6

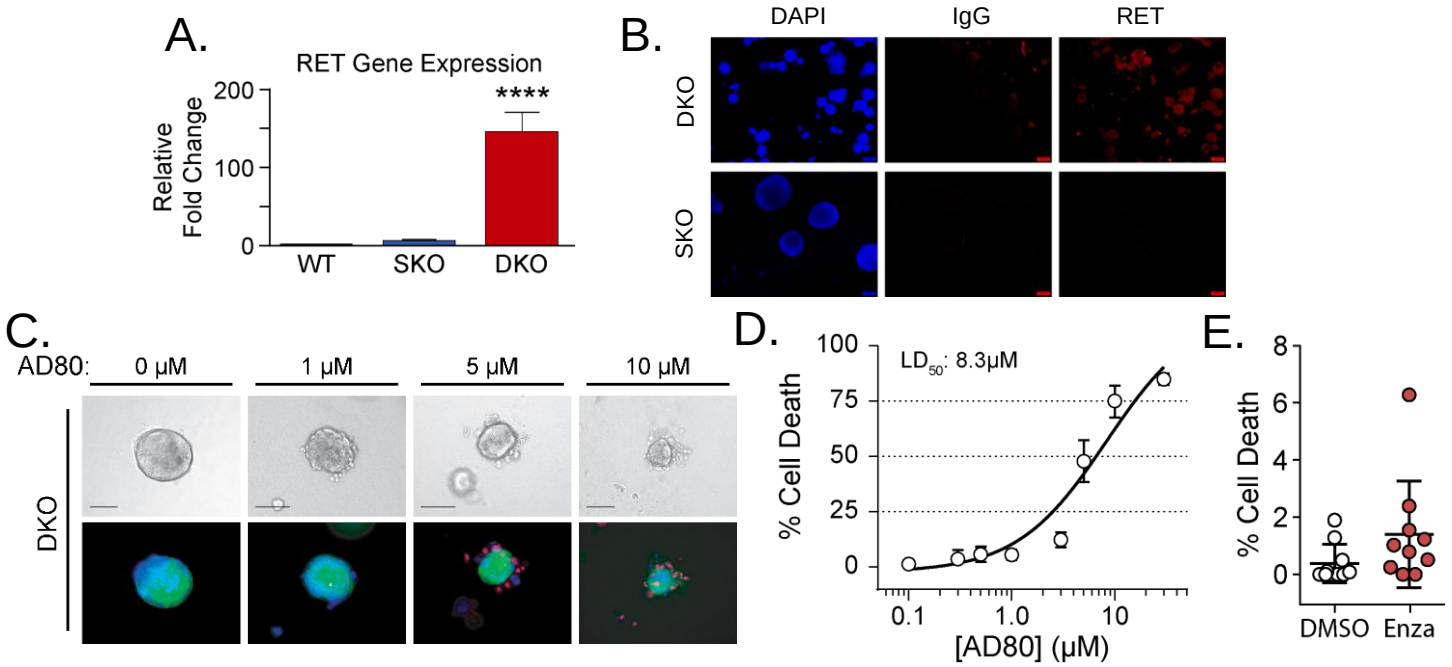


Figure 6. RET is increased in a mouse model of NEPC. A. RNAseq data of RET gene expression from wild type (WT), PTEN$^{-/-}$ and Rb$^{-/-}$ double knockout (DKO), or PTEN$^{-/-}$ single knockout (SKO) mouse prostate epithelium organoids. RET FKPM values were normalized to WT prostate tissue and expressed as fold change and analyzed by Student's t test. **B.** RET immunofluorescence staining in PTEN$^{-/-}$ and Rb$^{-/-}$ DKO derived organoids and PTEN$^{-/-}$ SKO organoids or IgG antibody control. DAPI staining of nuclear DNA is used to identify organoids. **C.** 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 =100µm. **D.** Dose response curve of DKO organoids treated with increasing concentrations of AD80. Circles represent the mean with error bars ± standard error. **E.** Percentage of PI positive cells in DKO organoids treated with DMSO or 10µM enzalutamide. Circles represent values from individual organoids. Horizontal bar represents the mean with error bars ± standard error.

Figure 7

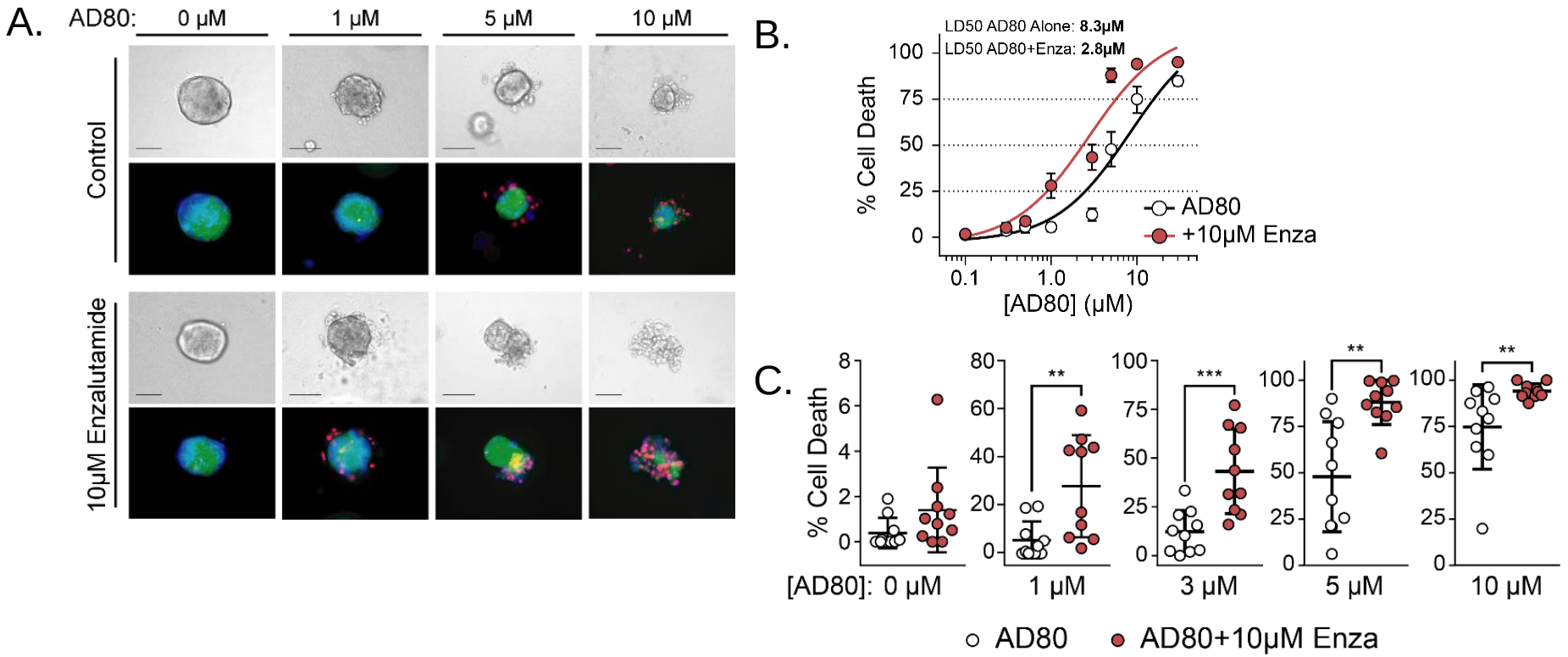


Figure 7. RET inhibition synergizes with enzalutamide in NEPC models. A. Brightfield images and corresponding immunofluorescence staining of GFP labeled-DKO organoids treated with the indicated concentrations of AD80 in the presence of DMSO (control) or 10 µM enzalutamide. Blue=DAPI staining of nuclei, Red=Propidium iodide staining of dead cells. Increasing concentrations of AD80 increase apoptosis, which is further enhanced by 10µM enzalutamide. Scale bar =100µm. **B.** Dose response curve of DKO organoids treated with increasing concentrations of AD80 alone or AD80 + 10 µM enzalutamide. The LD50 for AD80 alone was calculated to be 8.3µM, but this was reduced approximately three fold to 2.8 uM AD80 in the presence of 10 µM enzalutamide. **C.** Individual data points from the indicated concentrations of AD80 show an increasing incidence of apoptosis, which is further enhanced in the AD80+Enzalutamide group. ** $p<0.005$, *** $p<0.001$.

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