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TITLE: Mechanisms of Resistance to Androgen Deprivation Therapy in Advanced Castration-Resistant Prostate Cancer (CRPC)

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The overall hypothesis is that expression of the dipeptidase DPP4 is downregulated in prostate cancer (PCa) as a mechanism of resistance to androgen deprivation therapy (ADT). My overall objective is to demonstrate that DPP4 downregulation is a mechanism of ADT-resistance and PCa progression and to identify the specific pro-survival growth factor/cytokine targeted by DPP4 for degradation and its associated signaling cascade. Aim 1 will assess the effect of DPP4 downregulation and overexpression on the sensitivity of PCa xenografts to castration. Aim 2 will identify the pro-survival growth factor/cytokine targeted by DPP4 for degradation and the downstream signaling cascades effected. Aim 3 will extend the significance of DPP4 downregulation into primary PCa and CRPC clinical specimens and assess the interaction of DPP4 inhibition with ADT.

15. SUBJECT TERMS

DPP4, CD26, prostate cancer, castration-resistance, androgen deprivation therapy, growth factor, cytokine

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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

When men develop prostate cancer (PCa) that spreads or metastasizes to other parts of their body, the first and second line treatments used by doctors attempt to block the effects of the androgen hormones testosterone and dihydrotestosterone (DHT) on prostate cancer cells. This type of therapy is called androgen deprivation therapy or ADT because it deprives the prostate cancer cells of these important androgen hormones. Testosterone and DHT bind to the androgen receptor (AR) within cancer cells and stimulate the growth and progression of prostate cancer. Gonadotropin-releasing hormone agonists (GnRH-agonists), abiraterone, and enzalutamide are three drugs commonly used to block the pro-cancer hormone signaling that occurs through testosterone and DHT. GnRH agonists inhibit the testicular production of androgen hormones. Abiraterone inhibits an enzyme called CYP17A1, which decreases the levels of testosterone and DHT. Enzalutamide blocks the ability of testosterone and DHT to bind to androgen receptor (AR) and stimulate prostate cancer cells. These drugs are initially effective at stopping prostate cancer progression, but in nearly all men the cancer eventually becomes resistant. The subject of this research proposal is determining how downregulation of the gene *DPP4* and its protein product mediates prostate cancer resistance to ADT and how DPP4 inhibitors used to treat Type II diabetes influence prostate cancer progression. Over the past year I have shown that DPP4 downregulation is tightly associated with PCa progression in preclinical models and in clinical biopsy materials. In the VCaP xenograft model and in the majority of clinical cases, as PCa becomes resistant to ADT, AR signaling is restored. DPP4 is similar to PSA in that it is an AR-stimulated gene. However, in the resistant setting, while PSA expression is restored, DPP4 expression is not. This suggests that the continued downregulation of DPP4 might have functional significance in PCa survival, especially since DPP4 is known to degrade various pro-survival growth factors and cytokines. Of greater significance, I have also shown that inhibitors of DPP4 enzyme activity decrease the effectiveness of ADT. My overall hypothesis is that DPP4 expression is downregulated in PCa progression in order to increase local concentrations of prosurvival growth factors/cytokines to overcome androgen deprivation. As it will be difficult to identify therapies capable of increasing DPP4 protein expression within PCa cells, the next important step in this work will be to identify the pro-survival growth factor that is degraded by DPP4 and the kinase signaling cascade the growth factor activates to promote ADT resistance. This will allow us to target the growth factor and its associated receptor/kinase cascade directly to block ADT resistance.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

DPP4, CD26, prostate cancer, castration-resistance, androgen deprivation therapy, growth factor, cytokine

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Goal 1: Assess the functional significance of DPP4 downregulation in PCa xenograft setting.
Milestone: Production of stable cell lines capable of inducible knockdown and overexpression of DPP4 (Month 4) – 50% Complete
Milestone: Establishment of VCaP xenografts, PC-BID-1 and LuCaP-35 PDXs, and VCaP xenografts with stable inducible nonsense and DPP4 shRNA and stable inducible overexpression of DDP4 (Month 10-11) – 75% Complete
Milestone: Assess the functional significance of DPP4 downregulation in the PCa xenograft setting. (Month 15) – 50% Complete

Goal 2: Determine the signaling cascades effected by DPP4 downregulation/inhibition and the corresponding growth factors/cytokines targeted by DPP4 that are responsible for ADT resistance.
Milestone: Identification of signaling cascades effected by DPP4 downregulation/inhibition (Month 20) – 25% Complete

Goal 3: Determine the clinical significance of DPP4 expression and concurrent ADT/DPP4 inhibitor treatment on PCa progression. (Month 20) – 50% Complete

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

1. Major Activities: The major focuses over the past year have been to extend the preliminary results in xenograft model systems regarding the interaction of DPP4 inhibitors with castration, developing stable cell lines with inducible shRNA and cDNA to DPP4, and analysis of DPP4 expression in clinical material with clinicopathological correlation. In order to extend the xenograft results, studies have been performed in additional xenograft and PDX models on the interaction of sitagliptin treatment with castration on the development of castration-resistance. The development of a VCaP cell line stably expressing inducible shRNA to DPP4 has been completed and the establishment of the line with inducible cDNA to DPP4 is underway. Finally, DPP4 immunohistochemistry (IHC) has been optimized for FFPE clinical materials and DPP4 expression levels have been analyzed in primary PCa, Neoadjuvant-treated PCa, and metastatic CRPC.

2. Specific Objectives: My overall objective is to demonstrate the functional significance of DPP4 downregulation in mediating resistance to ADT in CRPC and identifying the mechanism by which this resistance occurs. My specific objectives include demonstrating the functional significance of DPP4 downregulation in preclinical models, identifying the specific growth factor/cytokine and associated signaling cascade that is upregulated in response to DPP4 downregulation, and correlating DPP4 expression/inhibition with PCa progression.

3. Significant Results and Key Outcomes:

DPP4 is downregulated in PCa progression both in preclinical models and clinical specimens. *DPP4* is an AR-stimulated gene and would be expected to decrease with initial ADT, but similar to other AR-stimulated genes such as PSA, its expression should be restored with castration resistance. However, as outlined in preliminary data for this award, this does not occur and *DPP4* expression remains downregulated. To further assess DPP4 downregulation in PCa progression, I submitted serial biopsies of VCaP xenografts prior to castration (Pre-Cx), at castration-resistance (CRPC), and at resistance to combined abiraterone and enzalutamide therapy (Abi/Enza Resistant) for RNA sequencing. The results of this sequencing confirmed the decreased expression of *DPP4* as VCaP xenografts progress. Interestingly, *DPP4* was not only one of the most decreased AR-stimulated transcripts, but was also one of the most decreased of all differentially expressed transcripts **(Figure 1A)**. These decreases were confirmed at the protein level by IHC and RPPA **(Figure 1B-C)**. Extending these results into several clinical RNAseq data sets from our group, I found *DPP4* expression levels to be decreased in castration-resistant samples compared to matched treatment-naïve controls **(Figure 2A)**. Moreover, IHC on tissue microarrays (TMAs) showed a striking decrease in DPP4 protein levels in CRPC (Avg. Immunoscore = 1.25) when compared to primary PCa (Avg. Immunoscore = 6.49) **(Figure 2B)**.

These results establish a clear correlation between the downregulation of DPP4 and PCa progression. Extending the preliminary results found in the VCaP xenograft to clinical specimens is a key outcome. Further, showing that expression of the AR-regulated *DPP4* remains low when AR signaling signaling is restored in CRPC is a significant finding. It suggests that the continued downregulation of DPP4 might have functional significance in PCa survival, and hints at the possibility of an altered AR cistrome in castration-resistance with functional consequences.

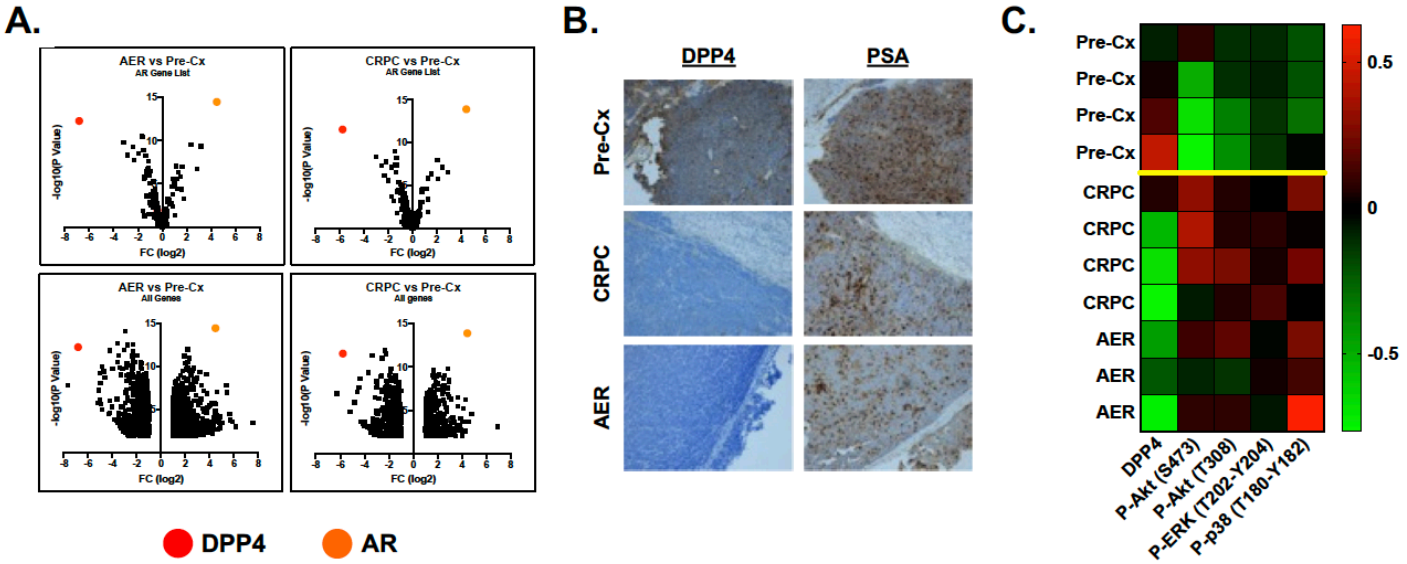
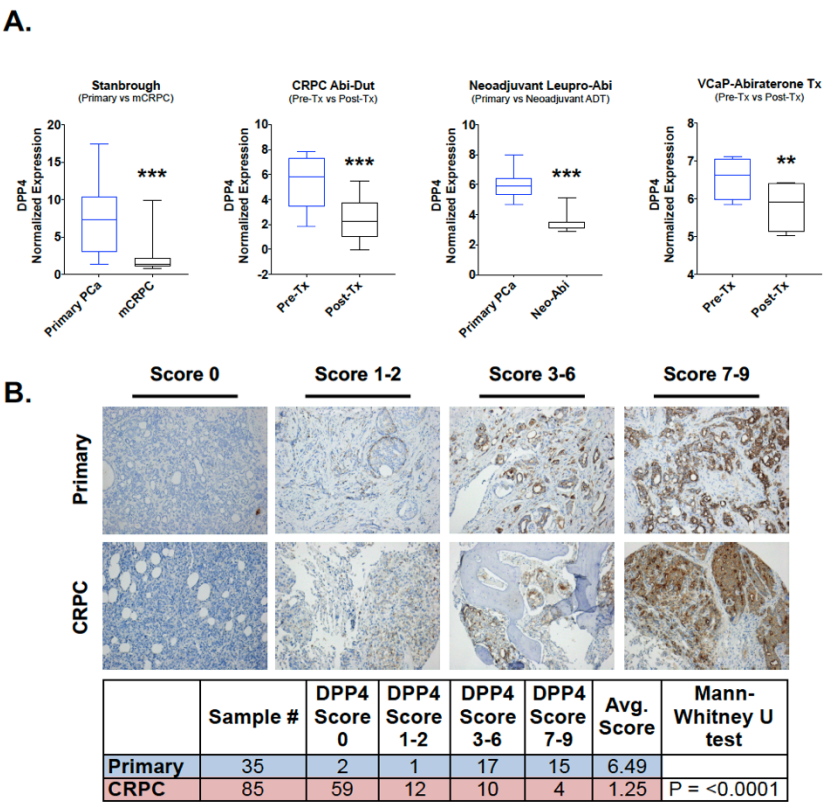


Figure 1 – DPP4 expression in AER VCaP xenograft serial biopsies. AER VCaP tumors were serial biopsied prior to cx (Pre-Cx), at tumor relapse (CRPC), and when tumors exceeded 2000mm³ (Abi/Enza resistant = AER). Initial cohort composed of 22 mice. **A)** Volcano plots of RNA sequencing data depicting AR target genes (267 AR gene signature from Mendiratta et al. PMID: 19289629) and all significantly differentially expressed genes (lower) in the AER vs. Pre-Cx and CRPC vs. Pre-Cx comparisons. The dots corresponding to *DPP4* and *AR* in each figure are highlighted. **B)** Representative images of DPP4 and PSA immunohistochemistry in serial biopsies of VCaP xenografts. **C)** RPPA data from the serial biopsies of 4 separate VCaP xenografts.

Inhibition of DPP4 enzyme activity decreases the effectiveness of ADT. DPP4 is a dipeptidase that is known to degrade many different growth factors and cytokines, including some that have been shown to promote prostate cancer survival/proliferation (IGF-1, NPY, SDF1-α). My hypothesis is that the decreases in DPP4 protein and associated decreases in DPP4 enzyme activity observed during castration-resistance result in increases in local concentrations of pro-survival growth factors that allow PCa to survive ADT. Given this hypothesis, inhibitors of DPP4 enzyme activity such as sitagliptin should mimic DPP4 downregulation and decrease the effectiveness of ADT. Preliminary studies treating VCaP xenografts that undergo castration with sitaglipitin supported this

hypothesis. These results have now been extended into a genomically different PCa cell line (LNCaP), and more significantly into a hormone-naïve patient derived xenograft (PDX), BID-PC-1 **(Figure 3)**. In particular, the BID-PC-1 PDX is extremely sensitive to castration and can be "rescued" by DPP4 inhibitor treatment. These results are a key outcome as they show that the effects of DPP4 inhibition are penetrant across PCa with different genomic backgrounds, VCaP (AR amplification, TMPRSS2:ERG fusion), LNCaP (PTEN deficient), and BID-PC-1 (hormone-naïve, BRCA2 deficient). As we obtain additional ca PDX models, I will continue to test the effects of DPP4 inhibition. DPP4 inhibitor treatment in these xenograft/PDX systems can also be used as another approach to identify the signaling cascades effected by DPP4 inhibition. Using this information we can then backtrack to the ligand DPP4 targets for degradation. This work also highlights significant implications regarding the interaction of DPP4 inhibitors used to treat Type II diabetes with the androgen deprivation therapies (leuprolide, abiraterone, enzalutamide, etc.) used to treat metastatic prostate cancer.

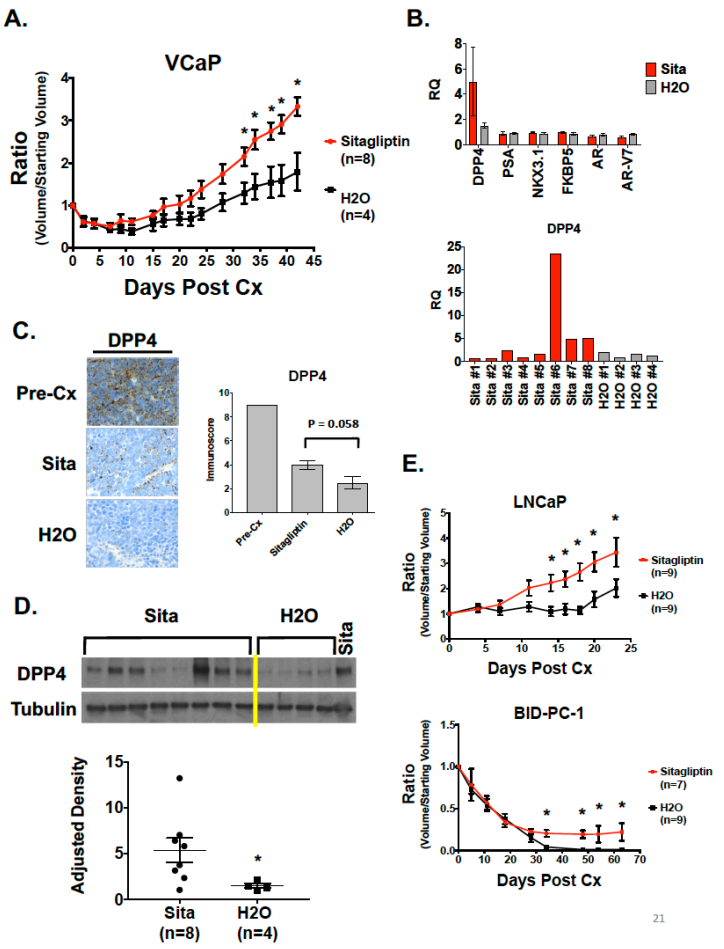


	Sample #	DPP4 Score 0	DPP4 Score 1-2	DPP4 Score 3-6	DPP4 Score 7-9	Avg. Score	Mann-Whitney U test
Primary	35	2	1	17	15	6.49	
CRPC	85	59	12	10	4	1.25	P = <0.0001

Figure 2 – DPP4 expression is decreased in ADT-resistant cliniral specimens. **A)** RNA expression levels of *DPP4* obtained through Affymetrix microarray (Stanbrough) or RNA sequencing (CRPC Abi-Dut, Neoadjuvant Leupro-Abi, and VCaP Abiraterone-Tx) of material from several clinical and preclinical studies comparing Pre-ADT samples to resistant Post-ADT samples.*** = P<0.0001, ** = <0.001, limma for RNA sequencing studies and two-tailed student's t-test for microarray study. Abi – Abiraterone, Dut – Dutasteride, Leupro – Leuprolide. **B)** Representative images of DPP4 immunohistochemistry and DPP4 immunoscoring from a series of hormone-naïve primary prostate cancer (Primary) and CRPC clinical sections.

DPP4 downregulation correlates with negative risk factors in primary PCa. DPP4 expression is strikingly decreased in the CRPC setting, but there are also a small fraction of primary prostate cancer that express little to no DPP4 **(Figure 2)**. To further characterize DPP4 expression in the primary setting I performed IHC on a mixed set of 20 low-grade and 20 high-grade PCa specimens from radical prostatectomies of hormone-naïve patients. While DPP4 protein was still expressed in all primary tumors, high-grade PCa (Gleason Score 8-10) showed a clear reduction in DPP4 protein levels compared to low-grade (Gleason 6) **(Figure 4)**. Further, when I dichotomized the samples by the H-score immunoscoring system into high and low DPP4 expressers **(Table 1)**. I found correlations between low DPP4 levels and a number of negative risk factors including Gleason score > 8, increased tumor volume, higher stage, margin positivity, presence of extra-prostatic extension (EPE), Pre-RP highest Gleason score >8, and increased percent of biopsy core positive for cancer. It is not yet clear what role DPP4 downregulation would play in the primary setting where androgen is plentiful and there is no need for additional pro-survival growth factors and cytokines. However, these DPP4-low primary tumors might represent a more aggressive PCa subtype that have altered AR signaling and that are less likely to respond to ADT therapy if the patient develops metastatic disease. I am expanding these studies into intermediate grade PCa (Gleason 7) disease to asses if DPP4 levels can differentiate intermediate favorable from intermediate unfavorable PCa.

Figure 3 – DPP4 inhibitor increases VCaP tumor resistance to castration. A) VCaP subcutaneous xenografts were grown in intact male mice until tumors reached 500mm^3, then mice were castrated (Cx) and immediately began treatment with sitagliptin (120 mg/kg) or vehicle administered in drinking water. Y axis is the ratio of tumor volume at a given time point divided by the tumor starting volume. Bars = standard error of the mean (SEM). * = P<0.05, Mann-Whitney U. **B)** RT-PCR of *DPP4* and the AR regulated genes *PSA*, *NKX3.*1, and *FKBP5*, as well as the transcripts for AR and AR-V7 in VCaP xenografts harvested at Day 42 of the experiment represented in panel A. Each column represents the expression levels of xenograft tumors from eight mice (Sita) or four mice (H2O), with RT-PCR performed on each in technical triplicate. Bars represent standard error of the mean. H2O = water, Sita = Sitagliptin, RQ = Relative Quantification **C)** Representative high power images of DPP4 immunohistochemistry from Sita and H2O-treated tumors and DPP4 (left) and immunoscoring of DPP4 protein expression (right). P =0.058, Mann-Whitney U. **D)** Western blot of cell lysate from Sita and H2O-treated tumors probed with anti-DPP4 antibody (above) and densitometric quantification of bands (below). Bars = standard error of the mean (SEM). * = P<0.03, Mann Whitney U. **E)** LNCaP and BID-PC-1 subcutaneous xenografts were grown in intact male mice until tumors reached 500mm^3, then mice were castrated (Cx) and immediately began treatment with sitagliptin (120 mg/kg) or vehicle administered in drinking water. Y axis is the ratio of tumor volume at a given time point divided by the tumor starting volume. Bars = standard error of the mean (SEM). * = P<0.05, Mann-Whitney U.



<u>VCaP cell line stably expressing shRNA to DPP4 has been established.</u> Using a lentiviral expression system I have created several VCaP cell lines that express inducible shRNAs, including a control nonsense shRNA and several shRNAs against DPP4. For the 8515 cell line, doxycycline induction induces dramatic DPP4 protein knockdown at as little as 0.1 µg/ml **(Figure 5)**. Xenografts of these cell lines are currently being expanded for use in experiments to identify the signaling cascades altered by acute DPP4 downregulation. Once candidate signaling pathways are identified, the levels of possible activating growth factors/cytokines can be determined and correlated with DPP4 expression levels.

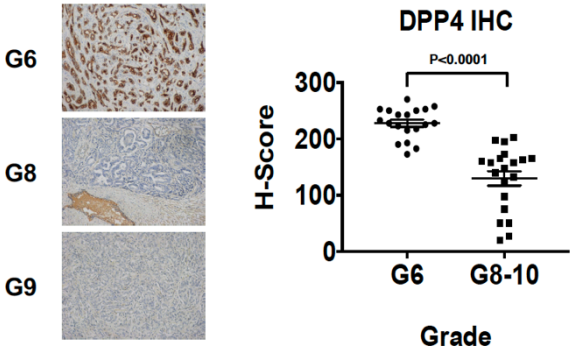


Figure 4 – DPP4 protein expression inversely correlates with tumor grade in hormone-naïve primary PCa. A) Immunohistochemistry for DPP4 protein in a selection of hormone-naïve primary prostate cancers. **B)** H-Score quantification of DPP4 IHC in 20 cases of low-grade and 20 cases of high-grade hormone-naïve primary prostate cancer. P<0.0001, Mann-Whitney U.

Table 1

	H-Score >190 (N=20)	H-Score < or =190 (19)	P	Test
Mean H-Score	228.1	124.1	p<0.0001	Mann-Whitney U
Grade LGvsHG				
LG	17 (85)	3 (15)	p<0.0001	Chi X2
HG	2 (11)	17 (89)		
Mean Gleason Score	6.5	8.737	p<0.0001	Mann-Whitney U
Age	58.4	62.79	p=0.0473	Mann-Whitney U
Wt (gram)	48.35	53.37	p=0.1177	Mann-Whitney U
Tumor volume (%)	13.75	26.67	p=0.0079	Mann-Whitney U
pT Stage				
pT2	18 (90)	3 (16)	p<0.0001	Chi X2
pT3	2 (10)	16 (84)		
Margin				
Positive	2 (10)	11 (58)	p<0.05	Chi X2
Negative	18 (90)	8 (42)		
EPE				
Positive	1 (5)	15 (79)	p<0.0001	Chi X2
Negative	19 (95)	4 (21)		
Pre-RP PSA (ng/ml)	5.796	7.521	p=0.5567	Mann-Whitney U
Pre-RP Highest Gleason Score	6.529	8.22	p<0.0001	Mann-Whitney U
# of (+) cores	3.75	5	p=0.3099	Mann-Whitney U
Highest CA % in (+)cores	47.94	72.5	p=0.0124	Mann-Whitney U
Race				
White	13 (65)	13 (70)	for White vs Black p=0.6819, Chi X2	
Black	3 (15)	2 (10)		
Hispanic	1 (5)	1 (5)		
Asian	1 (5)	0		
Native American	1 (5)	0		
Unknown	1 (5)	3 (15)		

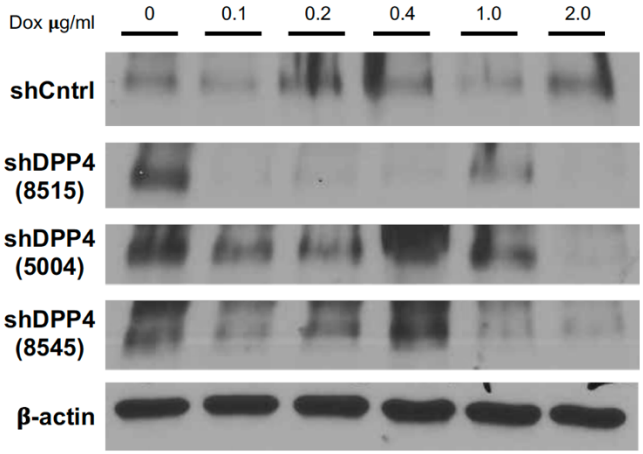


Figure 5 – VCaP cells stably transfected with an inducible shRNA against DPP4. Western blots showing DPP4 protein expression following doxycycline induction in four VCaP cell lines stably expressing different inducible shRNAs against DPP4 and a control shRNA.

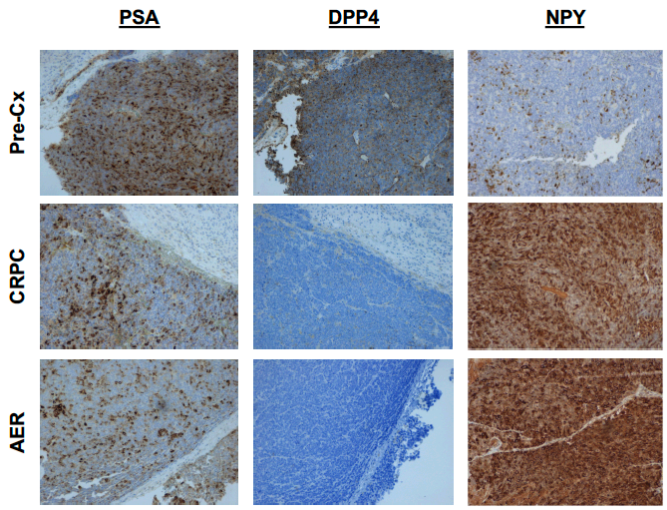


Figure 6 – DPP4 protein expression inversely correlates with NPY protein expression. Immunohistochemistry for PSA, DPP4, and NPY protein in representative serial biopsies of a VCaP xenograft at Pre-Cx, CRPC, and Abi/Enza resistance.

NPY is a possible pro-survival growth factor in PCa increased by DPP4 downregulation. While stable cell lines are being developed, I also took a likely candidates approach to identifying the growth factor DPP4 targets for degradation and the possible signaling cascades upregulated by DPP4 downregulation. NPY has been shown to positively effect prostate cancer proliferation and is a well characterized degradation target of DPP4. IHC for NPY in serial biopsies of VCaP xenografts at Pre-Cx, CRPC, and Abi/Enza resistance show an increase in NPY protein levels that inversely correlates with DPP4 expression **(Figure 6)**. In reviewing the RNAseq data on these tumors, NPY transcript levels also increase with progression making it possible that the observed increase in NPY protein might be by transcription alone. However, increased NPY protein levels might be a product of increased transcription as well as decreased degradation (via DPP4 downregulation). The xenograft studies using inducible knockdown and overexpression of DPP4 will be helpful in dissecting this.

4. Other Achievements: None

5. Stated Goals Not Met: For Aim 2, stable cell lines expressing an inducible DPP4 cDNA and xenografts derived from these cell lines have not yet been developed, but will be the main focus at the beginning of year two of this award. For Aim 3, with the assistance of my mentor Dr. Balk, I have attempted to develop a collaboration with several different Harvard Cancer Center and Harvard T.H. Chan School of Public Health epidemiologists who are part of our prostate SPORE program. Many of them have access to SEER type databases that would be ideal for the proposed studies. We have had several good discussions, but no results as yet. We plan to make headway on the epidemiological goals for CRPC outlined in Aim 3 during the next funding period. The goal of the collaboration will be to access patient data and look at the interaction of DPP4 inhibitors with ADT in men with Type II diabetes and metastatic PCa.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those

in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Through this project I have had extensive one-on-one work with my mentor, Steve Balk. This includes a weekly progress report style meeting and several ad-hoc meetings a week to brainstorm DPP4 hypotheses. I also present my work every 6 weeks to the lab group as a whole and one a year to the department as a whole.

I have had the opportunity to perform poster presentations and an oral presentation at several conferences and meetings including the Gordon Research Conference – Hormone Dependent Cancers 2019, The Prostate Cancer Foundation Annual Retreat – 2018, and the Multi-Institutional Prostate SPORE Retreat – 2019

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Results from these studies have been presented in poster presentation and oral presentation format at the Gordon Research Conference – Hormone Dependent Cancers, Aug. 2019, the Prostate Cancer Foundation Annual Retreat – Oct, 2018, and The Multi-Institutional Prostate SPORE Retreat – March, 2019.

Additionally, some of the results described above were published as a 1st author publication in the journal Cancer Research (Russo et al. 2018, PMID: 30242112)

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

The major focus of the next reporting period for both Aim 1 and Aim 2 will be using the cell lines with inducible shRNA and cDNA to DPP4 to both knockdown and overexpress DPP4 in the xenograft setting. Then using a combination of IHC, western blotting, RPPA, and possibly phosphoproteome mass-spec, I will determine those signaling cascades significantly altered by changes in DPP4 levels. This will narrow the candidates of possible growth factors targeted by DPP4 for degradation. Once the principle growth factor/signaling cascade activated by DPP4 downregulation is identified, I can initiate studies to directly target the signaling cascade with inhibitors that are available to stop xenograft progression. For Aim 3, I will continue to analyze the expression patterns of DPP4 protein in primary PCa samples extending my results to Gleason Score 7 PCa with clinicopathological correlation. I will also continue to develop collaborations with my epidemiologist colleagues in our prostate SPORE group to look at the interaction of DPP4 inhibitors with ADT. ***4.***

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

The published findings (Russo et al. 2018, PMID: 30242112) on the DPP4 inhibitor sitagliptin decreasing the effectiveness of castration to inhibit PCa xenograft and PDX growth has implications for the use of DPP4 inhibitors to treat diabetes in men newly diagnosed with metastatic prostate cancer who are starting ADT. The results suggest that DPP4 inhibitors interfere with ADT and men be treated with ADT should stop the use of DPP4 inhibitors and switch to a different diabetes medication. It is important to note that these results are in preclinical models and require further support from epidemiological data.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:
- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report

5. CHANGES/PROBLEMS: *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to Report

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  Russo JW, Gao C, Bhasin SS, Voznesensky OS, Calagua C, Arai S, Nelson PS, Montgomery B, Mostaghel EA, Corey E, Taplin ME, Ye H, Bhasin M, Balk SP. Downregulation of Dipeptidyl Peptidase 4 accelerates progression of castration-resistant prostate cancer. (2018) Cancer Research. 78(22):6354-6362. PMID: 30242112.
  Status of Publication: Published.
  Acknowledgement of Federal Support: Yes

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to Report

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Gordon Conference – Hormone Dependent Cancers (2019) – Oral Presentation

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to Report

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to Report

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to Report

- **Other Products**

  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name: *Mary Smith*
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID): *1234567*
Nearest person month worked: *5*

Contribution to Project: *Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support: *The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name: Joshua W. Russo
Project Role: Principal Investigator
Research Identifier:
Nearest person month worked: 10
Contribution to Project: Dr. Russo performed all the animal studies and in vitro work.
Funding Support: CDMRP PCRP Early Investigator Award, PCF Young Investigator Award, Mazzone Research Awards Program Development Award

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

The following grant has ended:

David Mazzone Research Awards Program
Development Award (Russo) 07/01/2018-06/30/2019 0 cal months/0%
David Mazzone Awards Program - $53,000/yr
DPP4-mediated Mechanisms of Resistance in Castration-Resistant Prostate Cancer
The overall goal is to determine the functional significance of DPP4 downregulation in prostate cancer progression.
Aim 1. Assess the functional significance of DPP4 downregulation in the PCa xenograft setting.
Aim 2. Determine the signaling cascades effected by DPP4 downregulation/inhibition and the corresponding growth factors/cytokines targeted by DPP4 that are responsible for ADT resistance.
Aim 3. Determine the clinical significance of DPP4 expression and concurrent ADT/DPP4 inhibitor treatment on PCa progression.
There is scientific overlap as this award will provide support for supplies and technical help needed to carry out the work and achieve the objectives of the DoD award. However, there is no budgetary overlap as this award will not provide salary for Dr. Russo.
David Mazzone Awards Program Administrator: Juan Carlos Hincapie 617-632-6155 jhincapie@parteners.org

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

9. APPENDICES:

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

-Attached first author manuscript, Russo et al, *Cancer Research*, 2018, PMID: 30242112

Published OnlineFirst September 21, 2018; DOI: 10.1158/0008-5472.CAN-18-0687

Priority Report

Cancer Research

Downregulation of *Dipeptidyl Peptidase 4* Accelerates Progression to Castration-Resistant Prostate Cancer

Joshua W. Russo[1], Ce Gao[2], Swati S. Bhasin[2], Olga S. Voznesensky[1], Carla Calagua[3], Seiji Arai[1,4], Peter S. Nelson[5], Bruce Montgomery[6], Elahe A. Mostaghel[5], Eva Corey[6], Mary-Ellen Taplin[7], Huihui Ye[3], Manoj Bhasin[2], and Steven P. Balk[1]

Abstract

The standard treatment for metastatic prostate cancer, androgen deprivation therapy (ADT), is designed to suppress androgen receptor (AR) activity. However, men invariably progress to castration-resistant prostate cancer (CRPC), and AR reactivation contributes to progression in most cases. To identify mechanisms that may drive CRPC, we examined a VCaP prostate cancer xenograft model as tumors progressed from initial androgen sensitivity prior to castration to castration resistance and then on to relapse after combined therapy with further AR-targeted drugs (abiraterone plus enzalutamide). AR activity persisted in castration-resistant and abiraterone/enzalutamide–resistant xenografts and was associated with increased expression of the AR gene and the AR-V7 splice variant. We then assessed expression of individual AR-regulated genes to identify those that persisted, thereby contributing to tumor growth, versus those that decreased and may therefore exhibit tumor suppressor activities. The most significantly decreased AR target gene was dipeptidyl peptidase 4 *(DPP4)*, which encodes a membrane-anchored protein that cleaves dipeptides from multiple growth factors, resulting in their increased degradation. DPP4 mRNA and protein were also decreased in clinical CRPC cases, and inhibition of DPP4 with sitagliptin enhanced the growth of prostate cancer xenografts following castration. Significantly, DPP4 inhibitors are frequently used to treat type 2 diabetes as they increase insulin secretion. Together, these results implicate DPP4 as an AR-regulated tumor suppressor gene whose loss enhances growth factor activity and suggest that treatment with DPP4 inhibitors may accelerate emergence of resistance to ADT.

Significance: These findings identify DPP4 as an AR-stimulated tumor suppressor gene that is downregulated during progression to castration-resistant prostate cancer, warning that treatment with DPP4 inhibitors, commonly used to treat type 2 diabetes, may accelerate prostate cancer progression following androgen deprivation therapy. *Cancer Res; 78(22); 6354–62. ©2018 AACR.*

Introduction

The standard treatment for metastatic prostate cancer is androgen deprivation therapy (ADT) to suppress androgen receptor (AR) activity, but men invariably progress despite castrate androgen levels (castration-resistant prostate cancer, CRPC). AR activity persists in most CRPC, with increased intratumoral androgen synthesis being a major mechanism driving this AR activity (1). AR activity in CRPC can be suppressed by agents such as abiraterone, which further decrease androgen synthesis, or by AR antagonists such as enzalutamide, but patients still invariably progress. A subset of these abiraterone/enzalutamide–resistant tumors express low or undetectable AR and some have neuroendocrine features (2, 3), but AR appears to be contributing to progression in most cases. Multiple mechanisms may contribute to persistent AR activity including alterations in the AR (*AR* gene amplification or activating mutations, expression of constitutively active AR splice variants, or AR posttranslational modifications), further increases in intratumoral androgen synthesis, and activation of multiple signaling pathways or epigenetic alterations that enhance tumor cell growth and may directly or indirectly enhance AR activity. However, the contribution of any single mechanism to resistance is unclear, and multiple mechanisms may contribute to resistance in a single patient.

Although most prostate cancers are initially AR-dependent, the critical genes and pathways regulated by AR remain unclear. One basis for this dependence is AR regulation of multiple genes involved in metabolic pathways (4, 5). However, in addition to its oncogenic properties, studies in model systems show that AR also can have tumor suppressor activity. The clinical significance

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

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of these observations is supported by an inverse relationship between AR activity and cell proliferation in CRPC clinical samples (6), and by recent clinical trials of rapid cycling between high and low serum testosterone concentrations in men with CRPC (7). Possible mechanisms for these responses include AR-mediated DNA damage, AR repression of genes such as *MYC*, and inactivation of multiple E2F-regulated genes through increased recruitment of pRb (8, 9). Alternatively, AR may repress growth through increasing expression of multiple genes involved in differentiation, consistent with its normal function in prostate epithelium. Significantly, several studies have indicated that the AR cistrome and transcriptome becomes reprogrammed during prostate cancer development and progression to CRPC, consistent with selective pressure to block AR's tumor-suppressive functions and potentially acquire new oncogenic functions (10). This study identifies dipeptidyl peptidase 4 (*DPP4*) as an AR-stimulated tumor suppressor gene whose expression is suppressed with progression to CRPC.

Materials and Methods

Cell lines, xenografts, and tissue samples

VCaP and LNCaP cells were from ATCC and used for subcutaneous xenograft injections within 4 passages. VCaP and LNCaP cell identities were confirmed by short tandem repeat (STR) profiling, and *Mycoplasma* testing was negative. To generate VCaP xenografts, 6-week-old male ICR SCID mice (Taconic Biosciences) were injected subcutaneously with 5×10^6 cells in 100% Matrigel. Xenografts were grown until 1,000 mm^3, then mice were castrated. For the abiraterone/enzalutamide–resistant VCaP xenograft model, when tumors in castrated mice exceeded 150% of their nadir volume, they were considered relapsed and mice were started on abiraterone acetate (30 mg/kg) + enzalutamide (50 mg/kg) in drinking water. Tumors were serial biopsied precastration, at tumor relapse (CRPC), and mice were sacrificed when tumors reached 2,000 mm^3 on dual abiraterone plus enzalutamide treatment (abiraterone/enzalutamide resistant; AER). For the sitagliptin studies, VCaP, LNCaP, and BID-PC-1 xenografts were grown to approximately 500 mm^3, then mice were castrated and immediately administered daily sitagliptin (120 mg/kg, SelleckChem) in drinking water. The BID-PC-1 xenograft was generated from a metastasis in a patient with BRCA2-deficient CRPC and has been passaged in noncastrated male immundeficient mice. Written informed consent was obtained from patients for the tissue analyses, and all studies involving human materials were carried in accordance with the U.S. Common Rule and approved by the Beth Israel Deaconess Medical Center (BIDMC) Instituional Review Board. All animal studies were approved by the BIDMC Institutional Animal Care and Use Committee.

Knockdown of DPP4 protein expression in VCaP cells was performed using a SMARTpool of ON-TARGETplus siRNAs targeting DPP4 (Dharmacon, catalog no. L-004181-00-0005). Neoadjuvant leuprolide–abiraterone samples were obtained from patients who underwent radical prostatectomy after neoadjuvant treatment in a phase II clinical trial (11). CRPC tissues were obtained from rapid autopsy specimens at BIDMC. Tissue analyses were in accordance with the Dana-Farber/Harvard Cancer Center Institutional Review Board. A further rapid autopsy tissue microarray (TMA) was obtained from University of Washington (Seattle, WA) in accordance with their Prostate Cancer Donor Program (6).

IHC

For IHC, 5-mm formalin-fixed, paraffin-embedded (FFPE) sections underwent epitope retrieval using Dako PT Link platform. Staining was on the Dako Link 48 autostainer, with amplification using EnVision FLEX rabbit linkers, and visualization using the EnVision FLEX high-sensitivity visualization system (Dako). Sections were stained for anti-AR (N20, Santa Cruz Biotechnology; 1:1,000), anti-ARV7 (RM7, RevMab; 1:100), anti-phospho-AR (S81; MilliporeSigma, 1:5,000), anti-PSA (FLEX polyclonal rabbit anti-human PSA, IR514, DAKO), and anti-DPP4 (D6D8K, Cell Signaling Technologies, 1:100). DPP4 antibody specificity was confirmed on cultured VCaP cells. After siRNA knockdown of DPP4 mRNA (Supplementary Fig. S1A), we found decreased DPP4 protein levels and expression by IHC (Supplementary Fig. S1B and S1C, respectively). Anti-DPP4 staining in clinical prostate samples showed strong membrane staining in luminal epithelium (Supplementary Fig. S1D). DPP4 positivity was defined by moderate-to-strong, punctate, membranous, and cytoplasmic staining. DPP4 immunointensity was scored as negative (0), weak (1), moderate (2), and strong (3), based on the most predominant intensity pattern. DPP4 percentage score was based on the percentage of tumor cells demonstrating the most predominant intensity pattern or stronger as: 0 (negative), 1 (1%–9%), 2 (10%–49%), and 3 (≥50%). DPP4 score (0–9) was based on the immunointensity score multiplied by the percentage score.

Gene expression analysis

RNA was isolated from FFPE blocks that contained greater than 90% tumor cell content by cutting approximately ten, 8-mm ribbons from each block and isolating the RNA with the RNeasy FFPE Kit (Qiagen). Quantitative RT-PCR amplification was with TaqMan One-Step RT-PCR reagents (Thermo Fisher Scientific) and results were normalized to coamplified β-actin. RNA sequencing (RNA-seq) was performed on two to three biological replicates. Sequencing libraries were generated using the NEB Ultra Directional RNA Library Prep Kit, and we obtained approximately $2.5–3.0 \times 10^7$ paired-end reads. Additional gene analysis techniques and RNA-seq analysis methods are mentioned in Materials and Methods. RNA-seq data have been deposited in the NCBI Gene Expression Omnibus (GEO) with the accession code GSE109708.

Statistical analysis

GraphPad Prism 7 Software (GraphPad Software Inc.) was used for all statistical analysis unless otherwise specified. All results are presented as the mean ± SEM. The Mann–Whitney *U* nonparametric test was used to test the statistical significance between gene expression of controls and experimentally manipulated samples unless otherwise specified. Values of $P < 0.05$ were considered statistically significant.

Results

AR activity persists in abiraterone/enzalutamide–resistant VCaP xenografts

VCaP xenografts were established and biopsies were taken precastration, when the tumors relapsed following castration (CRPC), and when they again relapsed following combined abiraterone (30 mg/kg/day) and enzalutamide (50 mg/kg/day)

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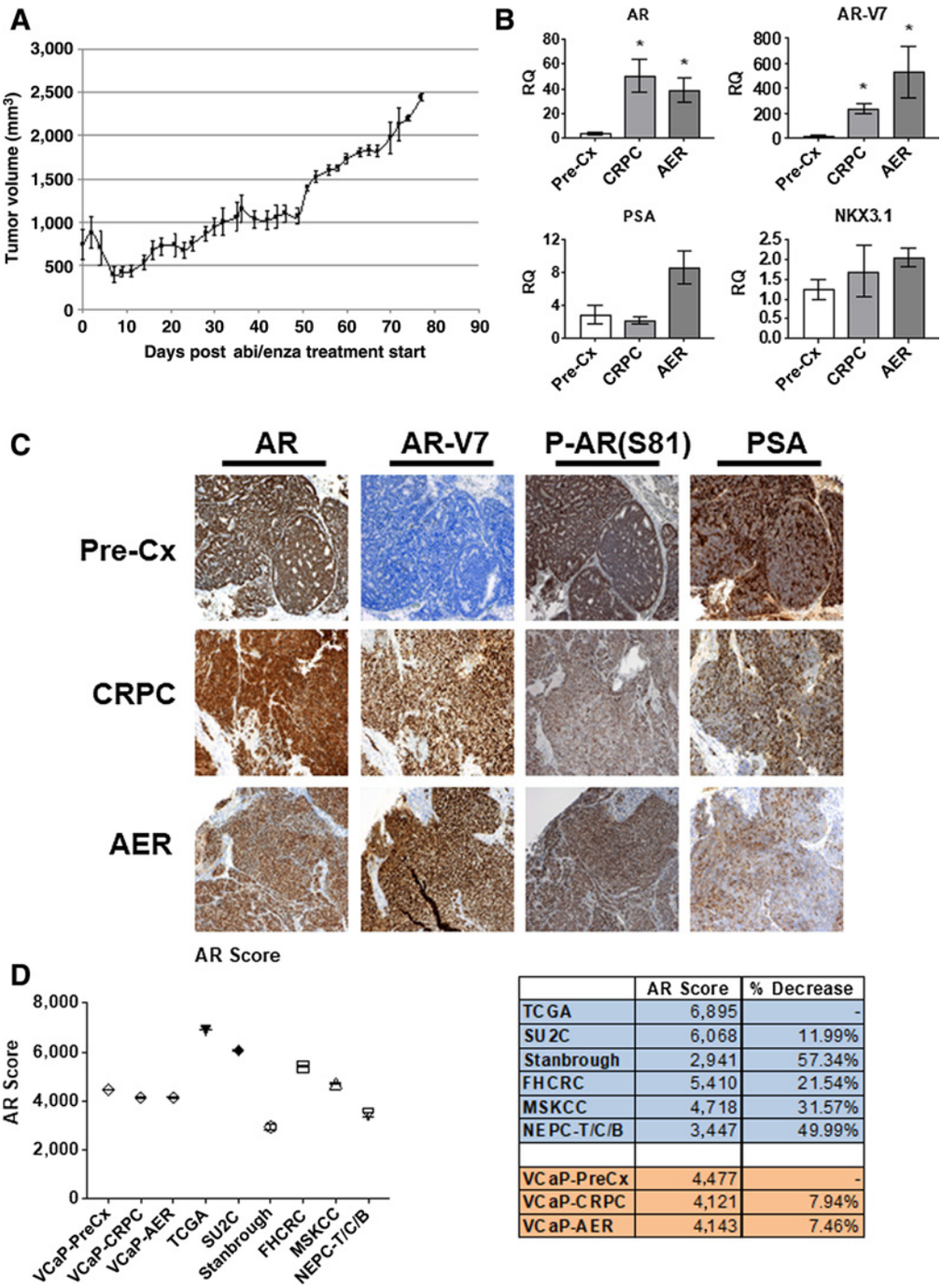

	AR Score	% Decrease
TCGA	6,895	-
SU2C	6,068	11.99%
Stanbrough	2,941	57.34%
FHCRC	5,410	21.54%
MSKCC	4,718	31.57%
NEPC-T/C/B	3,447	49.99%
VCaP-PreCx	4,477	-
VCaP-CRPC	4,121	7.94%
VCaP-AER	4,143	7.46%

Figure 1.

Abiraterone/enzalutamide–resistant (AER) VCaP xenograft tumors have restored AR signaling. **A,** VCaP CRPC xenografts are initially sensitive to abiraterone/enzalutamide (abi/enza), but recover tumor volume by day 30. The initial cohort was 22 mice. **B,** qRT-PCR of *AR, AR-V7, PSA,* and *NKX3.1* in serial biopsies of abiraterone/enzalutamide–resistant VCaP xenografts. Each column represents xenografts from four mice, with qRT-PCR in technical triplicate. Bars, SEM. *, $P < 0.03$, Mann–Whitney *U* test. RQ, relative quantification; Pre-Cx, precastration. **C,** IHC of AR, AR-V7, serine 81 phosphorylated AR [P-AR(S81)], and PSA in serial biopsies of a representative tumor. **D,** Left, AR score for available precastration and CRPC datasets. Bars depicting SEM are not visible due to small size. Right, the % decrease in the AR score from precastration state (TCGA, VCaP–Pre-Cx) to the CRPC state (SU2C, Stanbrough, FHCRC, MSKCC, NEPC-T/C/B, VCaP-CRPC, VCaP-AER) are listed. SU2C, Stand Up 2 Cancer; Stanbrough, from Stanbrough et al. (Supplementary Reference 3); FHCRC, Fred Hutchinson Cancer Research Center; MSKCC, Memorial Sloan Kettering Cancer Center; NEPC-T/C/B, Neuroendocrine Prostate Cancer-Trento/Cornell/Broad.

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treatment (abiraterone/enzalutamide–resistant; Fig. 1A). Analysis of AR and AR splice variant 7 (AR-V7) mRNA in serial biopsies showed increases as tumors progressed precastration to CRPC, with further increases in AR-V7 as tumors became abiraterone/enzalutamide–resistant (Fig. 1B). IHC similarly showed increased AR-V7 protein in the CRPC and abiraterone/enzalutamide–resistant tumors, although total AR protein was not substantially altered (Fig. 1C; Supplementary Fig. S2). AR phosphorylation on S81 (an indicator of AR transcriptional activity), also persisted in the CRPC and abiraterone/enzalutamide–resistant xenografts without significant alteration as tumors progressed (Fig. 1C; Supplementary Fig. S2). Consistent with persistent AR transcriptional activity, CRPC and abiraterone/enzalutamide–resistant xenografts also expressed the levels of the AR target genes *KLK3* (*PSA*) and *NKX3.1* that were at least equivalent to the levels in the precastration tumors (Fig. 1B and C; Supplementary Fig. S2). Moreover, based on RNA-seq and a curated list of 266 AR target genes, the strength of AR signaling was comparable (~8% decrease) between the precastration and CRPC/abiraterone/enzalutamide–resistant xenografts (Fig. 1D). There was a similar difference (~12% decrease) in the AR signaling scores in the clinical The Cancer Genome Atlas (TCGA; primary prostate cancer) versus the SU2C (CRPC) datasets, whereas the additional clinical CRPC datasets showed AR score decreases ranging between 21% and 57% relative to primary untreated prostate cancer. These data indicate that VCaP abiraterone/enzalutamide–resistant tumors had restored AR signaling despite maximal AR blockade.

Overlapping pathways mediate progression to CRPC and abiraterone/enzalutamide–resistant tumors

RNA-seq showed that 1,441 genes were significantly differentially expressed between abiraterone/enzalutamide–resistant and precastration tumors (Fig. 2A; Supplementary Table S1). About half of these were significantly differentially expressed between CRPC and precastration xenografts (47%, Supplementary Table S2). For most of the remaining genes, there was a trend toward altered expression in the CRPC xenografts (Fig. 2B), indicating that progression to abiraterone/enzalutamide resistance was driven largely by mechanisms that were already engaged during progression to CRPC. Consistent with this conclusion, comparison of the abiraterone/enzalutamide–resistant versus the CRPC xenografts identified a much smaller group of genes as being significantly altered (115 genes), with most similarly altered in CRPC (Supplementary Table S3). Moreover, two of the most enriched pathways in the abiraterone/enzalutamide–resistant versus precastration tumors were also enriched in the CRPC versus precastration tumors (axonal guidance signaling and glioblastoma multiforme signaling; Supplementary Fig. S3A). Finally, hierarchical clustering and principal component analysis both separately grouped the precastration samples (Supplementary Fig. S3B and S3C).

Using a more stringent cutoff [$\log_2(\text{FC}) \geq 2.5$ and $P \leq 1.00\text{E}{-}05$], 72 genes were altered in the abiraterone/enzalutamide–resistant versus precastration tumors (Fig. 2C), and these genes were all similarly altered in the CRPC versus precastration tumors (Supplementary Table S4). As expected, AR was among the most highly upregulated genes. Also markedly increased were 2 olfactory receptor genes (*OR51E1* and *OR51E2*) and the nephroblastoma overexpressed (*NOV*) gene, which have been linked previously to prostate cancer. Interestingly, the only other highly increased nuclear receptor was *NR3C2* (mineralocorticoid receptor), which we previously found increased in VCaP xenografts treated with single-agent abiraterone (12) and in relapsed tumors in men with CRPC being treated with abiraterone plus dutasteride (13).

AR-regulated *DPP4* gene expression is not restored in CRPC or abiraterone/enzalutamide–resistant tumors

While AR signaling was substantially restored in the CRPC and abiraterone/enzalutamide–resistant xenografts, we hypothesized that expression of AR-regulated genes that are critical for tumor growth would be most consistently and robustly restored, whereas those that are less critical (or exhibit growth-suppressing effects) may not be restored. Consistent with this hypothesis, volcano plots showed that AR target gene expression in the abiraterone/enzalutamide–resistant versus precastration xenografts was not restored in a symmetric fashion, with the *AR* gene being the most significant outlier among genes that are increased in the abiraterone/enzalutamide–resistant xenografts (Fig. 2D, top left; Supplementary Table S5). Conversely, the most significantly decreased gene in the abiraterone/enzalutamide–resistant xenografts was *DPP4*, which has previously been shown to be an androgen-stimulated gene (4, 14, 15). We further confirmed that *DPP4* gene expression was stimulated by DHT in VCaP and LNCaP cells (Supplementary Fig. S4A), and was decreased by enzalutamide (Supplementary Fig. S4B). A similar pattern was observed when comparing the CRPC versus precastration xenografts, indicating that AR fails to restore *DPP4* expression at this stage as well (Fig. 2D, top right; Supplementary Table S6). When this AR gene signature list was expanded to include all differentially expressed genes, *DPP4* was still among the most significantly downregulated genes (Fig. 2D, bottom left and right).

The loss of *DPP4* mRNA in the CRPC and abiraterone/enzalutamide–resistant tumors was confirmed by qRT-PCR (Fig. 3A). Moreover, IHC confirmed that DPP4 protein was markedly decreased in the CRPC and abiraterone/enzalutamide–resistant tumors (Fig. 3B). As DPP4 mediates the degradation of multiple growth factors, we next submitted tumor lysates for reverse-phase protein array analysis to determine whether there were clear differences in key prostate cancer–related signaling cascades. Indeed, there was increased activation of the PI3K, ERK–MAPK, and p38–MAPK pathways in the CRPC and abiraterone/enzalutamide–resistant tumors (Fig. 3C). Extending our results to clinical samples, previously published datasets also showed decreased *DPP4* mRNA in CRPC (Supplementary Fig. S5).

DPP4 protein is markedly reduced in CRPC clinical samples

We next performed IHC for DPP4 on untreated primary prostate cancer tissue, residual tumor from a neoadjuvant leuprolide–abiraterone trial (11), and sections of metastatic CRPC. Tumors from the neoadjuvant trial showed markedly decreased DPP4, whereas metastatic CRPC sections showed near complete absence of DPP4 (Supplementary Fig. S6). We further examined a metastatic CRPC TMA (6) using a DPP4 immunoscore to quantify DPP4 protein expression. Strikingly, 84% (71/85) of CRPC sections had a DPP4 immunoscore ≤ 2, with 69% (59/85) of CRPC sections being negative, whereas only 9% (3/35) of untreated primary prostate cancer had a score ≤ 2 (Fig. 3D). Overall, CRPC specimens exhibited a much lower average DPP4 immunoscore compared with untreated primary prostate cancer (1.25 and 6.49,

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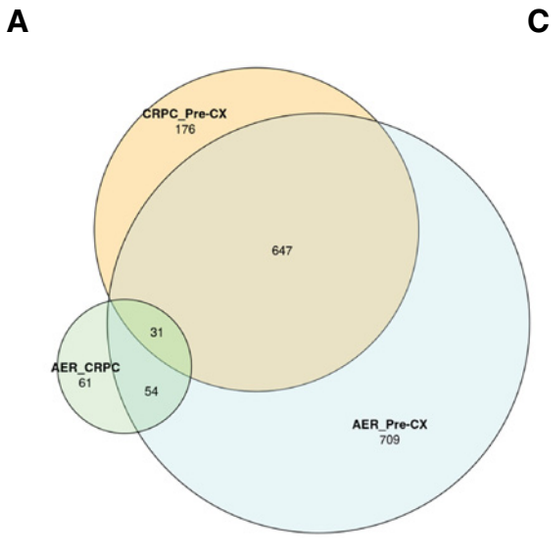
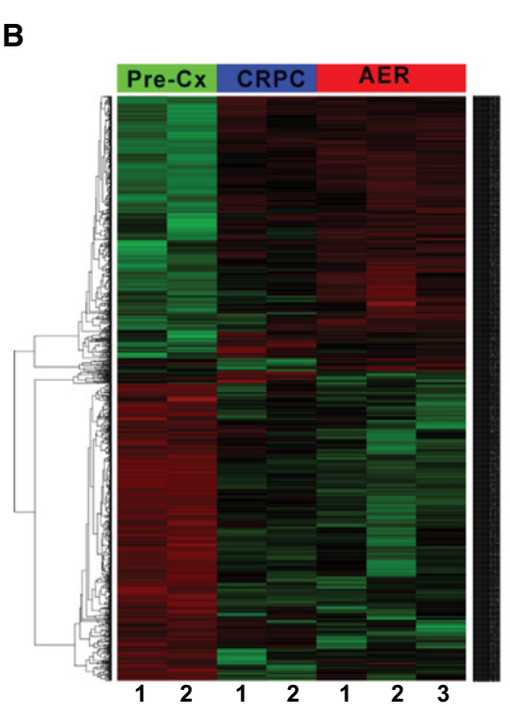


C

AER vs. Pre-Cx

Upregulated genes		
Gene	Log_2(FC)	*P* Value
TMEFF2	5.44	9.94E-08
OR51E1	5.39	1.39E-08
OR51E2	4.60	7.82E-08
AR	4.48	3.42E-15
BHLHE41	4.46	1.99E-06
NOV	4.42	1.74E-06
SCG2	4.16	1.62E-06
FOLH1	4.05	3.08E-06
FRAS1	3.98	3.30E-06
LOC100130417	3.84	4.98E-06
SDC2	3.80	1.29E-07
NR3C2	3.73	7.08E-10
EPHA4	3.53	5.79E-08
LRFN2	3.44	6.18E-06
DNAH7	3.28	5.44E-07
ANK2	3.27	1.35E-06
THBS1	3.20	4.41E-10
RTN1	3.10	9.74E-07
LINC02418	3.06	3.12E-06
COLEC12	2.97	2.17E-09
ADAM7	2.88	2.08E-07
SESN3	2.85	5.43E-09
VWA5B1	2.78	2.97E-09
KLF9	2.73	2.75E-09
NRXN2	2.69	1.51E-10
SAMD11	2.68	8.62E-07
PTGER2	2.67	4.50E-08
CACNA1C	2.63	2.54E-10
SATB1	2.61	1.37E-08

Downregulated genes		
Gene	Log_2(FC)	*P* Value
FAM189A2	-7.70	1.49E-08
DPP4	-6.83	5.40E-13
SH3RF2	-5.17	1.47E-06
CACNG4	-5.14	5.27E-08
VSTM2A	-5.01	5.87E-10
ST6GALNAC1	-5.00	7.85E-09
VSTM2A-OT1	-4.93	2.19E-06
TESC	-4.87	8.82E-11
HABP2	-4.84	9.82E-06
ALDH1A1	-4.74	3.71E-10
LUZP2	-4.62	1.80E-09
TRPM8	-4.23	1.32E-12
TMSB15A	-4.04	4.27E-06
LOC101929653	-3.98	1.85E-10
FRMPD3	-3.62	5.52E-07
SPOCK2	-3.57	3.09E-11
STEAP4	-3.56	1.78E-13
COL4A5	-3.49	5.26E-12
DIRAS2	-3.43	1.19E-10
CRISP2	-3.34	4.23E-06
ANKRD29	-3.33	8.85E-10
HLA-DMA	-3.19	1.60E-10
KRT14	-3.11	5.13E-06
NRCAM	-3.05	1.25E-12
LAMA3	-2.97	6.92E-15
UGT2B4	-2.96	9.56E-08
NNMT	-2.95	7.80E-08
SLC27A6	-2.93	1.54E-06
NTM	-2.88	4.30E-08
ESRRG	-2.86	1.00E-09
HGD	-2.85	1.30E-10
MCCC2	-2.84	4.74E-09
MCC	-2.80	4.26E-11
VWF	-2.77	7.72E-07
CUEDC1	-2.77	3.29E-07
ENSG00000213777	-2.77	3.00E-13
WISP1	-2.64	9.63E-06
TOX3	-2.62	8.55E-10
C4orf19	-2.62	2.52E-09
TG	-2.62	2.03E-09
ELOVL2	-2.61	6.56E-11
CNKSR2	-2.56	1.47E-09
MICALCL	-2.50	8.26E-07



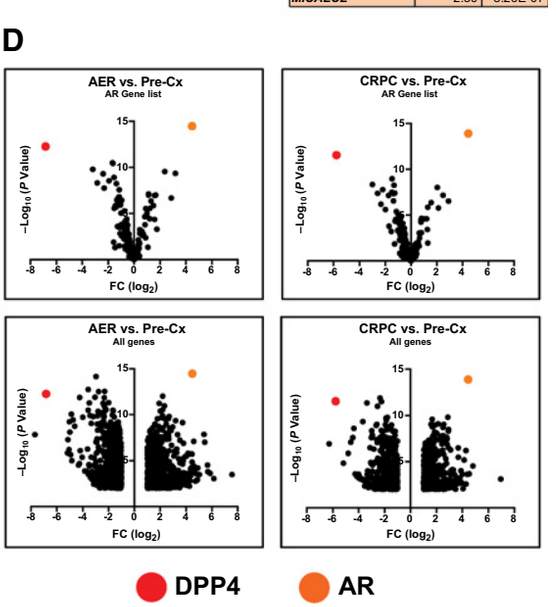


Figure 2.

Analysis of RNA-seq from serial biopsies. **A,** Venn diagram depicting the overlap between differentially expressed genes in precastration (Pre-Cx), CRPC, and abiraterone/enzalutamide–resistant (AER) serial biopsies of four different VCaP xenografts. **B,** Unsupervised clustering of precastration, CRPC, and abiraterone/enzalutamide–resistant xenografts based on differential gene expression. **C,** Differentially expressed genes between abiraterone/enzalutamide–resistant and precastration xenografts that meet the stringent criteria of $\log_2 FC > 2.5$ and $P < 1 \times 10^{-5}$. **D,** Volcano plots depicting AR target genes (266 AR gene signature from Mendiratta et al.; ref. 12), supplemented with a selection of DHT-responsive genes from Xu and colleagues (ref. 4; top), and all the significantly differentially expressed genes (bottom) in the abiraterone/enzalutamide–resistant versus precastration and CRPC versus precastration comparisons. *DPP4* and *AR* are highlighted.

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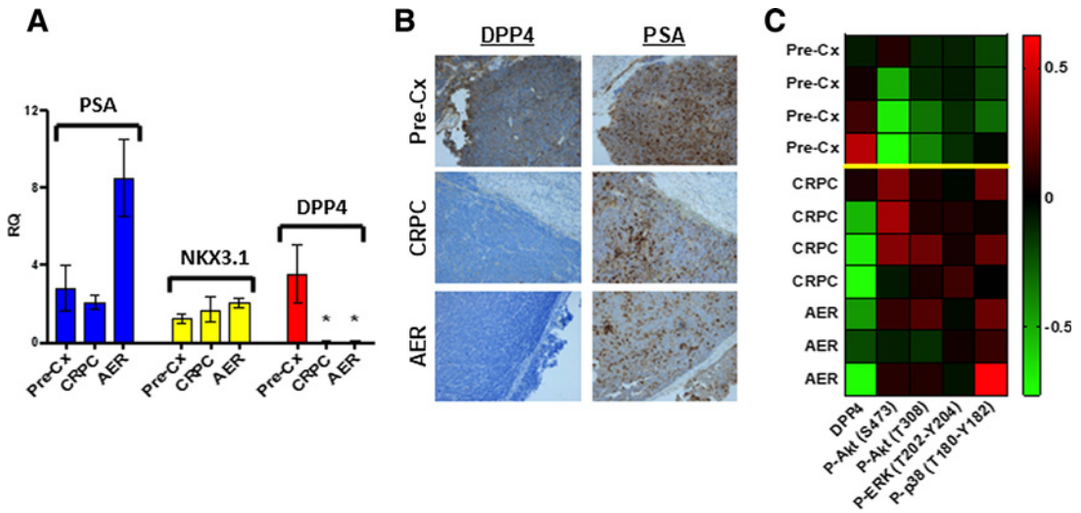
respectively, $P < 0.0001$), further supporting the decreased expression of DPP4 in CRPC.

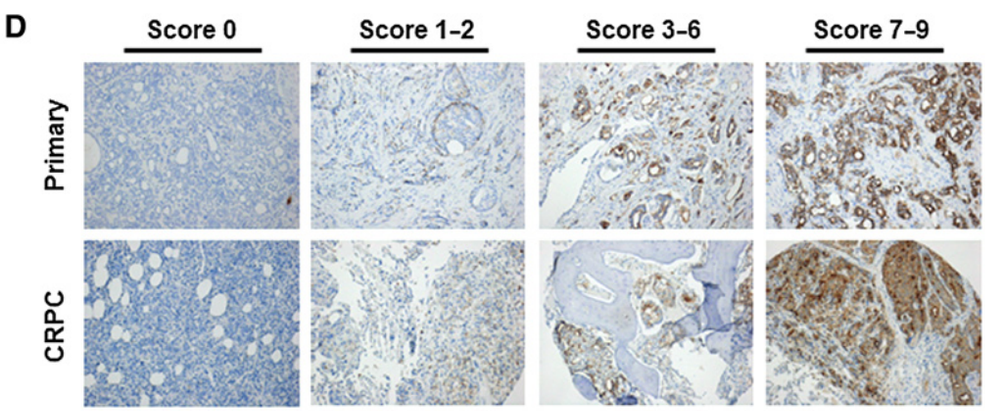
DPP4 downregulation is mediated by a reversible epigenetic mechanism

Several mechanisms of DPP4 downregulation in other contexts have been reported, including *DPP4* promoter/early exon 1 methylation (16, 17) and downregulation of a long noncoding RNA (lncRNA), lncRNA-OIS1 (18). Bisulfite conversion of DNA from four abiraterone/enzalutamide–resistant VCaP tumors showed that the *DPP4* promoter and early exon 1 regions were unmethylated (Supplementary Fig. S7). Furthermore, there was no difference in lncRNA-OIS1 between the precastration and abiraterone/enzalutamide–resistant serial biopsies of 5 tumors (Supplementary Fig. S8). Finally, to determine whether DPP4 downregulation is mediated through an irreversible genomic mechanism, we attempted to restore DPP4 expression in CRPC VCaP xenografts by treatment with high-dose testosterone. Treatment of castrated mice bearing CRPC VCaP xenografts with daily intraperitoneal injection of testosterone (200 mg/kg) for 3 days restored *DPP4* mRNA to precastration levels (Supplementary Fig. S9A) and also substantially increased DPP4 protein (Supplementary Fig. S9B and S9C), indicating that *DPP4* downregulation is epigenetic.

Inhibition of DPP4 activity increases *in vivo* resistance to castration

These findings suggested that DPP4 inhibitors may enhance the growth of prostate cancer after ADT. To test this hypothesis,





	Sample no.	DPP4 Score 0	DPP4 Score 1–2	DPP4 Score 3–6	DPP4 Score 7–9	Avg. Score	Mann–Whitney U test
Primary	35	2	1	17	15	6.49	
CRPC	85	59	12	10	4	1.25	$P = < 0.0001$

Figure 3.

DPP4 expression is decreased in abiraterone/enzalutamide–resistant (AER) and CRPC VCaP xenografts. **A,** qRT-PCR for *PSA, NKX3.1*, and *DPP4* expression in serial biopsies of VCaP xenografts. Each column represents the expression levels of xenograft tumors from four separate mice, with RT-PCR performed on each in technical triplicate. *, $P < 0.03$, Mann-Whitney U. RQ, relative quantification. **B,** Representative images of DPP4 and PSA in serial biopsies of VCaP xenografts. **C,** Reverse-phase protein array from serial biopsies of four separate VCaP xenografts (see Materials and Methods). **D,** Representative images of DPP4 IHC and immunoscoring from a series of hormone-naïve primary prostate cancer and CRPC clinical sections. Pre-Cx, precastration.

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androgen-sensitive VCaP xenografts were grown in intact male mice, followed by castration in combination with a DPP4 inhibitor (sitagliptin, 120 mg/kg/day) or control. Both groups initially responded to castration, but the sitagliptin-treated xenografts progressed more rapidly (Fig. 4A). AR expression and activity were comparable in the relapsed control and treated tumors harvested at approximately 6 weeks, suggesting that DPP4 inhibition was not acting primarily through AR, and consistent with it acting through enhanced growth factor stimulation (Fig. 4B). Although DPP4 expression was decreased in both the treated and control tumors, there was a trend toward higher DPP4 expression in the treated versus control xenografts (Fig. 4B–D). This is consistent with decreased selective pressure to downregulate the DPP4 expression in mice treated with sitagliptin. There also was a positive correlation between DPP4 protein levels and fold increase in tumor volume over 6 weeks in the treated xenografts (Supplementary Fig. S10), suggesting that tumors expressing the highest levels of DPP4 protein had the greatest increase in growth factors in response to the sitagliptin.

To determine whether these results could be extended to tumors with genomic alterations distinct from those in VCaP (*AR*-amplified, *TMPRSS/ERG* fusion), we examined the effects of sitagliptin on xenografts generated from LNCaP cells (*PTEN*-deficient) and on BRCA2-deficient patient-derived xenografts (BID-PC-1). Similar to the results with VCaP, treatment with sitagliptin decreased the efficacy of castration in the LNCaP xenografts (Fig. 4E, top). The BID-PC-1 patient-derived xenograft is extremely sensitive to ADT, and castration alone has led to complete responses in all mice examined for up to 12 months. In contrast, 3 of 7 tumors treated with sitagliptin had only partial responses, with one of these progressing by 2 months (Fig. 4E, bottom).

Discussion

As a transmembrane protease, DPP4 can target numerous growth factors/cytokines, and may have oncogenic or tumor suppressor properties (19). Its oncogenic functions may be related

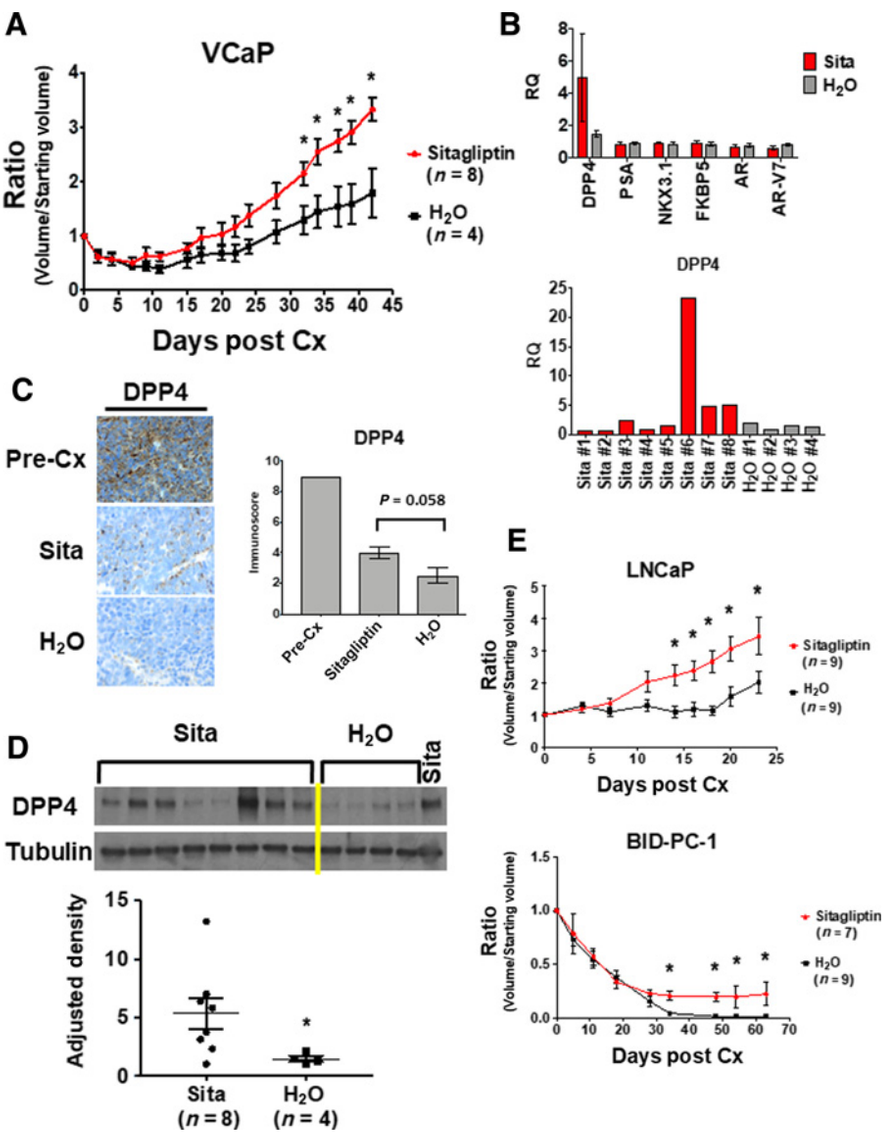

Figure 4.

DPP4 inhibitor increases resistance to castration *in vivo*. **A,** VCaP xenografts were grown in intact male mice until 500 mm^3, then mice were castrated (Cx) and immediately begun on treatment with sitagliptin (120 mg/kg/day) or vehicle. *y*-axis is the ratio of tumor volume at a given time point divided by starting volume. *, $P < 0.05$, Mann-Whitney U. **B,** qRT-PCR of indicated transcripts in xenografts harvested at day 42. Each column is expression in tumors from 8 mice (sitagliptin, Sita) or four mice (H_2O), with qRT-PCR performed in technical triplicate. Bottom, *DPP4* mRNA in individual tumors. RQ, relative quantification. **C,** Representative DPP4 IHC from sitagliptin and H_2O-treated tumors (left) and immunoscoring of DPP4 protein (right). $P = 0.058$, Mann-Whitney U. **D,** Sitagliptin and control tumor lysates probed with anti-DPP4 (top) and densitometric quantification (bottom). *, $P < 0.03$, Mann-Whitney U. **E,** Mice with LNCaP and BID-PC-1 xenografts were castrated and immediately begun on treatment with sitagliptin (120 mg/kg/day) or vehicle (*, $P < 0.05$, Mann-Whitney U).

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to suppression of antitumor immune responses, although it may also have an immuostimulatory scaffold function by anchoring adenosine deaminase (20). Previous studies in prostate cancer have indicated that DPP4 may enhance degradation of FGF2 and CXCL12 (21, 22), and a reduced serum DPP4 activity (due to a low molecular weight inhibitor) was found in men with metastatic prostate cancer (23). However, consistent with our IHC results, DPP4 levels are not decreased in untreated primary prostate cancer (24). Therefore, we hypothesize that there is no selective pressure to downregulate DPP4 in primary prostate cancer, but that the initial decrease in DPP4 and subsequent increase in growth factor levels after ADT is important for tumor cell survival. Strong selective pressure to keep DPP4 levels low would then result in the emergence of CRPC cells with generally restored AR function that have epigenetically silenced the *DPP4* gene. Consistent with this hypothesis, treatment with sitagliptin accelerated the progression of prostate cancer xenografts to castration resistance.

DPP4 inhibitors are used for type 2 diabetes as they block the degradation of GLP-1 and GIP-1, which promotes insulin secretion. Multiple studies have assessed for possible links between DPP4 inhibitors and cancer, but no consistent links have been found (25). This may indicate that DPP4 has only minimal effects on tumor development, or perhaps counterbalancing tumor suppressive (possibly immune) and oncogenic functions. However, although DPP4 inhibition may not have effects on prostate cancer development, previous epidemiologic studies have not addressed whether it impairs responses to ADT. This study supports a tumor suppressive function of DPP4 after ADT, and suggests that treatment with DPP4 inhibitors may decrease the efficacy of ADT.

Disclosure of Potential Conflicts of Interest

P.S. Nelson is a consultant/advisory board member for Janssen. No potential conflicts of interest were disclosed by other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.W. Russo, O.S. Voznesensky, S. Arai, P.S. Nelson, B. Montgomery, E.A. Mostaghel, M.-E. Taplin, H. Ye, M. Bhasin, S.P. Balk
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.W. Russo, C. Gao, S.S. Bhasin, O.S. Voznesensky, S. Arai, P.S. Nelson, B. Montgomery, M.-E. Taplin, H. Ye, M. Bhasin, S.P. Balk
Writing, review, and/or revision of the manuscript: J.W. Russo, P.S. Nelson, B. Montgomery, E.A. Mostaghel, E. Corey, M.-E. Taplin, H. Ye, M. Bhasin, S.P. Balk
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Study supervision: J.W. Russo, S.P. Balk

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Downregulation of *Dipeptidyl Peptidase 4* Accelerates Progression to Castration-Resistant Prostate Cancer

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