AWARD NUMBER: W81XWH-21-1-0404

TITLE: The Role of Ubiquitination in CRPC Transitioning to NEPC

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CONTRACTING ORGANIZATION: Baylor College of Medicine, Houston, TX

REPORT DATE: August 2022

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
August 2022	Annual	01Aug2021-31Jul2022

4. TITLE AND SUBTITLE

The Role of Ubiquitination in CRPC Transitioning to NEPC

5a. CONTRACT NUMBER
W81XWH-21-1-0404

5b. GRANT NUMBER
PC200445

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Baylor College of Medicine
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

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

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

castration-resistant prostate cancer patients rapidly become resistant to androgen receptor pathway inhibitors. About 20% of these patients develop a highly aggressive neuroendocrine prostate cancer. Currently no target therapy is available for neuroendocrine prostate cancer. Understanding the molecular mechanisms driving castration-resistant prostate cancer to become neuroendocrine prostate cancer would provide valuable insights into the development of target therapies.

We recently found that an oncogenic enzyme, TRAF4, is important for castration-resistant prostate cancer development. It is also gene amplified in 25% of neuroendocrine prostate cancer patients. The goal of this proposed research is to understand the role of TRAF4 in neuroendocrine prostate cancer development. We will also test the effect of a potential TRAF4 enzyme inhibitor on controlling castration-resistant prostate cancer progression to neuroendocrine prostate cancer.

15. SUBJECT TERMS
Neuroendocrine prostate cancer, ubiquitination, inhibitor

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	16	19b. TELEPHONE NUMBER *(include area code)*

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1. INTRODUCTION:

castration-resistant prostate cancer patients rapidly become resistant to androgen receptor pathway inhibitors. About 20% of these patients develop a highly aggressive neuroendocrine prostate cancer. Currently no target therapy is available for neuroendocrine prostate cancer. Understanding the molecular mechanisms driving castration-resistant prostate cancer to become neuroendocrine prostate cancer would provide valuable insights into the development of target therapies. We recently found that an oncogenic enzyme, TRAF4, is important for castration-resistant prostate cancer development. It is also gene amplified in 25% of neuroendocrine prostate cancer patients. The goal of this proposed research is to understand the role of TRAF4 in neuroendocrine prostate cancer development. We will also test the effect of a potential TRAF4 enzyme inhibitor on controlling castration-resistant prostate cancer progression to neuroendocrine prostate cancer.

2. KEYWORDS:

Neuroendocrine prostate cancer, ubiquitination, inhibitor, TRAF4

3. ACCOMPLISHMENTS:

What were the major goals of the project?

(1) Major goals of the project:

a. To investigate the role of TRAF4 in NEPC transdifferentiation.

Expected completion date: 20th month
Percentage of completion: 50%

b. To determine the role of TRAF4-targeted ubiquitination in NEPC transdifferentiation.
Expected completion date: 30th month
Percentage of completion: 5%

c. To evaluate a TRAF4 small molecule inhibitor and to determine its effects on NEPC transdifferentiation and tumor growth
Expected completion date: 36th month
Percentage of completion: 10%

(2) Accomplishments under these goals:

Major activities:

1) IACUC Approval at Baylor College of Medicine: application approved.
2) DOD Animal Care and Use Review Office (ACURO): application approved.

3) analyzed the expression levels of NEPC markers in TRAF4 overexpressing PC cells.

4) compared TRAF4-regulated gene signature with androgen-sensitive prostate cancer cells and neuroendocrine differentiated cells

5) Generated doxycycline inducible TRAF4 wild type and ubiquitin ligase deficient mutant expressing lentiviruses.

6) demonstrated TRAF4-mediated AR ubiquitination in CRPC PDXs.

Specific objective: To determine whether TRAF4 plays a role in neuroendocrine differentiation of prostate cancers.

Significant results:
Specific aim 1:To investigate the role of TRAF4 in NEPC transdifferentiation
Major Task 1: To determine whether TRAF4 overexpressed CRPC tumors resemble the "intermediate state" in the process of CRPCs transdifferentiation into NEPCs.

Subtask 1: Submit documents for ACURO approvals

Completed

Subtask 2: To determine the expression levels of NEPC markers in TRAF4 overexpressing tumor cells.

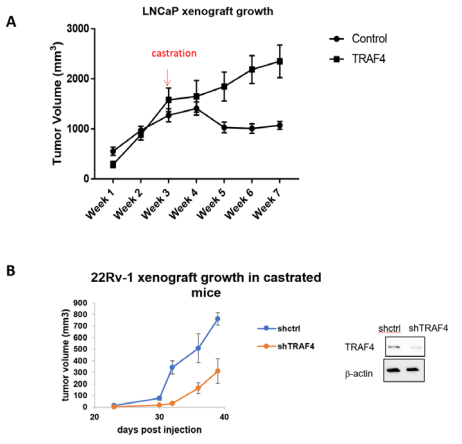

Fig 1: TRAF4 promotes CRPC development. (A) TRAF4 overexpressing LNCaP xenograft tumors are castration resistant (B) TRAF4 knockdown inhibits 22Rv-1 xenograft tumor growth in castrated mice.

We demonstrated that TRAF4 overexpression promotes LNCaP prostate cancer cell castration-resistant xenograft tumor growth (Fig. 1A). We also generated inducible TRAF4 shRNA expressing 22Rv-1 cells and control cells, and injected the cells into castrated SCID mice subcutaneously. We found that TRAF4 knockdown significantly reduced the xenograft tumor castration-resistant growth (Fig. 1B), further confirming the role of TRAF4 in promoting CRPC growth.

To determine whether TRAF4-promted CRPC development also regulates NEPC differentiation, we measured the levels of several neuronal differentiation markers in TRAF4 overexpressing prostate cancer cells, C4-2b and LNCaP cells. We found that several NEPC markers, including ENO2, NCAM1, BRN2 and ROBO1, are upregulated in TRAF4 overexpressing cells (Fig. 2A-D), suggesting that TRAF4 plays a role in upregulation of NEPC markers.

Subtask 3: To determine whether TRAF4-regulated gene signature resembles the intermediate state between CRPC and NEPC.
We analyzed the RNA-Seq data from TRAF4 overexpression and control LNCaP xenografts for differential expression, followed by the Broad Institute gene set enrichment analysis (GSEA) using the oncogenic gene sets from molecular signatures database (MSigDB). Genes enriched in top enriched PRD_EED (A), E2F1 (B), and MYC (C) pathways (Fig. 3A) with the gene expression values

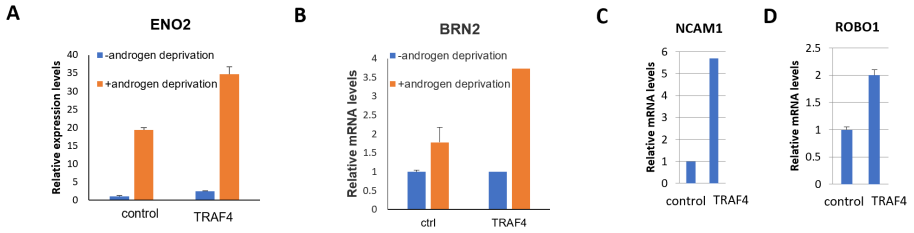

Fig. 2: TRAF4 overexpression increased NEPC marker gene expressions in prostate cancer cells.

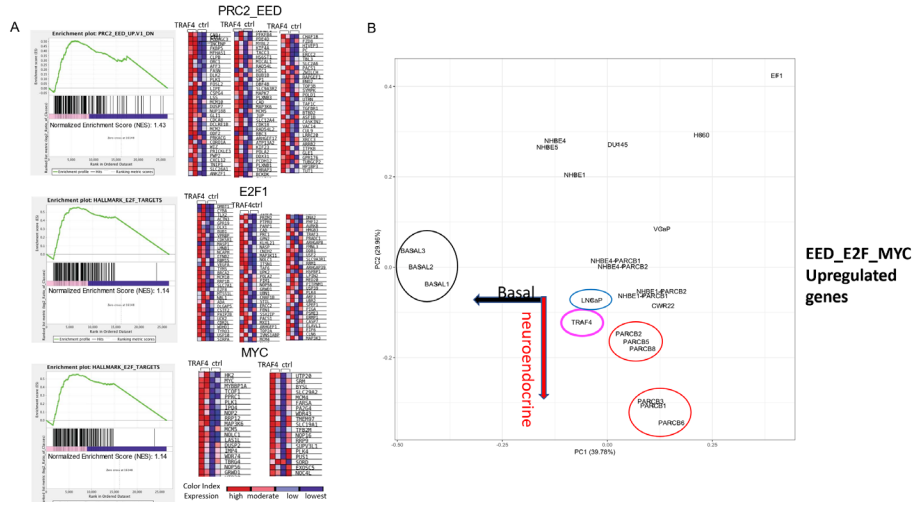

Fig. 3: TRAF4 induces a transitional state intermediate towards neuroendocrine prostate cancer lineages (A) Gene set enrichment analysis (GSEA) of the genes associated with TRAF4 overexpression in xenograft tumors. Genes enriched in PRD_EED, E2F1, and MYC pathways with the normalized enrichment scores (NES) are shown. (B) Partial least squares regression analysis (PLSR) of RNA-Seq transcriptome data from TRAF4 overexpressing xenograft tumors and 22 PrAd, SCPC and other NEPC specimens RNA-Seq dataset from Park Science 2018 & Beltran Nature Medicine 2016. Upregulated EED, E2F and MYC signature genes enriched from the RNA-Seq data for TRAF4 overexpressing xenograft tumors and engineered tumor lines and patient-derived prostate cancer cell lines were projected onto the PLSR plot.

were normalized with the 22 PrAd (Prostate cancer adenocarcinoma), SCPC (small cell prostate carcinoma) and other NEPC specimens RNA-seq dataset from Park Science 2018 & Beltran Nature Medicine 2016. To comprehensively understand the relationship between TRAF4 overexpressing xenograft tumors and the PrAd, SCPC and other NEPC subtypes, we normalized the RNA-Seq expression matrix of the upregulated EED, E2F and MYC signature genes from all the RNA-Seq datasets from Park et al. (Science 2018). This normalization work resulted in a set of 23 complete datasets that were subsequently projected onto the plot. To investigate the predictive linear-relationship between the TRAF4 overexpressing xenograft tumors and the PrAd, SCPC and other NEPC subtypes, a supervised learning approach partial least squares regression analysis (PLSR) was performed (Mevik et al., 2007). This supervised learning approach facilitates a better understanding of the underlying relationship and structure of TRAF4-driven upregulated gene signature among the subtypes of 22 PrAd, SCPC and other NEPC specimens. By analyzing the correlative relationship of TRAF4-driven tumors in the large-scale human prostate genomic datasets, we found that TRAF4 induces a transitional state intermediate towards neuroendocrine prostate cancer lineages (shown in Fig. 3B), suggesting that TRAF4 overexpression may have a potential to be tested as a novel biomarker and/or targeting utility of some subtypes of neuroendocrine prostate cancers.

Specific aim 3: To evaluate a TRAF4 small molecule inhibitor and to determine its effects on NEPC transdifferentiation and tumor growth

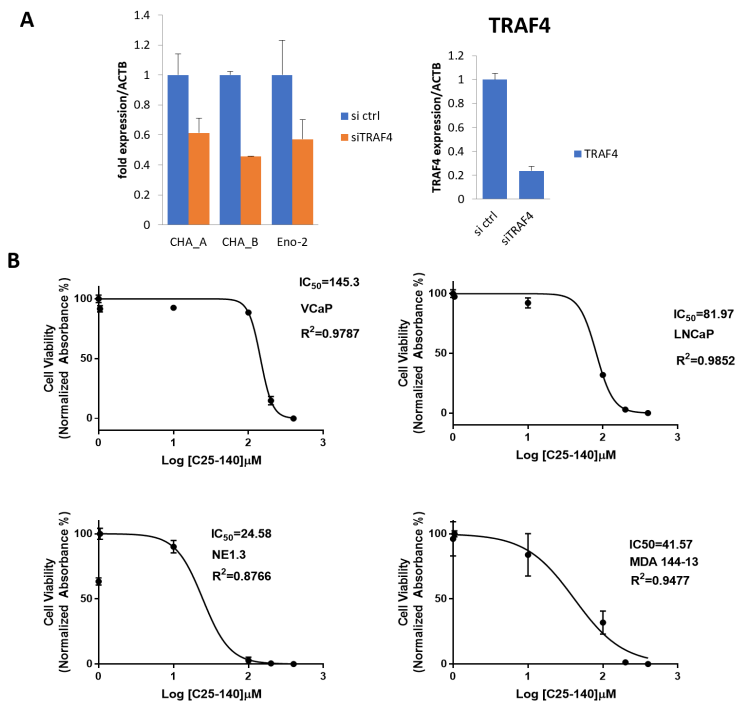

Fig. 4: TRAF4 inhibition regulates NEPC cell marker expression and cell viability (A) TRAF4 knockdown in NEPC cell NCI-660 decreased NEPC marker expressions. (B) NEPC cell lines (NE1.3 and MDA 144-13 are sensitive to TRAF4 inhibition compared to adenocarcinoma cell lines.

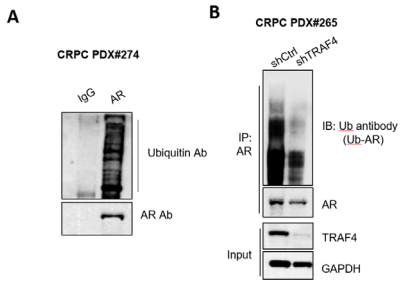

Fig. 5: TRAF4 mediates AR ubiquitination in CRPC and NEPC PDXs. (A) in vivo AR ubiquitination was performed in MDA PCa-274 NEPC PDX through immunoprecipitation using IgG or AR-specific antibody followed by Western blot analysis using a ubiquitin antibody. (B) In vivo ubiquitination in MDA PCa-265 CRPC PDX cells after TRAF4 knockdown using lentivirus shRNA or control shRNA. Immunoprecipitation was performed using an AR antibody followed by Western blot using a ubiquitin antibody. The input shows the TRAF4 protein levels in control and TRAF4 knockdown cells.

Major Task 6: To determine the effects of TRAF4 inhibition on NEPC cell proliferation and PDX tumor growth in vivo

Since TRAF4 overexpression increased NEPC marker expression in CRPC cells (Fig. 2), we also depleted TRAF4 in NEPC cells NCI-H660 using siRNA to determine whether TRAF4 affects NEPC marker gene expression in this cell line. As shown in Fig. 4A, TRAF4 knockdown indeed decreased several marker gene expressions. We are now in the process of testing the effect of TRAF4 inhibitor in regulating NEPC marker expressions. We found that a small molecule inhibitor, C25-140, inhibits in vitro TRAF4 auto-ubiquitination and in cell TRAF4-promoted AR ubiquitination (data not shown). To determine whether this inhibitor is effective in killing NEPC cells. We treated two NEPC cell lines, NE1.3 and MDA-144-13, and two androgen-sensitive prostate cancer cells, VCaP and LNCaP, with different concentrations of C25-140 and then measured the cell viability. As shown in Fig. 4B, we found that NEPC cell lines are more sensitive to the inhibitor treatment compared to androgen sensitive cell lines, suggesting that TRAF4 inhibition may be an effective approach in inhibiting NEPC cells.

We demonstrated previously that TRAF4 mediates non-classical AR ubiquitination in prostate cancer cell lines to promote CRPC development. To determine whether TRAF4 also mediates AR ubiquitination in prostate cancer PDXs, we immunoprecipitated AR from AR-positive NEPC PDX #274, which was obtained from MD Anderson Cancer Center, and then blocked with anti-ubiquitin-specific antibody. As shown in Fig. 5A, polyubiquitinated forms of endogenous AR were detected in this NEPC PDX. We also knocked-down TRAF4 in another PDX and found that TRAF4 depletion significantly decreased AR ubiquitination (Fig. 5B), suggesting that TRAF4-mediated AR ubiquitination also occurs in PDXs. Next, we will investigate whether TRAF4 inhibition decreases PDX tumor growth.

What opportunities for training and professional development has the project provided?

Nothing to report

How were the results disseminated to communities of interest?

Nothing to report

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

1. We will determine whether TRAF4 overexpressing cells are resistant to enzalutamide treatment
2. We will test the role of TRAF4 E3 ubiquitin ligase activity in promoting NEPC transdifferentiation using the TRAF4 ubiquitin ligase deficient mutant expressing stable cell line we generated.

4. 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

5. CHANGES/PROBLEMS:

Nothing to report

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

None

Changes that had a significant impact on expenditures

None

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

Significant changes in use or care of human subjects

None

Significant changes in use of biohazards and/or select agents

None

6. 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.

Nothing to Report

- **Website(s) or other Internet site(s)**

Nothing to Report

- **Technologies or techniques**

Nothing to Report

- **Inventions, patent applications, and/or licenses**

Nothing to Report

- **Other Products**

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: *Ping Yi*
Project Role: *PI*
Researcher Identifier (e.g. ORCID ID): 0000-0001-9433-6805
Nearest person month worked: *6*

Contribution to Project: *Dr. Yi designed and supervised the proposed research*

Name: *Lance Lumahan*
Project Role: *research technician*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *6*

Contribution to Project: *Mr. Lumahan performed the experiment proposed*.

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

8. **SPECIAL REPORTING REQUIREMENTS**
   COLLABORATIVE AWARDS:
   QUAD CHARTS:

9. **APPENDICES:**