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14. ABSTRACT It is now well-recognized that AR remains to be a critical player in castration-resistant prostate cancers. It was suggested that the function of AR in CRPC is not to turn on the same transcriptional targeted genes in the absence of androgen but to turn on a distinct set of genes independent of androgen. However, it was not clear what triggers the functional switch of AR. Here we report another pathway to bypass androgen dependency through AR ubiquitination. We found that TRAF4, a RING domain E3 ubiquitin ligase, is overexpressed in CRPCs. Its overexpression promoted androgen-independent cell growth. In this funding period we determined the underlying mechanism for TRAF4-promoted AR transcriptional activity on a different set of genes, which leads to androgen-independent growth. We also generated prostate-specific TRAF4 overexpression mouse strain to facilitate the study on the role of TRAF4 in CRPC development in vivo.					
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1. Introduction

It is now well recognized that AR remains active in castration-resistant prostate cancers (CRPCs). Post-translational modification, such as phosphorylation, plays a role in ligand-independent activation of AR. Ubiquitination is an important post-translational modification regulating protein degradation, trafficking, activity, and protein-protein interaction. Deregulation of the ubiquitin pathways has been implicated in a number of diseases including cancers. Targeting the ubiquitination system for cancer therapy has gained a broad interest. We recently found that TRAF4, a RING domain E3 ubiquitin ligase is highly expressed in CRPCs. Overexpression of TRAF4 promoted androgen-independent growth of prostate cancer cells and this function requires its E3 ubiquitin ligase activity. We further identified AR as a TRAF4 ubiquitin substrate using mass spectrometry and found that AR was able to regulate a different set of gene transcription when TRAF4 was overexpressed. In this study, we propose to test the hypothesis that TRAF4 mediated AR ubiquitination promotes CRPC development.

2. Keywords: TRAF4, AR, ubiquitination, UBE2C, CRPC

3. Accomplishments:

(1) Major goals of the project:

- a. To understand the function of TRAF4-mediated ubiquitination in castration-resistant prostate cancer cell proliferation
Expected completion date: 24th month
Percentage of completion: 100%
- b. To determine the role of TRAF4 in androgen-independent prostate cancer progression
Expected completion date: 24th month
Percentage of completion: 50%
- c. Determine the function of TRAF4 overexpression in CRPC development and metastasis using mouse tumor model
Expected completion date: 36th month
Percentage of completion: 15%

(2) Accomplishments under these goals:

Major activities: **Accomplishments under these goals:**

- Major activities: 1) Established the role of TRAF4-targeted AR ubiquitination in promoting CRPC-associated gene transcription.
2) Elucidated the underlying mechanism how AR ubiquitination promoted the ability of AR to regulate a different transcription program.
3) Initiated xenograft mouse studies to investigate the role of TRAF4 in regulating CRPC development.
4) Generated prostate-specific TRAF4 overexpression transgenic mouse strain.

Specific objective: To determine whether TRAF4-mediated AR ubiquitination plays a role in androgen-independent growth of prostate cancer cells.

Significant results:

Specific Aim 1: To determine the role of TRAF4-mediated ubiquitination in regulating androgen-independent growth

Subtask 3: To explore the mechanism for TRAF4-mediated androgen-independent growth

(1) TRAF4-mediated AR ubiquitination promotes AR activity on CRPC-associated genes.

In my last annual report, I found that TRAF4 promotes non-classical K27-linked ubiquitination of AR. I made a series of deletion and lysine mutation mutants of AR and demonstrated that K911 and K913 residues at the C-terminus of AR are TRAF4-targeted ubiquitination sites. Since we previously demonstrated that TRAF4 overexpression increased AR activity on CRPC-associated genes such as UBE2C and CDC20 (1), we then tested whether mutation of TRAF4-targeted AR ubiquitination sites could affect the ability of TRAF4 to promote transcription of these genes. A LNCaP cell line stably expressing flag-ARK911/913R mutant was generated (Fig. 1A). As shown in Fig. 1B, TRAF4 overexpression in parental LNCaP cells significantly increased UBE2C and CDC20 transcription levels when cells were cultured in androgen-deprived medium. In AR ubiquitination mutant cells, however, TRAF4 overexpression lost the ability to increase the transcription of these genes. In contrast, neither TRAF4 overexpression nor AR ubiquitination mutation had any effect on a classical AR target gene KLK3 transcription. These results suggest that the ability of TRAF4 to promote CRPC-

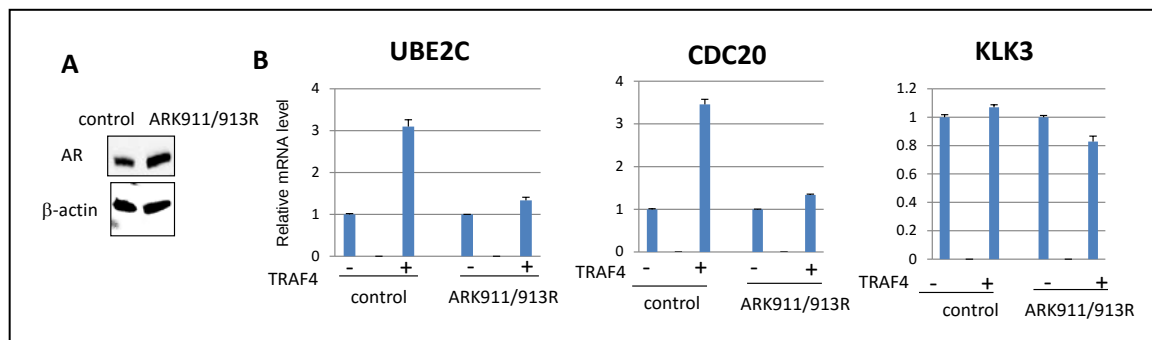


Fig. 1 TRAF4-targeted AR ubiquitination sites are important for TRAF4-promoted CRPC-associated gene transcription. (A) Expression of flag-ARK911/K913R mutant in LNCaP stable cells compared to parental cells. (B) Mutation of AR ubiquitination sites abolished TRAF4-mediated increase in UBE2C and CDC20 but not KLK3 transcription.

associated gene UBE2C and CDC20 transcription depends on AR ubiquitination.

(2) TRAF4 overexpression altered AR genomic binding sites

As mentioned above, UBE2C and CDC20 are CRPC-associated genes and are only regulated by AR in CRPC cells but not in normal androgen-dependent prostate cancer cells (1). Several lines of evidence have indicated that AR regulates a distinct transcriptional program in CRPCs (1-3). Since TRAF4 overexpression promoted AR transcription activity on these CRPC-associated genes, next we determined whether TRAF4 overexpression could alter AR genomic binding profile. A ChIP-seq analysis on the AR binding sites was carried out in TRAF4 overexpressing or vector control LNCaP cells cultured in androgen-deprived medium. As shown in Fig. 2A and B, TRAF4 overexpression indeed altered AR genomic binding profile. Majority of AR unique binding sites in TRAF4 overexpression cells were located in distal intergenic regions or intron regions (Fig. 2C). The top motif enriched for these unique binding sites was FoxA1 motif (Fig. 2D). We then compared multiple published FoxA1 ChIP seq datasets (either in LNCaP cells with or without androgen stimulation or in CRPC Abl cells cultured in androgen

deprived medium) with our AR ChIP seq with or without TRAF4 overexpression. Consistently, we found that more FoxA1 binding sites overlapped with new AR binding sites in TRAF4 overexpressing cells compared to those AR binding sites unique in control cells (Fig.2E). These

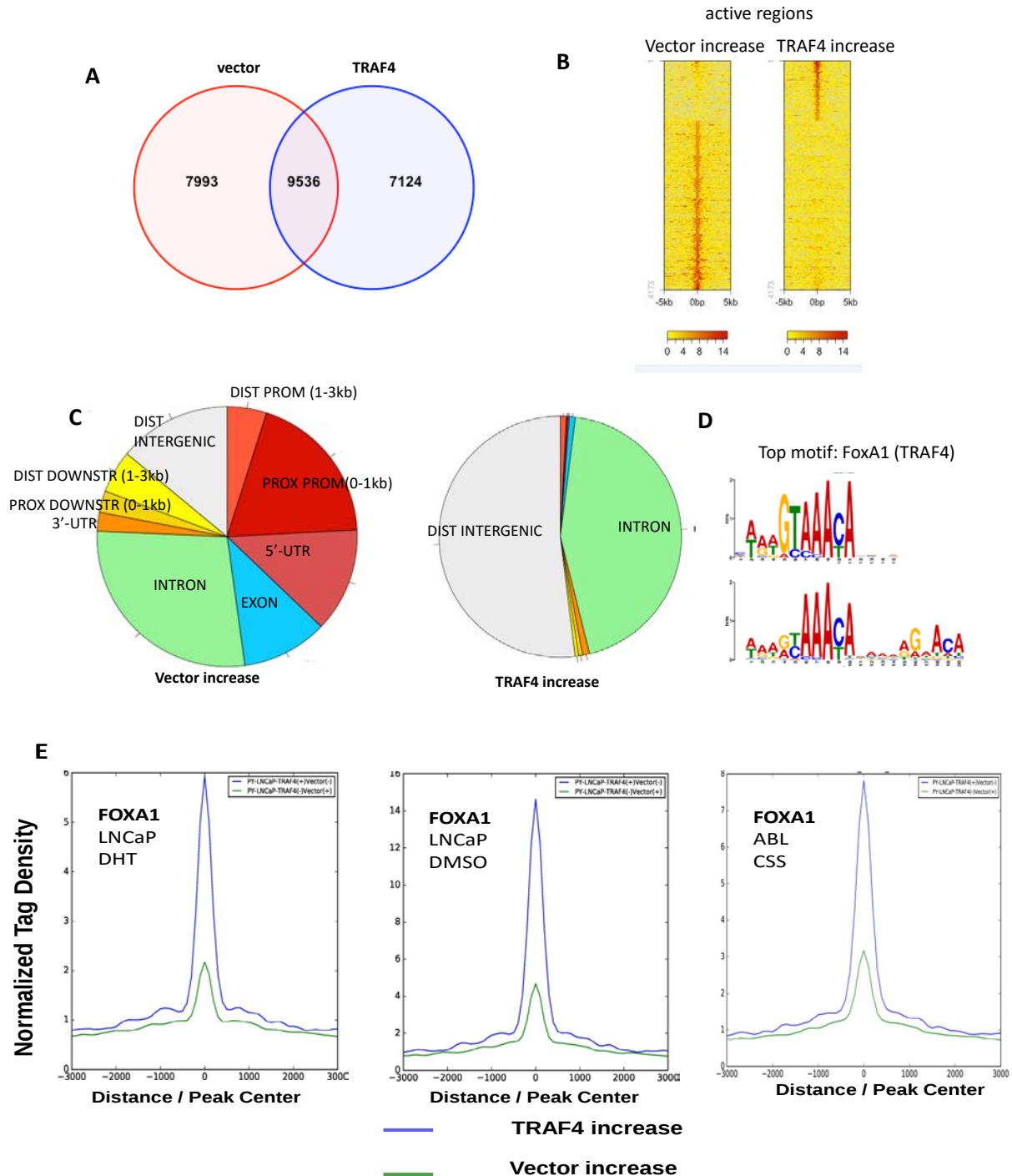


Fig. 2: TRAF4 overexpression altered AR genomic binding sites in LNCaP cells. (A) Venn diagram of AR binding sites in TRAF4 overexpressing LNCaP or vector control cells analyzed through AR ChIP seq data (B) heat map showing that AR regulated different active regions in TRAF4 or vector control overexpressing cells. (C) Majority of increased AR binding sites in TRAF4 overexpressing cells occurred in distal intergenic or intron regions. (D) FoxA1 binding motif was the top motif in AR binding sites upon TRAF4 overexpression (E) More FoxA1 binding sites from multiple datasets were enriched in unique AR binding sites in TRAF4 overexpressing LNCaP cells compared to unique sites in control cells.

results suggest that TRAF4 overexpression increases AR binding to FoxA1 binding sites located at distal enhancer regions that are normally not bound by AR.

(3) TRAF4 increased the interaction between AR and FoxA1

FoxA1 is a pioneer factor to facilitate AR binding to DNA. It was reported previously that FoxA1 plays a role in bring in AR to the UBE2C enhancer in androgen-independent prostate cancer cells (1). FoxA1 was found to be overexpressed in CRPCs and its overexpression facilitates AR chromatin binding resulting in a CRPC phenotype (4). To understand how TRAF4 regulated AR genomic binding, we examined the interaction between AR and FoxA1 in LNCaP cells. As shown in Fig. 3A, TRAF4 overexpression significantly increased the association between endogenous AR and endogenous FoxA1. Since TRAF4 targeted AR K911 and K913 residues for ubiquitination, we then tested whether TRAF4 is able to promote the interaction between this mutant and FoxA1. LNCaP cells stably expressing flag-AR wild type or flag-AR K911/913R were generated. As shown in Fig. 3B, we found that increased association of endogenous FoxA1 with wild type flag-AR but not with the ubiquitination defective mutant of AR when TRAF4 was overexpressed, suggesting that TRAF4-targeted AR ubiquitination is required for TRAF4-dependent increase on AR-FoxA1 interaction.

Altogether, these results suggest that TRAF4 overexpression promoted AR ubiquitination at its C-terminus, leading to increased interaction between AR and FoxA1, which increased AR binding to genomic sites that are associated with CRPC-related genes such as UBE2C to promote prostate cancer androgen independent growth.

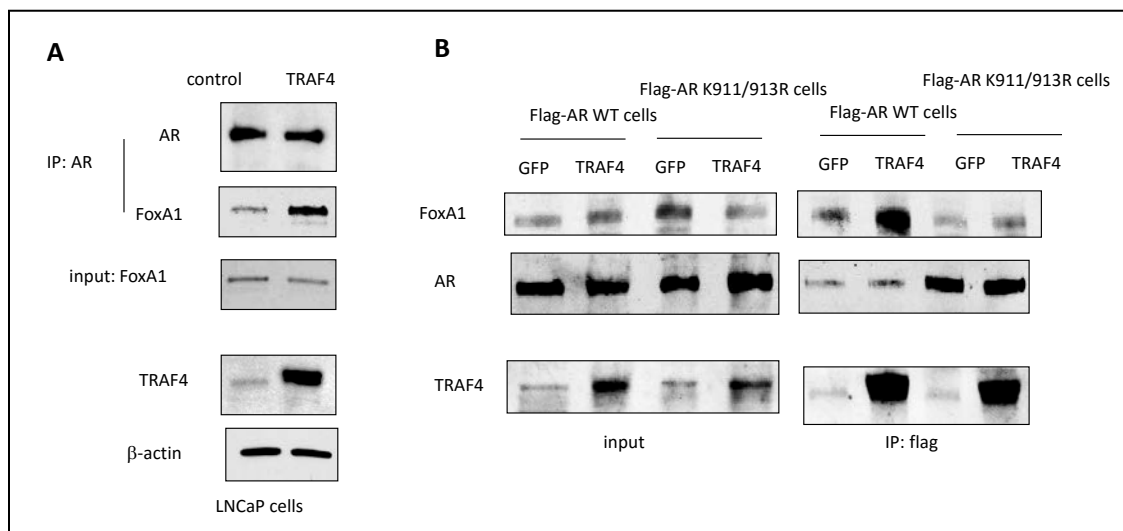


Fig.3 TRAF4 overexpression increased the interaction between AR and FoxA1. (A) Shown is the co-IP experiment in LNCaP cells with or without TRAF4 overexpression. AR was immunoprecipitation using specific AR antibody and the associated FoxA1 was detected in Western blot using FoxA1-specific antibody. (B) Mutation of TRAF4-targeted AR ubiquitination sites at K911 and K913 residues abolished TRAF4-mediated increase on AR and FoxA1 interaction. Shown is a co-IP experiment using an anti-flag specific antibody in flag-AR wild type or flag-AR K911/K913R expressing LNCaP cells.

Specific Aim 2: To determine the role of TRAF4 in androgen-independent prostate cancer progression

We found that overexpression of TRAF4 not only increased transcription of CRPC-associated genes such as UBE2C and CDC20 (Fig. 1B), but also promoted androgen-

independent growth of androgen-sensitive LNCaP cells (Fig.4A). In contrast, TRAF4 depletion significantly reduced the growth of androgen insensitive cell lines Abl and C4-2 cells (Fig. 4B and Fig. 4C). Currently we are in the process of testing the role of TRAF4 in promoting CRPC development using xenograft mouse models. LNCaP cells overexpressing TRAF4 or vector control mixed with matrigel were injected into SCID mice subcutaneously. After 3 weeks, palpable tumors were detected at the site of injection. We are now monitoring the growth of injected LNCaP cells and will determine the effect of castration on the tumor growth once tumors reach 0.5-0.6cm in diameter.

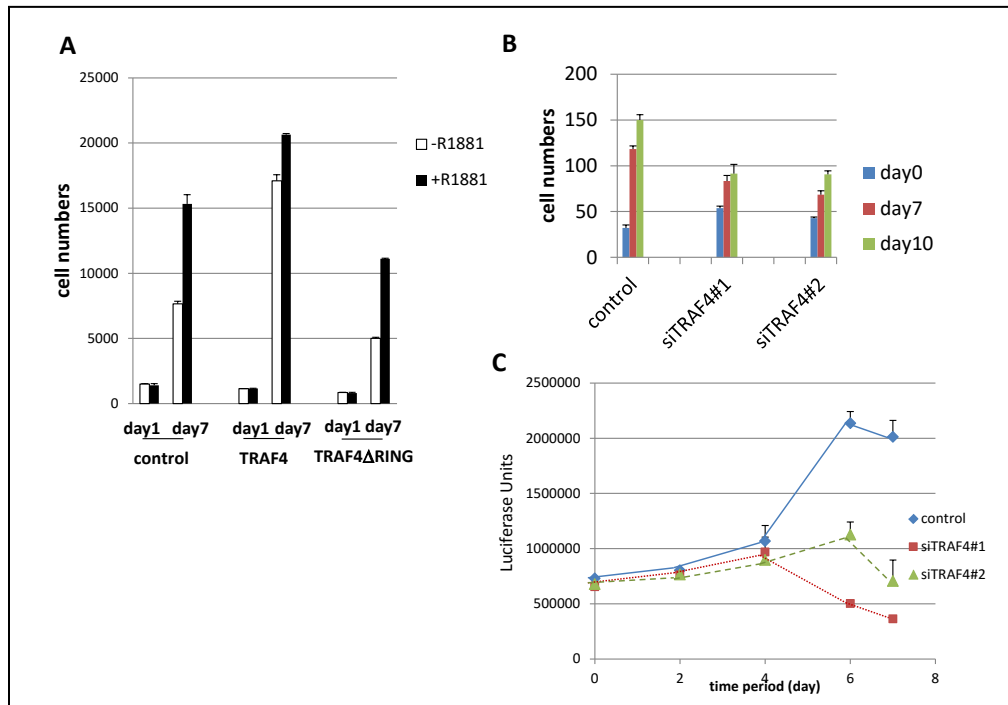


Fig. 4 TRAF4 is important for androgen-independent prostate cancer cell growth. (A) Overexpression of TRAF4 but not its ubiquitination defective mutant promoted LNCaP cell androgen-independent growth. (B-C)TRAF4 depletion inhibited the growth of CRPC cells Abl (B) and C4-2 cells.

Specific Aim 3: Determine the function of TRAF4 overexpression in CRPC development and metastasis using mouse tumor model

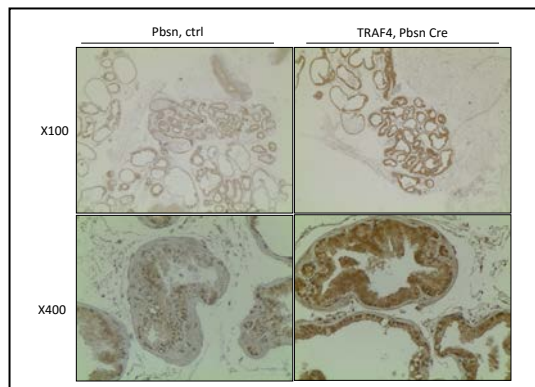


Fig. 5 Overexpression of TRAF4 in prostate epithelial cells in Probasin-cre/TRAF4 transgenic mouse. Shown is an IHC staining of mouse prostate using a TRAF4-specific antibody.

We have successfully inserted TRAF4 cDNA into a minigene, which was then inserted in the mouse embryonic stem cells. After embryo injection, we obtained a chimera with the TRAF4 minigene and the TRAF4 transgene is able to get germline transmission.

The prostate-specific expression of TRAF4 transgene is under the control of Cre recombinase driven by probasin (Pbsn) promoter. We crossed TRAF4 transgenic mice with Pbsn-cre mouse strain to obtain prostate-specific TRAF4 expressing mouse.

An immunohistochemistry was then performed on 5-month old Pbsn-cre mouse and Pbsn-cre/TRAF4 mouse prostate using

a TRAF4-specific antibody. As shown in Fig. 5, TRAF4 staining was detected in prostate epithelial cells in Pbsn-cre mouse, indicating endogenous TRAF4 expression in these cells. A much stronger TRAF4 staining was observed in Pbsn-cre/TRAF4 prostate epithelial cells. These results suggest that we have successfully generated a transgenic mouse strain with TRAF4 overexpression in prostate epithelial cells.

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(3) Opportunities for training and professional development this project provided:

This project provided me the opportunity to present my research at the Dan L. Duncan Comprehensive Cancer Center symposium.

(4) How were the results disseminated to communities of interest?

Nothing to report

(5) Plan to do during the next reporting period to accomplish the goals

- a. We will continue to determine the effects of TRAF4 on androgen-independent prostate cancer cell growth in vivo using xenograft mouse model
- b. We will cross the Pbsn-cre/TRAF4 transgenic mouse line with prostate-specific PTEN deletion mouse line to examine the role of TRAF4 in prostate cancer development.

4. Impact

(1) The impact on the development of the principal discipline of the project

We identified TRAF4 as a novel gene that promotes androgen-independent growth and metastasis of prostate cancer cells through AR ubiquitination. Depletion of TRAF4 reduced CRPC cell growth. Our study reveals a novel pathway regulating AR

post-translational modification important for CRPC progression and provides potential therapeutic targets.

(2) The impact on other discipline

Nothing to report

(3) The impact on technology transfer

Nothing to report

(4) The impact on society beyond science and technology

Nothing to report

5. Changes/Problems:

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

We were delayed in the Major Task 2 subtask 2. The problem was the LNCaP stable cells we injected into SCID mice were not in optimal conditions and mice did not develop tumor after injection. We generated new stable cell lines and re-injected the cells. Now we can detect palpable tumors after 3 weeks. We will monitor the tumor growth and determine the effects of TRAF4 overexpression on tumor growth following castration.

6. Products:

Publications, conference papers, and presentations:

Peer reviewed paper in revision: Singh, R., Karri, D., Shao, J., Dasgupta, S., Huang, S., Edwards, D.P., O'Malley, B.W. and Yi, P. **TRAF4-mediated NGF receptor TrkA ubiquitination regulates prostate cancer metastasis** J. Clin. Invest. (in revision)

7. Participants & other collaborating organizations

(1) Individuals have worked on the project

Name	Ping Yi	Ramesh Singh
Project Role	PI	Postdoctoral associate
Researcher Identifier	0000-0001-9433-6805	0000-0001-5052-7925
Nearest person month worked	3	6
Contribution to Project	Dr. Yi designed and supervised the proposed research	Dr. Singh performed the experiment proposed.
Funding support		

(2) Changes in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period:

Nothing to report

(3) Other organizations involved as partners:

Nothing to report

8. Special Reporting Requirements

9. Appendices