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PRINCIPAL INVESTIGATOR: Ping Yi

CONTRACTING ORGANIZATION: Baylor College of Medicine
HOUSTON, TX 77030

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Ping Yi

E-Mail:pyi@bcm.edu

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Baylor College of Medicine
One Baylor Plaza
Houston, TX 77030

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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 role of TRAF4 and its regulated AR targeted genes in CRPC cell growth. We found that TRAF4 promoted AR recruitment to these gene enhancers to promote CRPC development. We further identified TRAF4-mediated AR ubiquitination sites.

15. SUBJECT TERMS
NONE LISTED

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

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 tested the hypothesis that TRAF4 mediated AR ubiquitination promotes CRPC development.

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

TRAF4, AR, ubiquitination, CRPC, E2F, cAMP

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.

Major Task 1: To determine the role of TRAF4-mediated ubiquitination in regulating androgen-independent growth
Expected completion date: 24th month
Actual completion date: 36th month

Major Task 2: xenograft tumor studies with TRAF4 overexpression prostate cancer cells in castrated mice

Expected completion date: 24th month
Actual completion date: 36th month

Major Task 3: Generate TRAF4 overexpression CRPC mouse models
Expected completion date: 36th month
Actual completion date: 48th month

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.

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

Key accomplishments:

(1) TRAF4 overexpression in androgen-dependent prostate cancer promotes cell growth in the absence of androgen. Its ubiquitin ligase activity is required for promoting androgen-independent cell growth. (details see appended manuscript)

(2) Androgen receptor was found as a TRAF4-mediated ubiquitination substrate through mass spectrometry. The ubiquitination site was identified at the C-terminal tail of AR (details see appended manuscript).

(3) TRAF4 overexpression selectively upregulates CRPC-associated AR target gene transcription, such as UBE2C and CDC20 (details see appended manuscript).

(4) TRAF4-mediated AR ubiquitination alters AR genomic binding profile(details see appended manuscript).

(5) Ubiquitinated AR has increased affinity with transcription factor FoxA1 and binds to enhancers of genes regulating cAMP signaling. TRAF4 overexpression upregulates intracellular cAMP levels (details see appended manuscript).

Major Task 2: xenograft tumor studies with TRAF4 overexpression prostate cancer cells in castrated mice

Key accomplishments:

(1) TRAF4 depletion inhibits the growth of androgen-independent prostate cancer cells LNCaP C4-2 and LNCaP Abl cells. Depletion of TRAF4-promoted AR selectively regulated gene, UBE2C, also reduces castration-resistant prostate cancer cell growth (details see appended manuscript).

(2) TRAF4 overexpression promotes castration-resistant tumor growth of LNCaP mouse xenografts (details see appended manuscript).

Major Task 3: Generate TRAF4 overexpression CRPC mouse models

Key accomplishments:

(1) Generated TRAF4 minigene.
We have successfully inserted TRAF4 cDNA into a minigene consisting of a ubiquitous CAGGS (a hybrid chicken β-actin and cytomegalovirus) promoter with a floxed STOP cassette inserted between the promoter and the TRAF4 gene to silence TRAF4 expression.

(2) Generated prostate-specific TRAF4 overexpressing mouse.
After embryo injection, we obtained a chimera with the TRAF4 minigene and the TRAF4 transgene is able to get germline transmission.

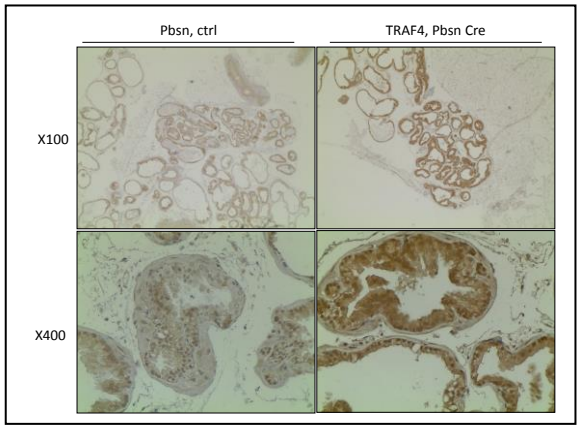

Fig. 1 Overexpression of TRAF4 in prostate epithelial cells in Probasin-cre/TRAF4 transgenic mouse. Shown is an IHC staining of mouse prostate using a TRAF4-specific antibody.

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. 1, 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.

To examine the role of TRAF4 overexpression in prostate cancer development, we crossed TRAF4 overexpression flox mice (TRAF4/+) with probasin cre; PTEN-/- mice, trying to obtain prostate specific TRAF4 overexpression and PTEN deletion mice (TRAF4/+;cre/+;PTEN-/+). However, all pups we got from breeding were either TRAF4/+;PTEN-/+ or PTEN-/+. We then set up more breeding cages to increase the possibility of obtaining desired mice. It is not clear yet why we did not get TRAF4/+;cre/+;PTEN-/+ mice from this breeding.

(3) Determined the expression levels of TRAF4 in human prostate cancers (details see appended manuscript).

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.

(1) This project provided me and my lab member the opportunity to attend AACR prostate cancer meeting and AACR annual meeting.

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.

The results were disseminated through peer-reviewed journal publications.

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

Nothing to report

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).

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.

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:*

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 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).*

Singh, R., Karri, D., Shen, H., Shao, J., Dasgupta, S., Huang, S., Edwards, D.P., Ittmann, M.M., O'Malley, B.W., **Yi, P.** TRAF4-mediated ubiquitination of NGF receptor TrkA regulates prostate cancer metastasis. *J Clin Invest.* (2018) 128, 3129-3143. Status: 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.*

Singh, R., Shao, J., Karri, D., Dasgupta, S., O'Malley, B.W. and Yi, P. TRAF4-mediated AR Ubiquitination and Castration-Resistant Prostate Cancer *Innovative Minds in Prostate Cancer Today Young Investigators Meeting*

*Singh, R., Karri, D., Shen, H., Shao, J., Dasgupta, S., Huang, S., Edwards, D.P., Ittmann, M.M., O'Malley, B.W., **Yi, P.** TRAF4-mediated ubiquitination of NGF receptor TrkA regulates prostate cancer metastasis. *AACR annual meeting 2018*

- **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	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		

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.

Nothing to report

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.

TRAF4-mediated non-proteolytic ubiquitination of androgen receptor promotes castration-resistant prostate cancer

Ramesh Singh[1], Hong Shen[1], Subhamoy Dasgupta[2], Meng Huang[1], Dileep Karri[3], Bokai Zhu[4], Cristian Coarfa[1], Jun Qin[1], Bert W. O'Malley[1] and Ping Yi[1*]

[1]Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX, USA

[2]Current address: Department of Cell Stress Biology, Roswell Park Comprehensive Cancer Center, Buffalo, NY, USA

[3]Current address: University of Texas Southwestern Medical School, Dallas, TX, USA

[4]Current address: Department of Medicine, University of Pittsburg, Pittsburgh, PA, USA

*To whom correspondence should be addressed: pyi@bcm.edu

Abstract

Castration-resistant prostate cancer poses a major clinical challenge with androgen receptor (AR) remains to be a critical player. Several lines of evidence indicate that AR induces a distinct transcriptional program after androgen deprivation in CRPCs. However, the mechanism driving AR binding to a distinct set of genomic loci in CRPC remains unclear. We demonstrate here that atypical ubiquitination of AR mediated by an E3 ubiquitin ligase TRAF4 plays an important role in this process. TRAF4 is highly expressed in CRPCs and promotes CRPC development. It mediates K27-linked ubiquitination at the C-terminal tail of AR and increases its association with pioneer factor FOXA1. Consequently, AR binds to a distinct set of genomic loci enriched with FOXA1 and HOXB13 binding motifs to drive different transcriptional programs including olfactory transduction pathway. Through upregulation of olfactory receptor gene transcription, TRAF4 increases intracellular cAMP levels and boosts E2F transcription factor activity to promote cell proliferation under androgen deprivation condition. Altogether, these results reveal a new post-translational mechanism driving AR-regulated transcriptional program switch to provide survival advantage for prostate cancer cell under castration condition.

Introduction

Prostate cancer (PCa) is the most commonly diagnosed malignancy and the second leading cause of cancer death among men in United States [1]. Androgen regulates normal and malignant prostate tissue growth via activation of androgen receptor (AR) signaling and plays an essential role in the initiation and progression of PCa. Androgen deprivation therapy (ADT) is the first line treatment choice for locally advanced or metastatic PCa. Despite initial responses to ADT, nearly all patients eventually develop castration-resistant prostate cancer (CRPC). It is now well recognized that CRPC continues to be dependent on the AR signaling [2, 3]. In CRPC, AR signaling is reactivated through different mechanisms including AR amplification or

overexpression, AR point mutations, expression of constitutively active AR splice variants, and intratumoral androgen synthesis. Several lines of evidence also indicate that AR indeed regulates a distinct transcription program in CRPC to promote castration-resistant cancer growth [4-6]. However, the underlying mechanism triggering the switch of AR-regulated transcription program is largely unknown.

AR belongs to a family of ligand-activated nuclear receptors. It consists of an N-terminal domain, which has a constitutively active activation function, a conserved central DNA-binding domain (DBD), an interdomain linker or hinge, and a C-terminal ligand-binding domain (LBD), which has a ligand-dependent activation function. Upon binding to androgen, AR translocates to the nucleus and binds to specific DNA sequences known as androgen-responsive elements (AREs) at the AR target gene promoters/enhancers, thereby regulating transcription of androgen-responsive genes. AR transcriptional activity is regulated by post-translational modifications such as phosphorylation, acetylation, sumoylation, and ubiquitination [7-9]. Ubiquitination is an important post-translational modification brought about by ubiquitin-activating enzymes (E1s), ubiquitin conjugating enzymes (E2s), and ubiquitin ligase enzymes (E3s). Ubiquitin (Ub) is a highly conserved protein of 76 amino acids that becomes covalently attached to lysine residues of target proteins. Apart from proteasomal degradation, protein ubiquitination can also lead to various non-proteolytic cellular functions, including endocytosis, endosomal sorting, subcellular localization, kinase activation and DNA repair [10].

AR ubiquitination has been shown to regulate AR activity and contribute to CRPC progression [11, 12]. The mechanisms identified so far are mainly through ubiquitination targeted inactive AR/corepressor complex degradation [11], or non-canonical ubiquitination-mediated recruitment of ubiquitin-binding domain containing AR coactivator [12]. However, only a small subsets of AR targeted gene transcription are regulated by these mechanisms [11, 12]. Here we demonstrate that another RING finger domain E3 ubiquitin ligase, TRAF4, promotes CRPC development. It

mediates non-classical K27-linked AR ubiquitination at its C-terminal end, increases AR interaction with pioneer transcription factor FOXA1, and subsequently altering AR genomic binding profile. Upon TRAF4 overexpression, ubiquitinated AR switches its transcription program and upregulates a set of CRPC-associated genes to promote CRPC progression. Our study reveals an important role of post-translation modification in governing AR-regulated gene switch in CRPCs.

Results

TRAF4 promotes castration-resistant prostate cancer growth

We recently found that TRAF4 is highly expressed in metastatic prostate cancers and plays an important role in prostate cancer metastasis [13]. Most of these metastatic patients were treated with androgen-deprivation therapy before developing metastasis [14-18]. We also found that the TRAF4 protein level is higher in androgen-insensitive LNCaP-Abl cells compared androgen-sensitive LNCaP prostate cancer cells (Fig. S1). To understand whether TRAF4 overexpression plays a role in the development of prostate cancer castration-resistance, we examined the growth of TRAF4 stably overexpressed LNCaP cells or control LNCaP cells in the absence or presence of androgen R1881. LNCaP cells depend on androgen for cell growth. We indeed observed that R1881 in the cell culture promoted control LNCaP cell growth compared to in the absence of R1881 (Fig. 1A). Overexpression of TRAF4 markedly increased cell growth in the absence of R1881. The addition of R1881 had a minimal increase on TRAF4 overexpressed cell growth, suggesting that TRAF4 overexpression promotes androgen-independent cell growth. Since TRAF4 is a RING domain containing E3 ubiquitin ligase, we next investigated whether TRAF4-promoted androgen-independent cell growth requires its E3 ubiquitin ligase activity. We recently generated a RING domain deletion mutant of TRAF4 overexpressing LNCaP cell line, which expresses similar levels of the mutant TRAF4 compared to the wild type TRAF4

expressing stable cell line [13]. In contrast to the wild type TRAF4 expressing stable cells, the mutant stable cells were still androgen-sensitive (Fig. 1A), suggesting that the ubiquitin ligase activity of TRAF4 is critical for its ability to promote androgen-independent growth.

To substantiate the importance of TRAF4 in CRPC development in vivo, we injected TRAF4 overexpressing or control LNCaP cells into NOD/SCID mice subcutaneously. Comparable tumor growth rate was observed between the two cell lines. When tumors reach approximately 1cm in diameter, mice were castrated. Tumors derived from control cells regressed and stopped growing in response to castration (Fig. 1B). In contrast, tumors derived from TRAF4 overexpressing cells continue to grow, suggesting that TRAF4 overexpression promoted castration resistant growth of prostate cancer cells in vivo.

TRAF4 overexpression promotes upregulation of E2F pathway

To understand how TRAF4 promotes CRPC, we performed a RNA seq analysis on TRAF4-overexpressing castration-resistant xenograft tumors and control castration-responsive tumors. GSEA enrichment analysis using the oncogenic gene datasets from MSigDB' was then carried out to identify pathways regulated by TRAF4 overexpression. The top three pathways enriched in TRF4 overexpressing CRPC tumors are E2F, Myc, and Polycomb repressive complex 2 (PRC2) regulated gene pathways (Fig. 2A). All of these pathways have been linked to CRPC [5, 19-24]. There are cross-talks between these pathways. Myc is known to modulate the cell cycle regulator E2F activity and expression [25, 26]. E2F also regulates the expression of Myc and the Polycomb group gene EZH2 [27-30].

Activation of the E2F pathway has been found in multiple CRPC models as well as human CRPCs [19, 20, 24, 31, 32]. In our RNA seq analysis, around 100 E2F target genes were also up-regulated in TRAF4 overexpressing CRPC tumors. To test whether TRAF4 regulates E2F activity, we transiently transfected an E2F-driven luciferase reporter plasmid into LNCaP cells

with or without co-transfection of increasing concentrations of TRAF4 expression plasmid. The expressed luciferase reporter activity was then monitored to measure E2F transcriptional activity. As shown in Fig. 2B, increasing concentrations of TRAF4 marked increased E2F driven luciferase reporter activity, suggesting that TRAF4 indeed enhances E2F activity.

We next examined whether inhibition of E2F activity could abolish TRAF4-promoted androgen-independent cell growth. Palbociclib is a selective cycline-dependent kinase CDK4/6 inhibitor. It inhibits CDK4/6-mediated Rb phosphorylation, therefore preventing E2F activation. As shown in Fig. 2C, TRAF4 overexpressing LNCaP cells grow faster in androgen-deprived medium compared to control cells. Treatment of Palbociclib reduced TRAF4 stable cell growth to the level of control cells. These results suggest that E2F activation is an important downstream effector regulating TRAF4-promoted androgen-independent growth.

TRAF4 promoted AR ubiquitination through the non-classical K27 ubiquitin linkage

Since TRAF4 E3 ubiquitin ligase activity is essential for promoting androgen-independent cell growth, we then try to identify TRAF4 ubiquitin substrate that is important for mediating this function. Flag-tagged TRAF4 was immunoprecipitated using a flag antibody from TRAF4 stably expressing LNCaP cells. Proteins interacting with flag-TRAF4 were then identified using mass spectrometry. Interestingly, AR was found among the proteins identified. AR is a well-known key player in driving CRPC [33-35].We found that TRAF4 is highly expressed in AR-positive but not AR-negative metastatic patient-derived xenograft (PDX) compared to in vitro cultured cell lines, LNCaP and PC3 (Fig. 3A), implying a possible link between TRAF4 and AR. Furthermore, knockdown of AR abolished TRAF4-enhanced E2F transcriptional activity (Fig.3B), suggesting that AR mediates the effect of TRAF4 on E2F activity.

To investigate whether TRAF4 plays a role in regulating AR function, a co-IP experiment was carried out in 293T cells transfected with flag-AR with or without HA-TRAF4 co-transfection. As

shown in Fig. 3C, an association of flag-AR and HA-TRAF4 was detected. We next performed an in vitro ubiquitination assay to determine whether AR is a TRAF4-mediated ubiquitination substrate. Purified AR protein was incubated with UBE1 (E1 ubiquitin activating enzyme), UbcH5a (E2 ubiquitin conjugating enzyme), and HA-ubiquitin, in the absence or presence of purified TRAF4 protein, for an in vitro ubiquitination assay. As shown in Fig. 3D, the presence of TRAF4 substantially increased AR ubiquitination in vitro, suggesting that AR is a TRAF4-targeted substrate. We further confirmed that TRAF4 wild type but not the RING domain mutant promoted AR ubiquitination in cells (Fig. 3E).

There are 7 lysine residues in the ubiquitin. Different ubiquitin lysine linkages could mediate different functions, including protein turnover, protein-protein interaction, and cellular trafficking. TRAF4 was previously reported to mediate K48- or K63-linked ubiquitination [36, 37]. We recently also found that TRAF4 is able to promote protein ubiquitination through non-classical K27 and K29-linkages [13]. To determine the type of ubiquitin linkage for AR, we co-transfected flag-AR, TRAF4, with wild type HA-tagged ubiquitin or different ubiquitin mutants. These ubiquitin mutants either have all seven lysine residues mutated to arginine (K0), or only have one lysine residue with all other six lysine mutated to arginine (K6, K11, K27, K29, K33, K48, or K63). As shown in Fig. 3F, AR is polyubiquitinated only in the presence of wild type or K27 ubiquitin, suggesting that TRAF4 mediates AR ubiquitination through K27 linkage.

Identification of TRAF4-targeted AR ubiquitination site

We next try to identify TRAF4-targeted AR ubiquitination sites. We first determined the functional domain at which the ubiquitination sites are located. A flag-tagged full-length wild type AR, an AR splicing variant ARV7 which lacks a LBD, or two AR deletion mutants (Mut 600-920 which lacks the NTD, and Mut 669-920 which only contains the LBD) were transiently co-transfected with TRAF4, HA-tagged ubiquitin into cells. As shown in Fig. 4A, ARV7 or the NTD

deletion mutant of AR (1-600) had barely detectable levels of ubiquitination compared to full-length or the LBD containing AR mutants, suggesting that TRAF4-targeted AR ubiquitination occurs at the LBD. We further narrowed down the ubiquitination target region and generated a series of C-terminal deletion mutants. We found that deletion of the last 53 amino acids at the C-terminus of AR abolished TRAF4 WT but not its RING deletion mutant –mediated ubiquitination (Fig 4B). There are four lysine residues located in this region. Each of these lysine residues was mutated to arginine. We found that K913 mutation abolished AR ubiquitination, suggesting that this residue is TRAF4-targeted ubiquitination site (Fig 4C).

To confirm that K913 is a bona fide ubiquitination target site, we performed a mass spectrometry to determine ubiquitination modification from AR protein. 6XHis-tagged ubiquitin, purified TRAF4, UBE1 and UbcH5a were incubated with recombinant AR protein purified from baculovirus in the in vitro ubiquitination assay. His-tagged ubiquitinated AR was then captured using a nickel affinity column followed by mass spectrometry analysis. Although this method cannot distinguish the AR ubiquitination pre-existed in cells or modified in vitro, we did find K913 among a total eight ubiquitinated lysines (Fig S2). These results suggest that K913 is a TRAF4-targeted ubiquitination site.

TRAF4 overexpression selectively upregulates CRPC-associated AR target gene transcription

To investigate the potential role of TRAF4 on AR function, we generated two TRAF4 stable LNCaP cells with low (T1) and high (T2) TRAF4 expression (Fig. S3). The AR protein levels were not significantly changed in control, T1, or T2 cells (Fig. S3), suggesting that TRAF4 overexpression did not alter AR protein turnover. We then examined the AR targeted gene transcription in these cells in either complete culture medium, which contains androgen, or

androgen-deprived medium. Unexpectedly, we did not find any major effects of TRAF4 overexpression on the expression of canonical AR target genes KLK3 (PSA) and TMPRSS2 (Fig. 5A). We even observed an inhibitory effect of TRAF4 overexpression on another AR target gene SGK expression (Fig. 5A right panel). It was previously reported that AR regulates a distinct transcriptional program in androgen-independent compared to androgen-dependent prostate cancer cells [4]. Several M-phase cell cycle genes, such as UBE2C and CDC20, are regulated by AR selectively in androgen-independent prostate cancer cells. Overexpression of UBE2C in CRPC is well-documented [4, 38-40] and it was reported to be important for androgen-independent prostate cancer cell growth [4]. However, it is still not clear what triggers the switch of AR-regulated transcription program in androgen-independent cells. We found that UBE2C and CDC20 were significantly up-regulated in TRAF4 overexpressing cells in both complete culture medium and androgen-deprived medium (Fig.5B and Fig S4A). The levels of these gene transcriptions were higher in high TRAF4 expressing T2 cells compared to low TRAF4 expressing T1 cells, suggesting that TRAF4 expression levels influence the transcription of UBE2C and CDC20.

To determine whether increased transcription of UBE2C and CDC20 was dependent on AR, we knocked-down AR or TRAF4 in control LNCaP or T2 cells. In control cells, neither AR knockdown nor TRAF4 knockdown has any effect on UBE2C transcription (Fig. 5C). This is consistent with the previous report that AR does not regulate UBE2C transcription in androgen-sensitive prostate cancer cells [4]. In TRAF4 overexpressing cells, however, up-regulation of UBE2C transcription was abolished upon AR or TRAF4 knockdown. Similar result was also observed for CDC20 transcription (Fig S4B). In contrast to UBE2C and CDC20 transcription, KLK3 and TMPRSS2 transcriptions were not differentially regulated in control and T2 cells. Their transcriptions were significantly reduced upon siAR treatment in both cells (Fig S5). These

results suggest that TRAF4 overexpression switches on AR function on activating selective CRPC-associated gene transcriptions.

To further confirm that TRAF4 plays a role in regulating UBE2C and CDC20 transcription in CRPC cells, we knocked down TRAF4 in androgen-independent C4-2 cells. As shown in Fig. 5D and Fig S4C, siTRAF4 treatment indeed reduced UBE2C and CDC20 transcription. It was previously reported that AR binds to UBE2C distal enhancer region in Abl cells but not in LNCaP cells [4]. We found that TRAF4 depletion significantly reduced AR recruitment to the UBE2C enhancer but not the KLK3 (PSA) enhancer in Abl cells (Fig. S6A and S6B). These results suggest that TRAF4 plays a role in regulating AR genomic binding to CRPC-associated gene enhancers. Consistent with its role in regulating AR-mediated CRPC-associated gene transcription, TRAF4 depletion markedly decreased the growth of CRPC cells, LNCaP-Abl and C4-2 (Fig. 5E and Fig. S4D, respectively).

TRAF4 overexpression alters AR genomic binding profile

Since TRAF4 promotes the ability of AR to turn on genes that are normally not regulated by AR in androgen-sensitive prostate cancer cells, it is likely that TRAF4 overexpression alters AR genomic binding profile. To examine this, we performed an AR ChIP seq analysis in control LNCaP and TRAF4 overexpressing stable cells under androgen-deprived condition. The AR binding peaks in the two cell lines did not completely overlap (Fig.6A). TRAF4 overexpression causes about 50-60% loss of AR binding peaks and 40-50% gain of new AR genomic binding sites. We analyzed the top gained AR binding peaks upon TRAF4 overexpression ($\log_2$ TRAF4/vector >1) and found that majority of them are located 5-500kb away from transcriptional start sites (Fig.S7A), suggesting that these new binding sites are not resided in proximal promoter regions but are instead likely located at enhancer regions. The top transcription factor binding motifs among these new sites are not ARE but Forhead domain family and

homeodomain family transcription factor binding motifs (Fig.6B). FOXA1 and HOXB13 are known pioneer factors that co-localize with AR and facilitate AR cistrome reprogramming in prostate tumors [41-43]. Although shared AR binding peaks between control and TRAF4 stable cells contain significant numbers of FOXA1 binding motifs, we noticed that even more FOXA1 binding sites were co-localized with AR binding sites in TRAF4 stable cells compared to the control cells (Fig. 6C and Fig.S7B). A similar result was also found for the HOXB13 binding sites (Fig. S7C). These results suggest that TRAF4 overexpression alters AR genomic binding profile and increases AR binding to FOXA1 and HOXB13 binding sites.

FOXA1 protein was previously reported to physically interact with AR [44]. To examine whether TRAF4 overexpression could regulate the interaction between endogenous AR and FOXA1, we performed a co-IP experiment using an AR-specific antibody in LNCaP cells infected with GFP or TRAF4 expressing adenovirus. As shown in Fig.6D, more FOXA1 was associated with AR when TRAF4 was overexpressed, suggesting that TRAF4 increased the AR-FOXA1 interaction. We next determined whether TRAF4-mediated AR ubiquitination is important for TRAF4-promoted AR-FOXA1 interaction. A flag-tagged AR wild type or AR K911/K913R mutant expressing LNCaP stable cell line was generated. Cells were treated with siRNA against AR to minimize the effect of endogenous AR followed by GFP or TRAF4 expressing adenovirus infection. A co-IP experiment was then performed using a flag-specific antibody. As shown in Fig.6E, TRAF4 overexpression significantly increased the association of FOXA1 with flag-AR WT but not flag-AR K911/913R mutant, suggesting that AR ubiquitination is important for TRAF4-enhanced AR-FOXA1 interaction.

TRAF4 promotes AR binding to olfactory receptors and regulates cAMP signaling

To understand how alteration of AR genomic binding profile affects androgen-independent cell growth, we performed KEGG pathway analysis on top gained AR binding sites in TRAF4 stable

cells (log_2 TRAF4/vector >2). Interestingly, olfactory receptor (OR) transduction is the most enriched pathway (Fig.7A). ORs are the largest family of G-protein coupled receptors which stimulate intracellular cAMP signaling upon activation [45-47]. In addition to olfactory epithelium, ORs have been reported to express in a number of non-olfactory tissues and cancerous tissues including prostate cancer [48-53]. Ectopically overexpression of ORs promotes prostate cancer progression, metastasis and neuroendocrine differentiation [54-57].

To determine whether TRAF4 can regulate OR expression via AR, we first performed a ChIP experiment to validate the binding of AR to the enhancer/promoter region of several OR genes in control or TRAF4 overexpressing LNCaP cells. As shown in Fig.7B, strong AR binding to these OR gene enhancer/promoter regions was observed in TRAF4 cells but not in control cells, confirming that TRAF4 overexpression indeed increased AR binding to these regions.

We next performed a qPCR analysis to examine whether transcription of these OR genes are indeed altered in TRAF4 overexpressing cells. Significantly higher expression levels of these genes were found in TRAF4 stable cells compared to those in control cells (Fig.7C). Importantly, knockdown of AR reduced OR gene expression levels in TRAF4 cells (Fig.7D). These results suggest that TRAF4-promoted AR binding to OR gene enhancer region upregulates these gene transcription.

To investigate whether upregulation of these OR gene expressions has any functional impact to the cells, we examined the levels of intracellular cAMP in control or TRAF4 cells with or without OR agonist β-ionone stimulation. We found that β-ionone treatment did not have significant impact on cAMP levels in control cells (Fig.7E). This is likely due to the low expression levels of ORs in LNCaP cells. However, it dramatically increased the cAMP levels in TRAF4 stable cells. These results confirm that TRAF4 overexpression upregulates OR gene expression and imply a role of TRAF4 in regulating intracellular cAMP signaling.

Discussion

AR remains active and alters its transcriptional program in CRPCs [2-6]. Understanding the event triggering the switch of AR-regulated transcriptional profile under castration condition is critical for effective treating CRPCs. Here we demonstrate that TRAF4-mediated atypical AR ubiquitination plays an important role in switching AR genomic binding profile.

TRAF4 is frequently overexpressed in advanced prostate cancers [58]. We demonstrate that its overexpression converts androgen-sensitive prostate cancer cells into castration-resistant cells both in vitro and in vivo (Figure 1). Importantly, the E3 ubiquitin ligase activity of TRAF4 is required for this function. Ubiquitination is a reversible post-translational modification. Different ubiquitin linkages (K6, K11, K27, K29, K33, K48 and K63) formed at the target protein could have distinct structural conformations and serve as an ubiquitin code for interacting with different readers, leading to different outcomes of ubiquitinated proteins [59]. We report here that TRAF4 promotes AR ubiquitination primarily through atypical K27-linkage. Unlike K11 and K48-linked ubiquitination, which mainly functions as a protein degradation signal [60], we found that TRAF4-mediated AR ubiquitination does not promote AR turnover. Instead, it dramatically increases the association of AR with pioneer factor FoxA1. Consequently, ubiquitinated AR binds to a number of new genomic locus that are enriched with FoxA1 or HoxB13 binding motifs. These results indicate a role of ubiquitination as a switch to alter AR genomic binding profile.

Our pathway analysis on top new genomic binding sites occupied by AR upon TRAF4 overexpression surprisingly reveals olfactory transduction pathway. In another independent non-stringent pathway analysis we also found that cAMP pathway, a downstream signaling stimulated by olfactory receptor, as the second most-enriched pathway bound by AR. Olfactory receptors are expressed in a number of human tissues outside of nasal epithelium. Its overexpression has been linked to tumor growth, invasion, metastasis, inflammation and

apoptosis. We found that TRAF4 promotes AR binding to several OR gene enhancers and upregulates their expression levels in prostate cancer cells. Consistent with OR gene upregulation, TRAF4 overexpressing cells also have higher levels of intracellular cAMP and respond to OR agonist stimulation in contrast to control cells.

Although multiple mechanisms are involved in castration-resistant prostate cancer, E2F pathway has emerged as a central pathway in this process [5, 19, 20, 24]. Disruption of RB/E2F axis was also frequently observed in the transition to CRPCs [32]. Consistently, cell cycle proliferation gene signature predicts poor outcome of patients with prostate cancer [61]. We found that E2F activity is significantly augmented upon TRAF4 overexpression. This increase is dependent on AR. It is likely that under androgen deprivation condition TRAF4 overexpression promotes AR atypical ubiquitination and the ubiquitinated AR binds to a distinct genomic loci to induce different transcriptional programs including OR gene expressions. Consequently, it increases intracellular cAMP levels and activates E2F transcriptional activity. Ultimately, these pathway activations facilitate cell survival and proliferation, leading to CRPC.

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Figure legends

Fig 1: TRAF4 overexpression promotes CRPC development. (A) Overexpression of TRAF4 wild type but not its E3-ubiquitin ligase defective mutant (ΔRING) promotes androgen-independent cell growth. Upper panel, schematic representation of wild type TRAF4 and its mutant. Bottom panel, control or TRAF4 overexpressing LNCaP stable cell numbers in the absence or presence

of 10nM of synthetic androgen R1881 at day 1 and day 7. (B) TRAF4 overexpressing LNCaP xenograft tumors are castration-resistant. Bottom panel, time periods for xenograft tumor studies.

Fig 2: TRAF4 overexpression drives E2F pathway activation to promote CRPC. (A) E2F, Myc and Polycomb repressive group 2 are top pathways enriched in TRAF4 overexpressing xenograft tumors. Shown is RNA seq analysis in LNCaP xenograft tumors after castration. (B) TRAF4 increases E2F transcriptional activity. Shown is an E2F-driven luciferase reporter assay in cells transiently transfected with the luciferase reporter and a TRAF4 expressing plasmid. (C) Inhibition of E2F activation abolishes TRAF4-promoted androgen-independent growth. Control or TRAF4 overexpressing LNCaP cells were grown in androgen-deprived culture medium treated with Palbociclib or vehicle. MTS assay was then carried out to monitor the cell growth.

Fig 3: TRAF4 mediates atypical K27-linked AR polyubiquitination. (A) TRAF4 is highly expressed in AR-positive human PDXs compared to AR-negative PDXs. Shown is TRAF4 mRNA levels in prostate cancer cell line and human PDXs using real time qPCR. (B) AR is important in mediating the effect of TRAF4 on E2F activity. Shown is an E2F-driven luciferase reporter activity in the absence or presence of siRNA against AR. (C) TRAF4 interacts with AR in cells. Flag-tagged AR was transiently transfected into 293T cells in the absence or presence of HA-tagged TRAF4 co-transfection. A co-IP experiment was then carried out using an HA-specific antibody. (D) TRAF4 promotes AR in vitro ubiquitination. Purified recombinant AR was incubated with UBE1, Ubch5a, HA-tagged ubiquitin in the absence or presence of purified TRAF4 protein. An immunoprecipitation was then carried out using an AR-specific antibody followed by Western blot analysis using an HA-specific antibody to detect AR ubiquitination. (E) TRAF4 wild type but not its RING domain deletion mutant promotes AR ubiquitination in cells. Flag-tagged AR and HA-tagged ubiquitin, V5-tagged wild type TRAF4, RING domain deletion mutant or vector control were transiently transfected into 293T cells followed by

immunoprecipitation using a flag-specific antibody. The levels of AR ubiquitination were measured using an HA-specific antibody in a Western blot analysis. (F) TRAF4 mediated AR ubiquitination mainly occurs through the K27-linkage. K0 represents all lysine residues in ubiquitin were mutated. K6-K63 represents the ubiquitin mutant with all lysine mutations except the depicted number of lysine.

Fig 4: TRAF4 mediates AR ubiquitination at the K913 residue. (A) TRAF4 mainly targets AR LBD for ubiquitination. Upper panel, schematic representation of wild type, splicing variant or deletion mutant of AR. Bottom panel, the levels of AR or its mutant ubiquitination in cells. (B) Deletion of the last 53aa in AR abolished TRAF4-mediated ubiquitination. Bottom panel, the amino acid sequence of the last 53aa which contains 4 lysine residues. (C) K913R mutation abolished TRAF4-mediated AR ubiquitination.

Fig 5: TRAF4 promotes upregulation of selective AR-targeted CRPC-associated genes. (A) TRAF4 overexpression does not activate AR activity on classical AR targets under androgen-deprived condition. T1, low TRAF4 expressing LNCaP cells. T2, high TRAF4 expressing LNCaP cells. (B) TRAF4 overexpression up-regulates UBE2C gene expression. (C) AR depletion abolishes TRAF4-upregulated UBE2C expression. (D) TRAF4 depletion in CRPC cells decreases UBE2C expression. (E) Depletion of TRAF4 or UBE2C inhibits CRPC LNCaP-Abl cell growth.

Fig 6: TRAF4 overexpression alters AR genomic binding profile and increases its interaction with FOXA1. (A) Venn diagram and distribution of AR genomic binding peaks in TRAF4 overexpressing cells or control LNCaP cells. Cells were cultured in androgen-deprived medium for 2 days followed by ChIP using an AR-specific antibody. ChIP seq analysis was then performed. (B) Top gained AR binding peaks in TRAF4 overexpressing cells are enriched with forkhead domain family and homeodomain family binding motifs. (C) AR gained more binding at

the FOXA1 binding sites in TRAF4 overexpressing cells compared to control cells. (D) TRAF4 overexpression enhances the association between AR and FOXA1. LNCaP cells were infected with adenovirus expressing TRAF4 or GFP control. A co-IP experiment was then carried out using an AR-specific antibody followed by a Western blot analysis using a FOXA1-specific antibody to detect AR-associated FOXA1. (E) AR ubiquitination mutation abolished the TRAF4-promoted AR-FOXA1 interaction. Show is a co-IP experiment in flag-AR wild type or ubiquitination mutant expressing cells. Cells were treated with siAR to reduce the levels of endogenous AR.

Fig 7: TRAF4 promotes AR binding to OR enhancers and upregulates OR gene expression and intracellular cAMP levels. (A) Olfactory transduction is the top enriched pathway in AR gained genomic binding loci upon TRAF4 overexpression. Shown is the KEGG pathway analysis in top AR gained peaks (log_2 TRAF4/vector >2). (B) TRAF4 promotes AR binding to several OR gene enhancers. ChIP experiments were performed in TRAF4 overexpressing LNCaP cells or control cells using an AR-specific antibody to independently validate ChIP seq results. (C) TRAF4 overexpression upregulates several OR gene expression. Shown are the mRNA levels of ORs through real time qPCR analysis. (D) AR depletion significantly reduced OR gene expression. (E) TRAF4 overexpression significantly increases intracellular cAMP levels both in the absence or presence of OR agonist.

Supplementary figure legends:

Fig S1: TRAF4 protein level is higher in androgen-insensitive prostate cancer cells. Shown is a Western blot analysis using TRAF4-specific antibody. LNCaP-Abl is a LNCaP derivative grown in androgen-deprived culture medium.

Fig S2: K913 is an ubiquitination target site identified in mass spectrometry. Baculovirus expressed recombinant AR was purified and incubated with TRAF4, UBE1, UbcH5a for in vitro

ubiquitination assay followed by mass spectrometry to identify ubiquitination targeted sites. Shown is a coomassie blue staining and corresponding ubiquitination sites identified from each band.

Fig S3: TRAF4 overexpression does not alter AR protein turnover. Left panel, TRAF4 expression levels in TRAF4 high (T2) or low (T1) expressing LNCaP cells. Right panel, AR expression levels in TRAF4 high or low expressing cells.

Fig S4: TRAF4 upregulates CDC20 gene expression through AR. (A) TRAF4 overexpression up-regulates CDC20 gene expression. (B) AR depletion abolishes TRAF4-upregulated CDC20 expression. (C) TRAF4 depletion in CRPC cells decreases CDC20 expression. (D) Depletion of TRAF4 inhibits CRPC LNCaP-C4-2 cell growth.

Fig S5: TRAF4 depletion does not significantly change classical AR target expression. (A) The levels of AR (left panel) or TRAF4 (right panel) after siRNA treatment. (B) AR depletion but not TRAF4 depletion significantly reduced classical AR target expression levels.

Fig S6: TRAF4 depletion abolished AR binding to UBE2C enhancer (left panel) but not the PSA enhancer (right panel) in LNCaP-Abl cells. Shown is a ChIP experiment using an AR-specific antibody.

Fig S7: AR gained more FOXA1 and HOXB13 binding sites in TRAF4 overexpressing cells. (A) Distribution of gained AR binding peaks from transcription start site (TSS). (B) AR gained more FOXA1 binding sites in TRAF4 overexpressing cells in multiple datasets. (C) AR gained more HOXB13 binding sites in TRAF4 overexpressing cells.

Materials and Methods

Reagents and antibodies. Primary antibodies were as follows: anti-TRAF4 (catalog sc-10776), anti–HA-probe (catalog sc-805), anti-Ub (catalog sc-8017), anti-GAPDH (catalog sc-32233), anti-AR (catalog sc-816), anti-β-Actin (catalog 47778), from Santa Cruz Biotechnology Inc, Anti-FoxA1 (catalog 58613) was purchased from Cell Signaling technology. Anti-AR (catalog 39781) was purchased from active motif. HRP-conjugated secondary anti-mouse (catalog 1706516) or anti-rabbit (catalog 1706515) antibodies were obtained from Bio-Rad. Monoclonal ANTI-FLAG M2-peroxidase (HRP) antibody (catalog 8592A), EZview Red ANTI-FLAG M2 Affinity Gel (catalog F2426) were obtained from Sigma-Aldrich. TRAF4 adenovirus (catalog VH819961) was obtained from Vigene Biosciences. GFP adenovirus was produced in the Gene Vector Core at Baylor College of Medicine.

Cell lines. The human prostate cancer cell lines LNCaP, LNCaP-abl, LNCaP-C4-2, VCaP, 22Rv1 and HEK293T cells were obtained from ATCC. LNCaP, VCaP and 22Rv1 cells were maintained in RPMI 1640 medium containing 10% FBS, 2 mM l-glutamine, 100 µg/ml streptomycin, and 100 U/ml penicillin at 37°C and 5% CO2. LNCaP C4-2 cells were grown in DMEM-Ham's F-12 with 10% fetal bovine serum, 5 mg/ml insulin, 13.65 pg/ml triiodothyronine, 5 mg/ml apo-transferrin, 0.244 mg/ml d-biotin, and 25 mg/ml adenine. LNCaP Abl cells were grown in RPMI 1640 containing 5% charcoal stripped and 2 mM glutamax. The human embryonic kidney epithelial cell line HEK293T was maintained in DMEM supplemented with 10% FBS. For androgen-independent experiments, cells were maintained in culture medium with 5% charcoal stripped serum supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin for the stated periods of time.

Reverse transcription and quantitative real-time PCR. Total RNA was extracted from the indicated cells by using an RNeasy Mini Kit (QIAGEN). RNA concentration and purity were measured by a Nano-Drop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific). 2μg total RNA was used to generate cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Real-time PCR was performed using SYBR green PCR master mix (Life Technologies, Thermo Fisher Scientific). Primers used are listed in Supplemental Tables 1 and 2. For all RT-qPCR analysis, β-actin was used to normalize RNA input, and expression levels were calculated according to the comparative Ct method (ΔΔCT).

Construction of expression vectors and AR mutants. The AR cDNA was cloned into FLAG-tagged pSG5 expression vector. All AR deletion as well as lysine mutants were also cloned into FLAG-tagged pSG5 expression vector. In addition, TRAF4 was cloned into HA-tagged pCM5 expression vector. We also obtained vector control and TRAF4 cloned into lentiviral pLV vector (Vector Builder, Chicago, IL, USA). WT ubiquitin, and its mutant constructs were obtained from Addgene. TRAF4 shRNA was cloned into pLenti6/TR vector (Thermo Fisher Scientific). The E2F-responsive plasmid pE2F-TA-Luc was from CLONTECH Laboratories, Inc. (Palo Alto, CA). Primers used for cloning are listed in Supplemental Tables 1 and 2.

Transfection and lentivirus infection. Cells were transfected with plasmid DNA using Lipofectamine 3000 and siRNA using Lipofectamine RNAiMAX transfection reagent (both from Thermo Fisher Scientific) following the manufacturer's protocol. Virus packaging was performed in HEK293T cells after cotransfection of plasmid with the packaging plasmid psPAX2 and envelope plasmid pMD2.G using Lipofectamine 3000.

Viruses were harvested 48 hours after transfection, and viral titers were determined. Target cells were infected with recombinant lentivirus-transducing units in the presence of 8 μg/ml Polybrene (Sigma-Aldrich).

Immunoblotting. Cells were harvested and protein was extracted from cells as previously described (PMID 30057199). Cells were lysed by IP buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate and 1% protease inhibitor cocktails) on ice for more than 20 min. Cell lysate was centrifuged for 10 min at 13,000 rpm at 4°C, and the supernatant was quantified by BCA protein quantification assay. Equal amounts of protein sample were added into 4x sample buffer and boiled for 5 min. The sample was subjected to SDS-PAGE analysis and transferred to nitrocellulose membrane. The membrane was blocked by 5% milk for 1 h at room temperature and incubated with primary antibody at 4°C overnight. The next day, the membrane was washed three times with 1x TBST and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The protein bands were visualized by SuperSignal West Pico Stable Peroxide Solution (Thermo Fisher Scientific). Endogenous GAPDH or β-Actin was used as the internal control.

Cell proliferation (MTS) assay. The CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) reagent (catalog G358A) was obtained from Promega, and the assay was performed according to the manufacturer's instructions. Briefly, cells were seeded in a 96-well plate and treated with or without specified reagents for times indicated. The plate was incubated at 37°C in a humidified, 5% CO2 atmosphere. 20 μl CellTiter 96 AQueous One Solution Reagent was added to each well containing 100 μl

media and again incubated for 3 hours. Absorbance was measured at 490 nm using a microplate reader.

Co-immunoprecipitation (coIP) Cells were harvested and lysed by IP lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate and 1% protease inhibitor cocktails) on ice for more than 20 min. Cell lysate was centrifuged for 10 min at 13,000 rpm at 4°C, and supernatant was quantified by BCA protein quantification assay. The protein was then incubated with primary antibody and protein A/G agarose beads (Thermo Fisher Scientific) with rotating at 4°C overnight. The next day, the beads were washed at least three times with IP wash buffer on ice, and then subjected to western blotting analysis.

In vitro Ubiquitination assay. FLAG-AR was transiently transfected into 293T cells. The protein was then purified from 293T cell lysates using anti-FLAG M2 beads and eluted from the beads using 3X FLAG peptide (Sigma-Aldrich). The purified protein was incubated with 100ng UBE1, 150 ng UbcH5a, and 5 μg HA-ubiquitin (Boston Biochem) in the absence or presence of 500 ng TRAF4 (Novus Biologicals) with ubiquitination buffer (50 mM Tris-Cl, pH 7.4, 2 mM ATP, 5 mM MgCl2, 2 mM DTT) at 30°C for 90 minutes. The incubation mixture was then subjected to immunoprecipitation using an anti-FLAG antibody, followed by Western blot analysis using an anti-HA antibody.

Castration surgery and CRPC tumor study. All animal experiments were performed in accordance with the Institutional Animal Care and Use Committees (IACUCs) at Baylor College of Medicine. For in vivo studies, 5-to 6-week-old male SCID mice (The Jackson Laboratory) were used for experimental castrate resistant prostate cancer

study. TRAF4 overexpressing or control LNCaP cells were injected into NOD/SCID mice subcutaneously. When tumors reach approximately 1cm in diameter, mice were were anesthetized using isoflourane and surgically castrated using standard surgical technique.

E2F luciferase reporter assay Cells were co-transfected with 500ng of pE2F-TA-Luc plasmid, 100 ng of pRL-Renilla (encoding the Renilla luciferase gene for standardization), and empty expression vector or increasing amount of TRAF4 expression vectors. Cells were harvested 48 h after transfection and firefly luciferase activity was measured on a Synergy LX multimode reader (BioTek, USA) using a Dual Luciferase Reporter Assay (Promega) and according to the manufacturer's protocol. Firefly luciferase experimental reporter was normalized to Renilla.

RNA Seq Total RNA from tumor tissues was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. The quantity and integrity of the total RNA were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and 1% agarose gel electrophoresis. Equal amounts of total RNA from each tissue was used for library preparation. Only the total RNA samples with RIN values ≥6.8 were used for constructing the cDNA libraries. All libraries were sequenced using the Illumina Platform PE150 Q30≥80% with a paired-end read length of 150 bp. The library construction and sequencing were performed by the Novogene Corporation (Beijing, China).

ChIP assay ChIP assays were performed using the ChIP-IT Express Kit (Active Motif) as previously described (PMID 25489091). Briefly, cells were were cross-linked with 1%

formaldehyde and lysed to release chromatin. The chromatin was sonicated, quantified, and was incubated with specific antibody or normal rabbit IgG (nonspecific antibody control) overnight at 4°C. The immune complexes were precipitated with ChIP-IT protein G magnetic beads, followed by extensive washing as recommended by the manufacturer. The chromatin–protein–antibody complexes were eluted, and the DNA–protein cross-links were reversed; then the chromatin DNA pulled down by the antibody was purified with the chromatin IP DNA purification kit (Active Motif).The specific protein-binding genomic DNA sequences of the genes of interest were detected by real-time PCR using SYBR green PCR master mix (Life Technologies, Thermo Fisher Scientific). The sequences of the qPCR primer sets used in our experiments are shown in Table S2. The abundance of the detected DNA (relative concentration) was calculated and normalized to each of its total input (before immunoprecipitation) amounts, respectively or as fold enrichment. The normalized relative DNA concentration in each sample was expressed as the fold change over its respective control. Each experiment was repeated at least three times, and the results were analyzed for statistical significance using the paired-sample t-test. The differences between samples with or without treatment were considered significant if the P value was less than 0.05.

cAMP assay cAMP levels were determined by cAMP enzyme immunoassay kit following the manufacturer's instructions (Cayman Chemical Co., Ann Arbor, MI, USA, Item no. 581001). Intracellular cAMP was determined in LNCaP cells with and without TRAF4 overexpression using acid extraction method. Briefly, cells were plated on a 6-well plate, left over night and exposed with different concentrations of β-ionone for 15 minutes. After treatment, the medium was removed and 300 µl of 0.1 M HCl was added.

These plates were then incubated at room temperature for 20 minutes, cells were directly scraped off, transferred into a fresh tube and centrifuged. Fifty microliter of this extract supernatant were measured in the cAMP ELISA.

Statistics. Unless otherwise indicated, all results represent mean ±SEM, and statistical comparisons between different groups were performed using the 2-tailed Student's t test or 1-way ANOVA with multiple comparisons corrections. For all statistical analyses, differences of $P < 0.05$ were considered statistically significant, and experiments were repeated at least 3 times. GraphPad Prism software version 4.0/7.0 (GraphPad Software) was used for data analysis.

Study approval. All animal experiments were approved by the Institutional Animal Care and Use Committees (IACUCs) at Baylor College of Medicine. Human tissue samples were obtained from the Human Tissue Acquisition and Pathology Core of the Dan L. Duncan Comprehensive Cancer Center and were collected from fresh radical prostatectomy specimens after obtaining written informed consent under an Institutional Review Board–approved protocol.

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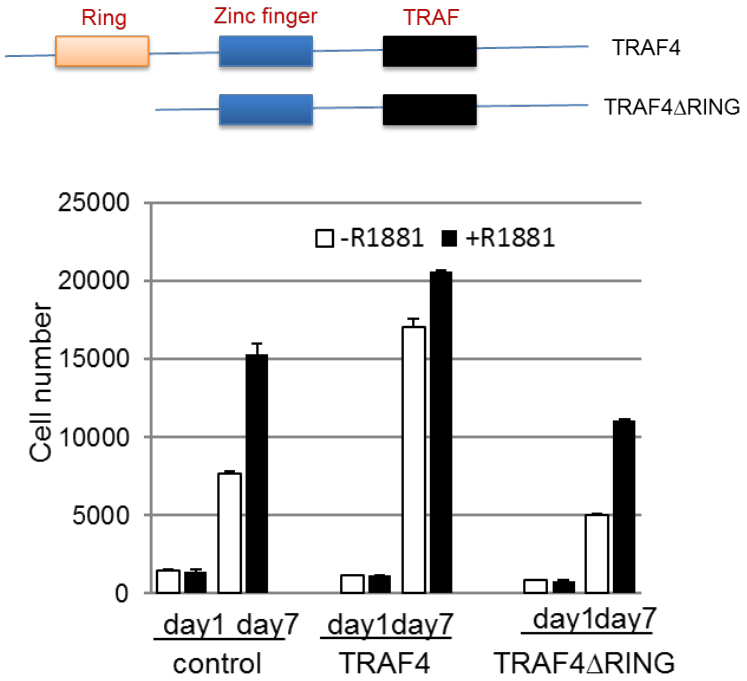
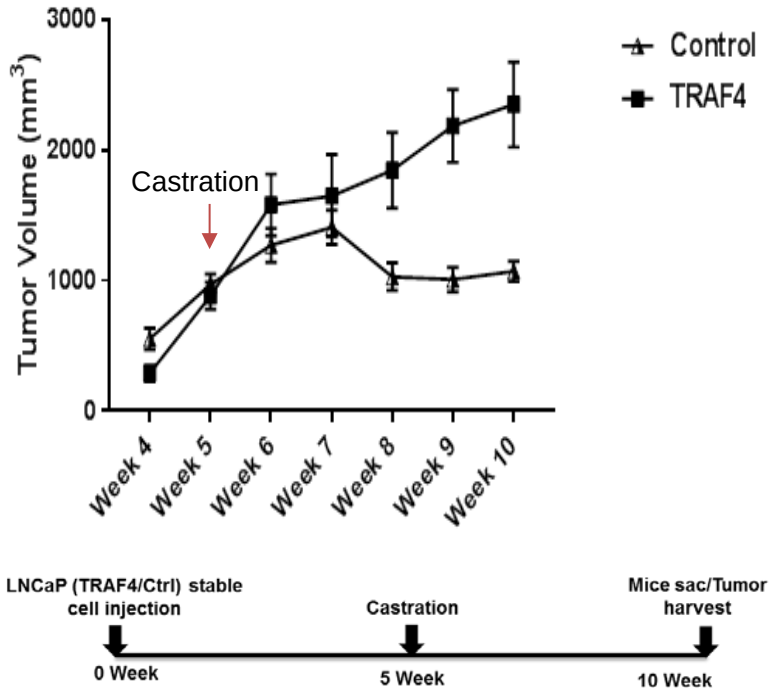
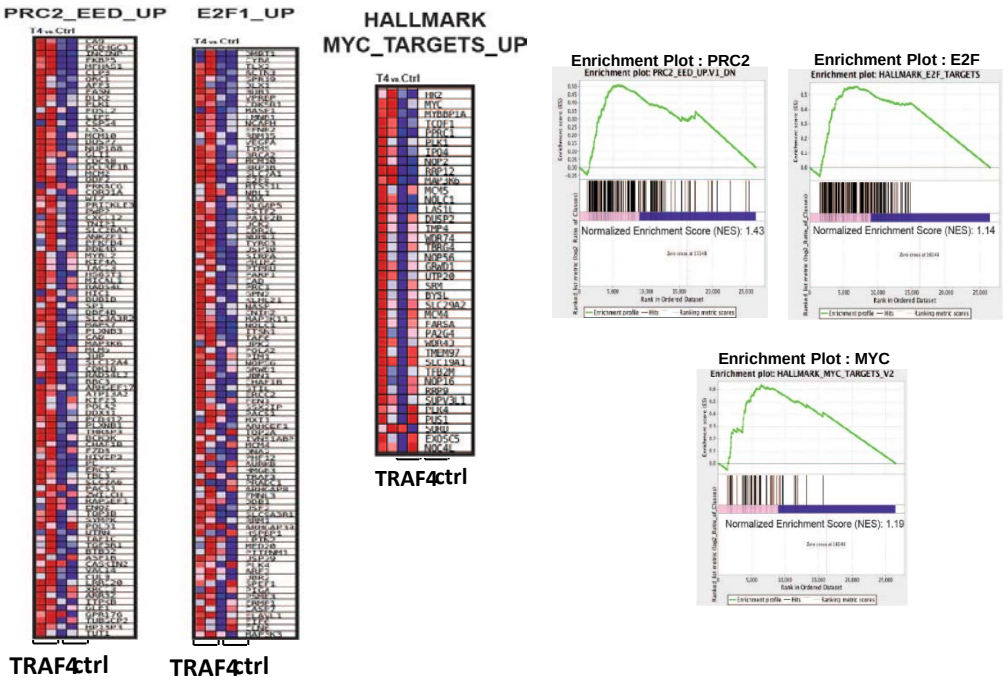
A.


Ring
Zinc finger
TRAF
TRAF4
TRAF4ΔRING
25000
20000
15000
10000
5000
0
Cell number
-R1881
+R1881
day1 day7
control
day1day7
TRAF4
day1day7
TRAF4ΔRING

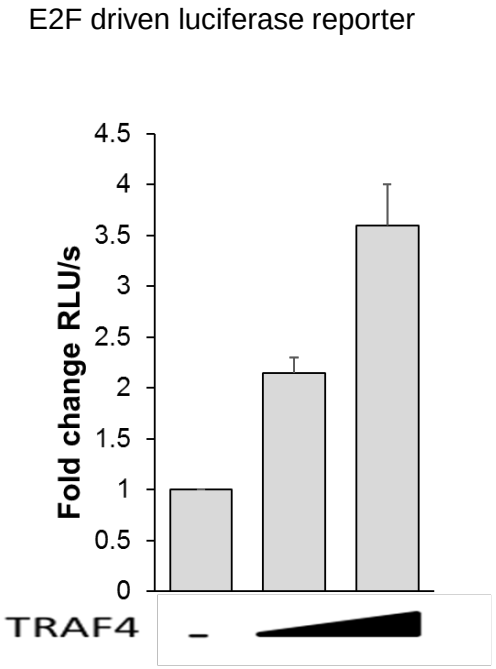
B.


3000
2000
1000
0
Tumor Volume (mm3)
Control
TRAF4
Castration
Week 4
Week 5
Week 6
Week 7
Week 8
Week 9
Week 10
LNCaP (TRAF4/Ctrl) stable cell injection
Castration
Mice sac/Tumor harvest
0 Week
5 Week
10 Week

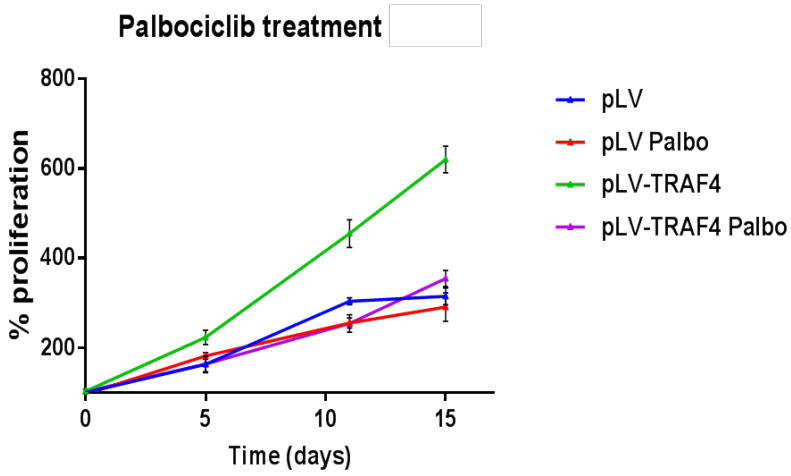
A.

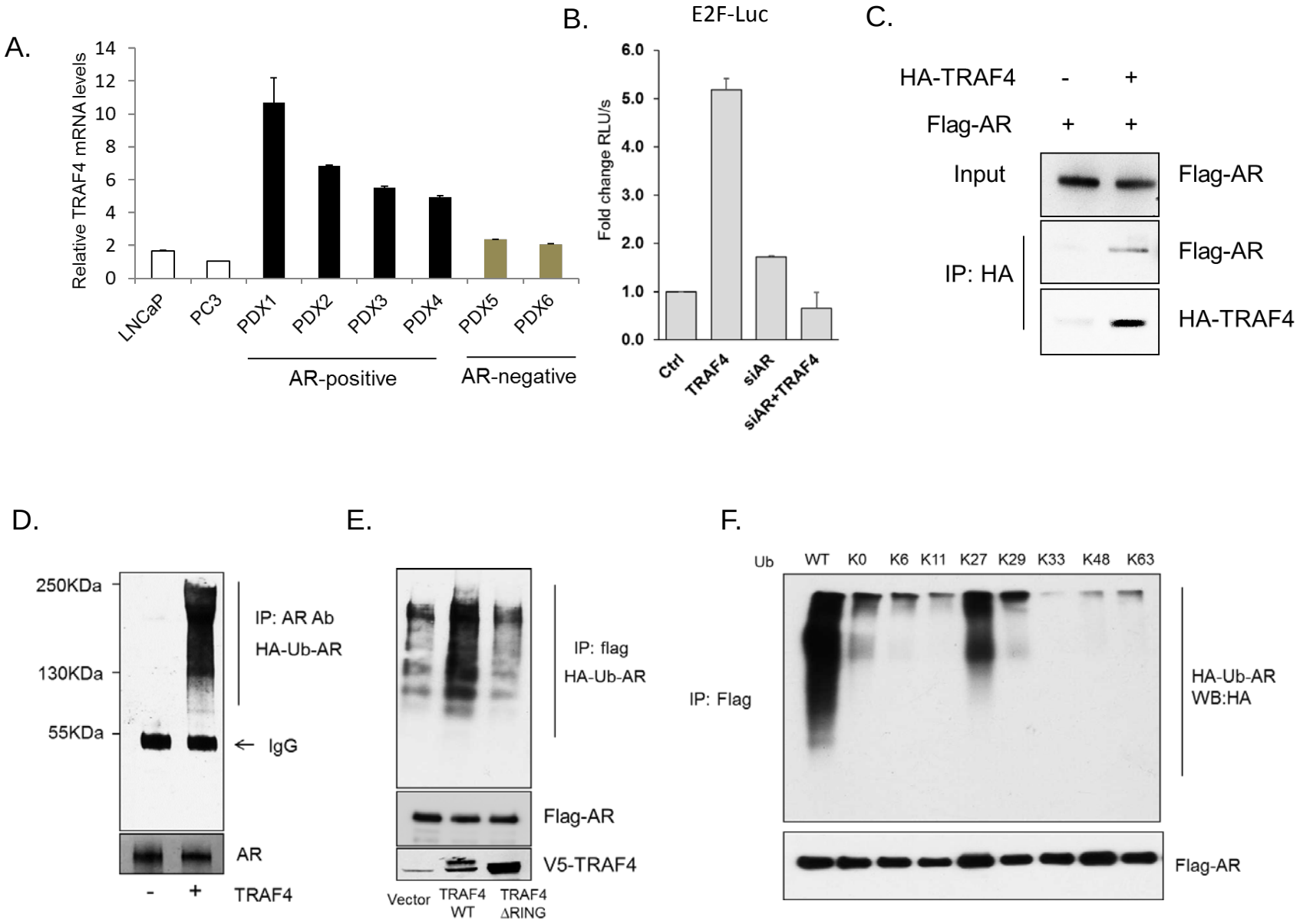

PRC2_EED_UP
E2F1_UP
HALLMARK
MYC_TARGETS_UP
T4 vs Ctrl
TRAF4
ctrl
TRAF4
ctrl
TRAF4
ctrl
Enrichment Plot : PRC2
Enrichment plot: PRC2_EED_UP.V1_DN
Normalized Enrichment Score (NES): 1.43
Enrichment Plot : E2F
Enrichment plot: HALLMARK_E2F_TARGETS
Normalized Enrichment Score (NES): 1.14
Enrichment Plot : MYC
Enrichment plot: HALLMARK_MYC_TARGETS_V2
Normalized Enrichment Score (NES): 1.19

B.

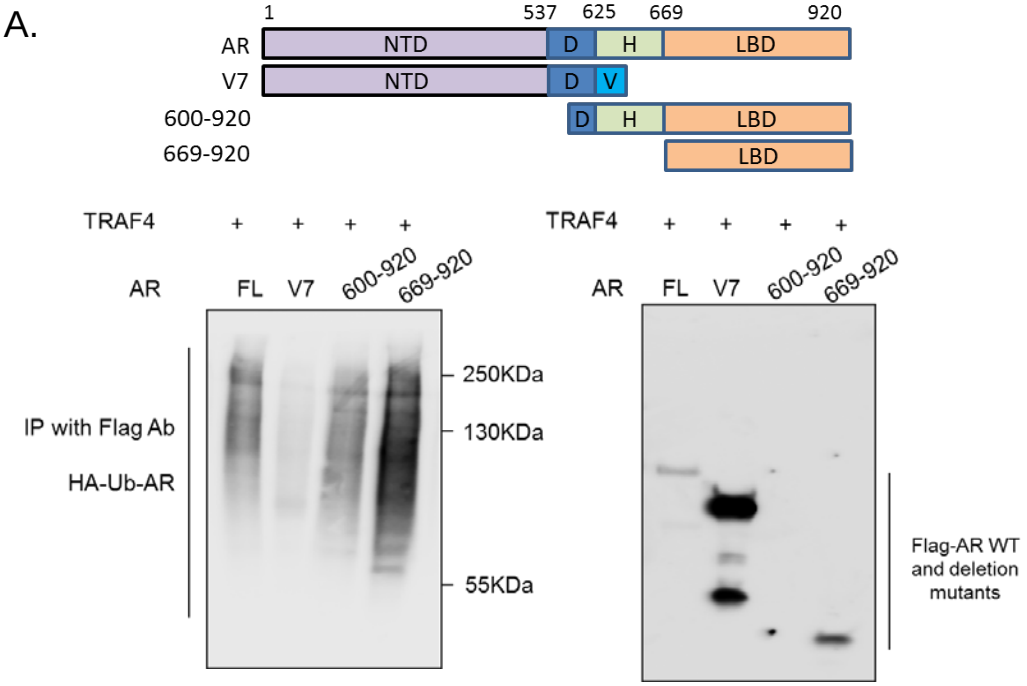

E2F driven luciferase reporter
Fold change RLU/s
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
TRAF4
–
+

C.

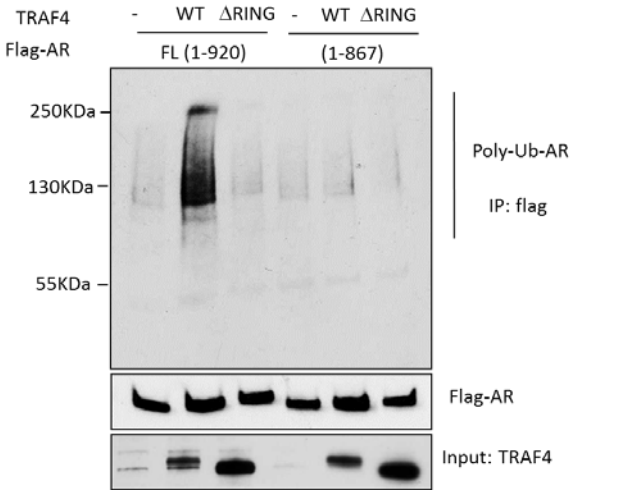

Palbociclib treatment
% proliferation
800
600
400
200
0
5
10
15
Time (days)
pLV
pLV Palbo
pLV-TRAF4
pLV-TRAF4 Palbo


A.
Relative TRAF4 mRNA levels
14
12
10
8
6
4
2
0
LNCaP
PC3
PDX1
PDX2
PDX3
PDX4
PDX5
PDX6
AR-positive
AR-negative
B.
E2F-Luc
Fold change RLU/s
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Ctrl
TRAF4
siAR
siAR+TRAF4
C.
HA-TRAF4 - +
Flag-AR + +
Input
Flag-AR
IP: HA
Flag-AR
HA-TRAF4
D.
250KDa
130KDa
55KDa
IP: AR Ab
HA-Ub-AR
← IgG
AR
- +
TRAF4
E.
IP: flag
HA-Ub-AR
Flag-AR
V5-TRAF4
Vector
TRAF4 WT
TRAF4 ΔRING
F.
Ub
WT
K0
K6
K11
K27
K29
K33
K48
K63
IP: Flag
HA-Ub-AR
WB:HA
Flag-AR

A.



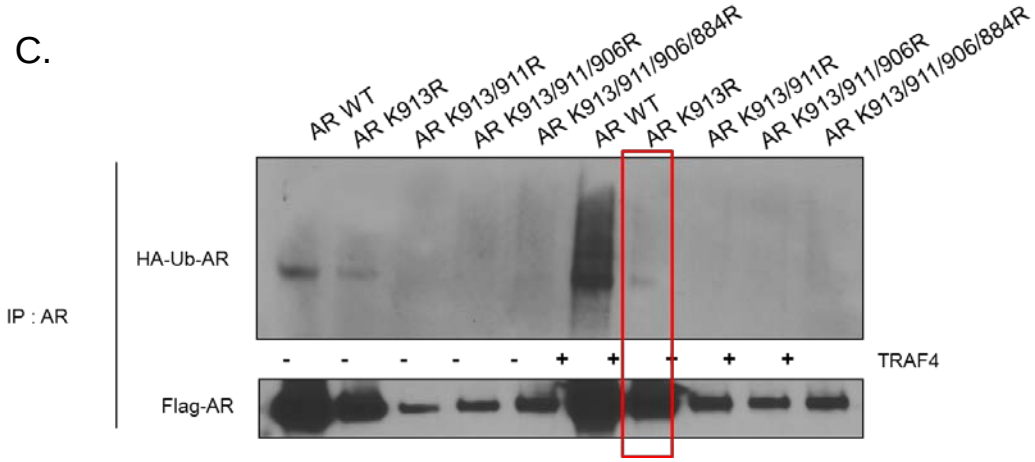
B.



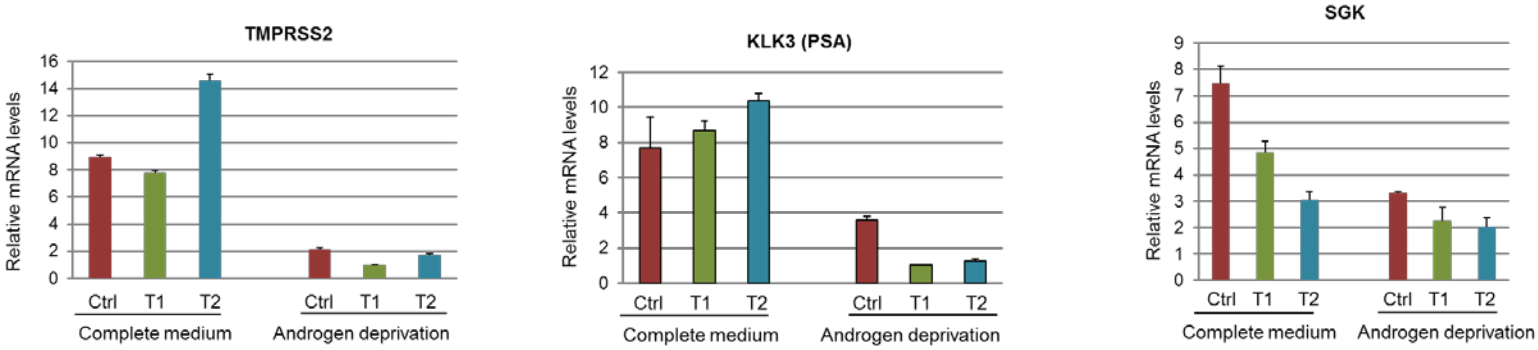
884 906 911 913

QPIARELHQFTFDLLIKSHMVSVDFPEMMAEIISVQVPKILSGKVKPIYFHTQ

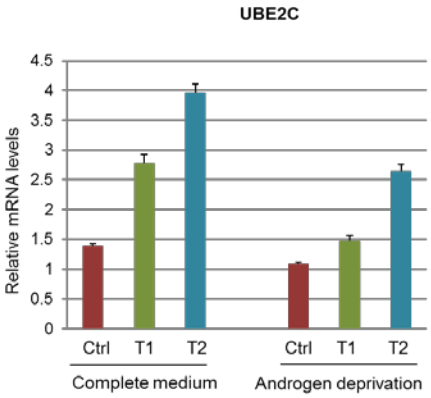
C.



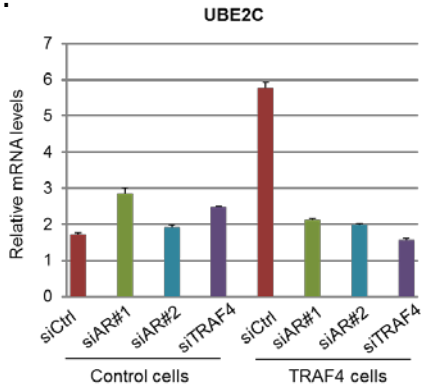
A.


TMPRSS2
Relative mRNA levels
Ctrl T1 T2 Ctrl T1 T2
Complete medium
Androgen deprivation
KLK3 (PSA)
Relative mRNA levels
Ctrl T1 T2 Ctrl T1 T2
Complete medium
Androgen deprivation
SGK
Relative mRNA levels
Ctrl T1 T2 Ctrl T1 T2
Complete medium
Androgen deprivation

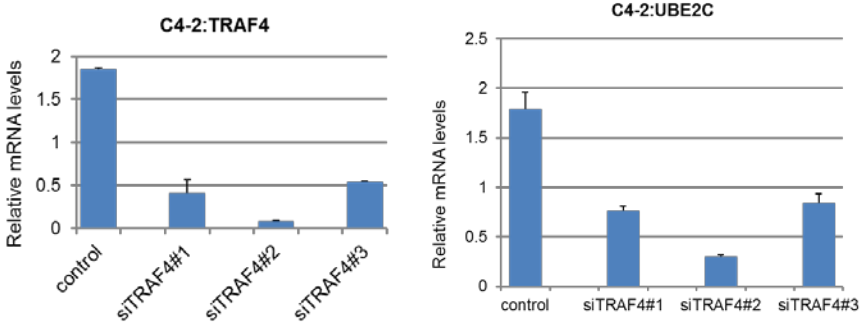
B.


UBE2C
Relative mRNA levels
Ctrl T1 T2 Ctrl T1 T2
Complete medium
Androgen deprivation

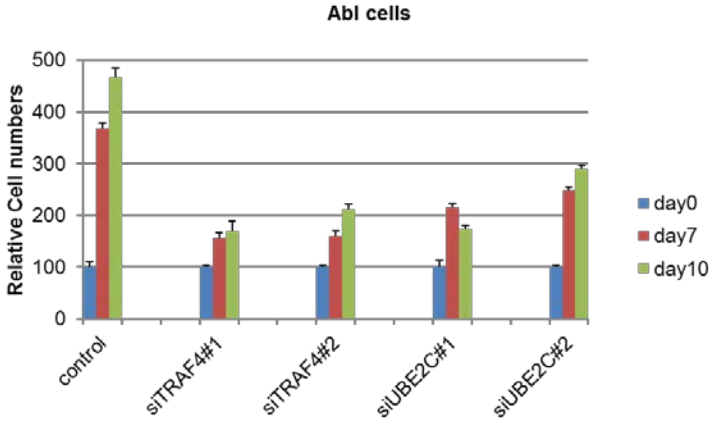
C.


UBE2C
Relative mRNA levels
siCtrl siAR#1 siAR#2 siTRAF4 siCtrl siAR#1 siAR#2 siTRAF4
Control cells
TRAF4 cells

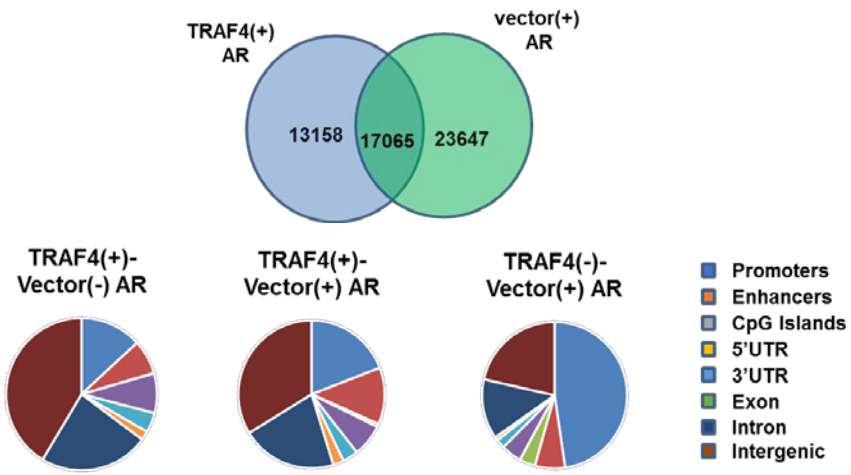
D.


C4-2:TRAF4
Relative mRNA levels
control siTRAF4#1 siTRAF4#2 siTRAF4#3
C4-2:UBE2C
Relative mRNA levels
control siTRAF4#1 siTRAF4#2 siTRAF4#3

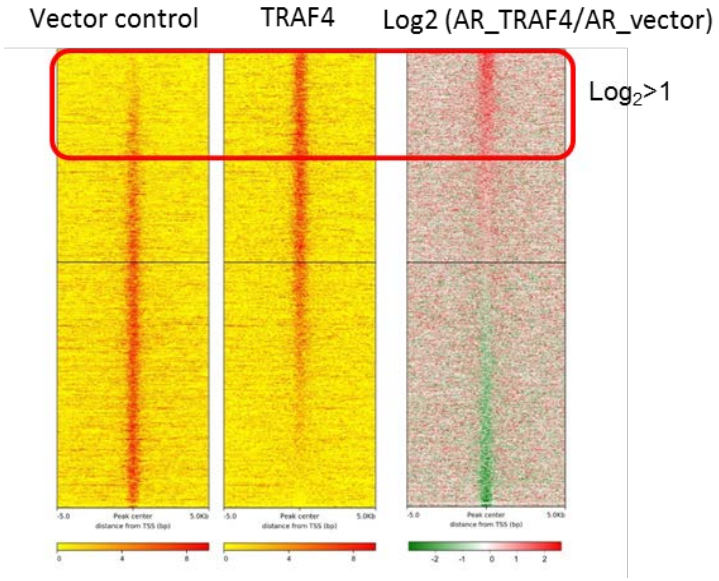
E


Abl cells
Relative Cell numbers
control siTRAF4#1 siTRAF4#2 siUBE2C#1 siUBE2C#2
day0
day7
day10

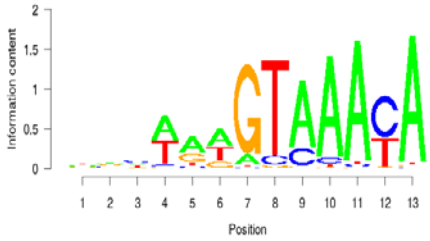
A.



B

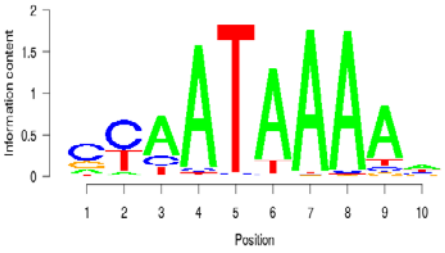


Forkhead domain family



Z score=-21.738

Homeodomain family



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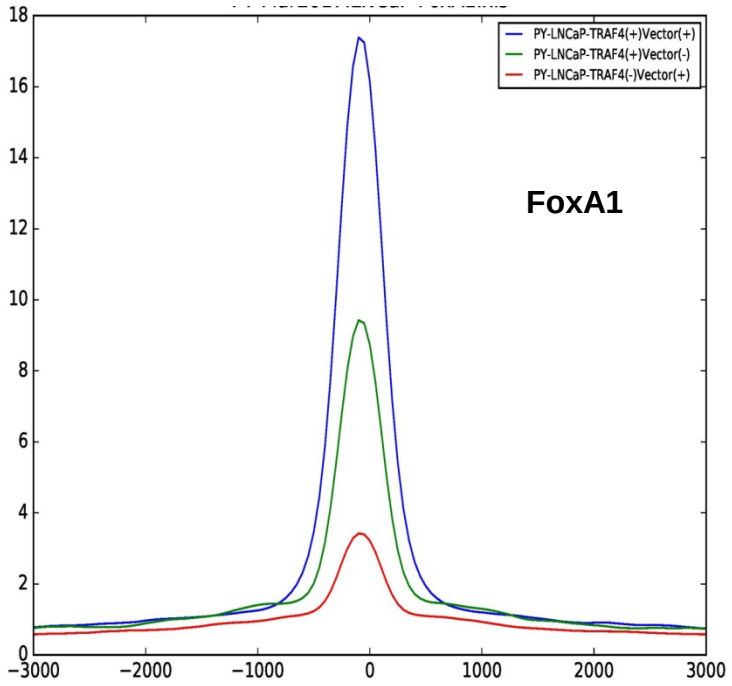
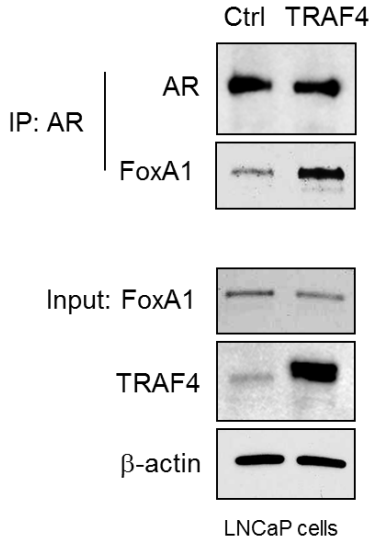
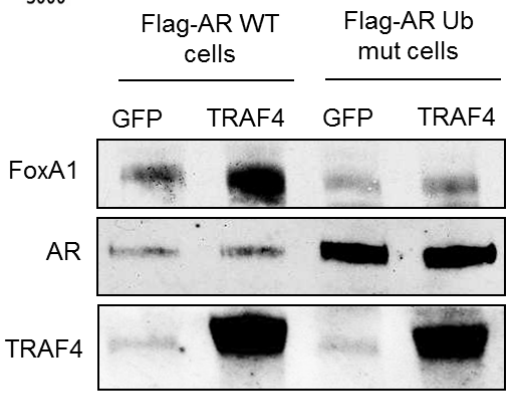
C

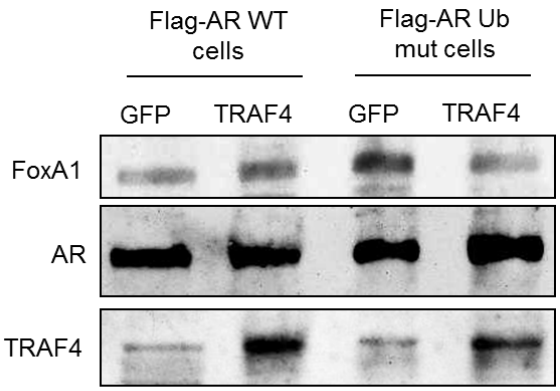

PY-LNCaP-TRAF4(+)Vector(+)
PY-LNCaP-TRAF4(+)Vector(-)
PY-LNCaP-TRAF4(-)Vector(+)
FoxA1
18
16
14
12
10
8
6
4
2
0
−3000
−2000
−1000
0
1000
2000
3000
Distance from Peak Center
TRAF4(+)Vector(+) AR
TRAF4(+)Vector(-) AR
TRAF4(-)Vector(+) AR

D.

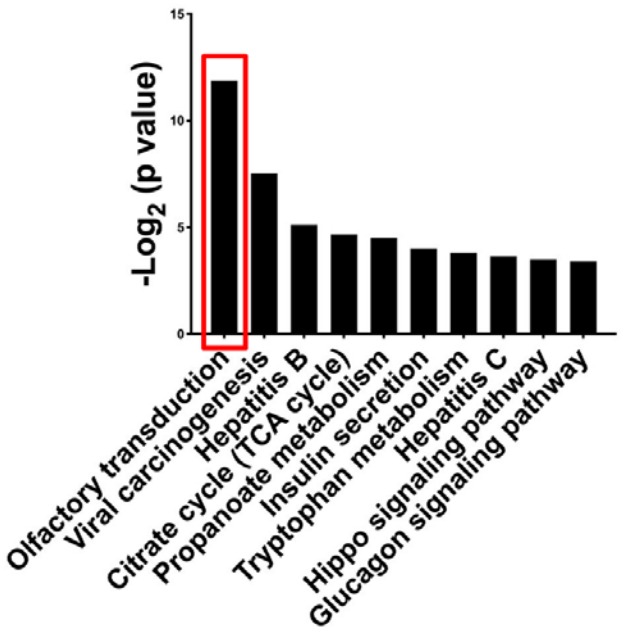

Ctrl
TRAF4
IP: AR
AR
FoxA1
Input: FoxA1
TRAF4
β-actin
LNCaP cells

E.

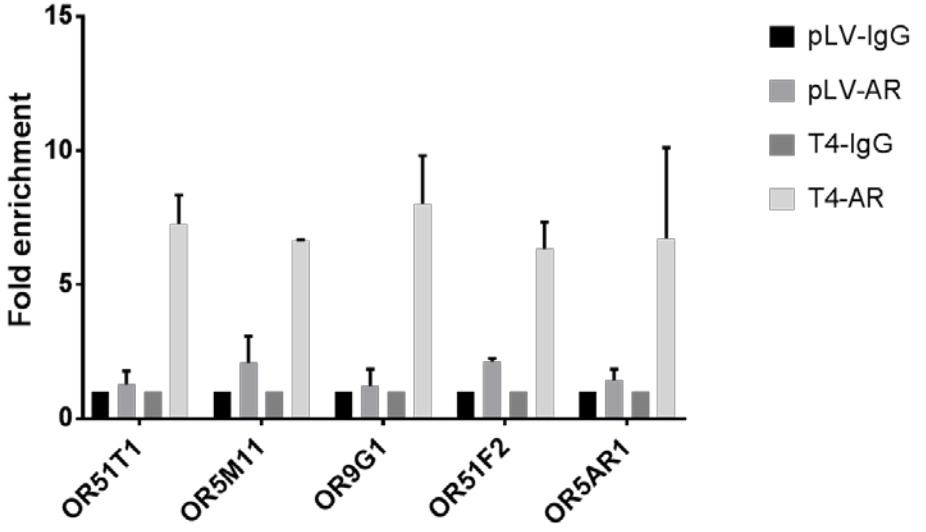

Flag-AR WT cells
Flag-AR Ub mut cells
GFP
TRAF4
GFP
TRAF4
FoxA1
AR
TRAF4
IP: flag


Flag-AR WT cells
Flag-AR Ub mut cells
GFP
TRAF4
GFP
TRAF4
FoxA1
AR
TRAF4
Input

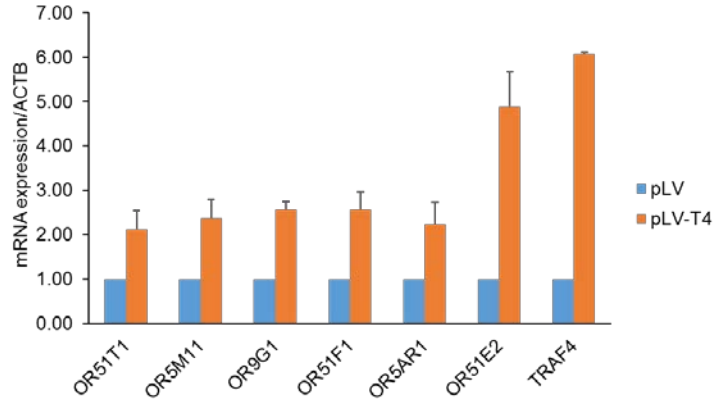
A.


-Log2 (p value)
15
10
5
0
Olfactory transduction
Viral carcinogenesis
Hepatitis B
Citrate cycle (TCA cycle)
Propanoate metabolism
Insulin secretion
Tryptophan metabolism
Hepatitis C
Hippo signaling pathway
Glucagon signaling pathway

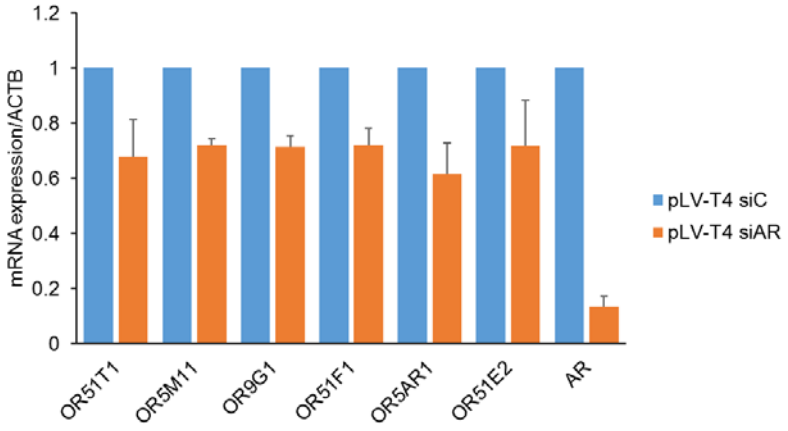
B.


Fold enrichment
15
10
5
0
OR51T1
OR5M11
OR9G1
OR51F2
OR5AR1
pLV-IgG
pLV-AR
T4-IgG
T4-AR

C.


7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
mRNA expression/ACTB
pLV
pLV-T4
OR51T1
OR5M11
OR9G1
OR51F1
OR5AR1
OR51E2
TRAF4

D.


1.2
1
0.8
0.6
0.4
0.2
0
mRNA expression/ACTB
pLV-T4 siC
pLV-T4 siAR
OR51T1
OR5M11
OR9G1
OR51F1
OR5AR1
OR51E2
AR

E.

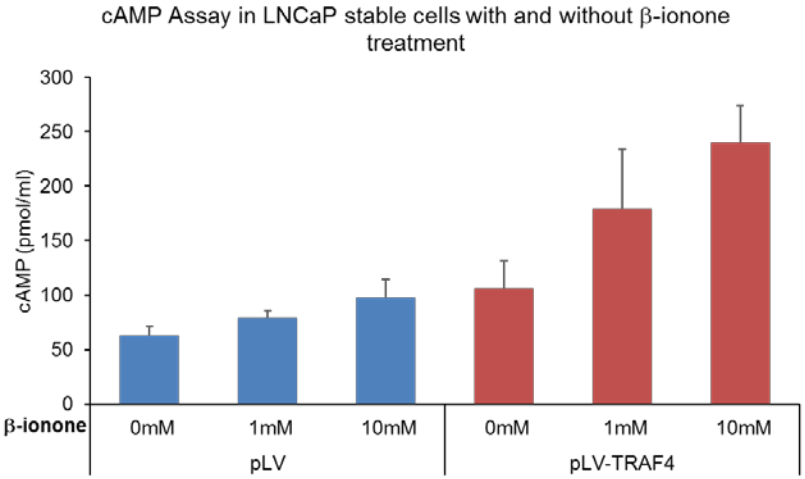

cAMP Assay in LNCaP stable cells with and without β-ionone treatment
300
250
200
150
100
50
0
cAMP (pmol/ml)
β-ionone
0mM
1mM
10mM
0mM
1mM
10mM
pLV
pLV-TRAF4

Fig S1

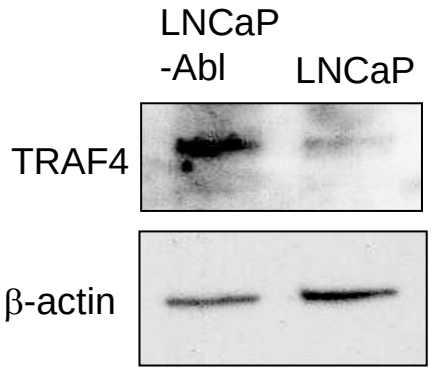
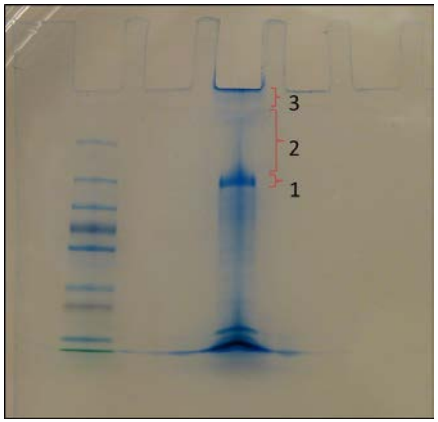


Fig S2



1-K182
2-K301, K388, K778, K913
3-K222, K301, K823

Fig S3

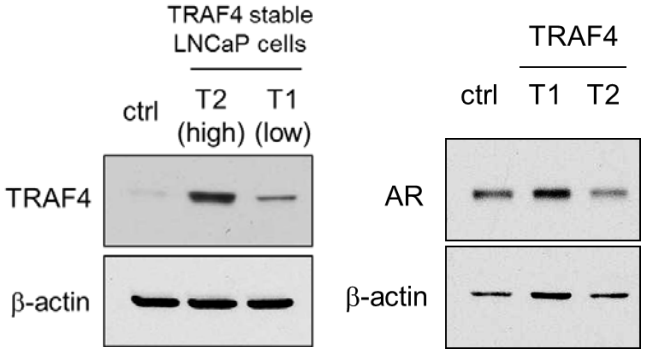
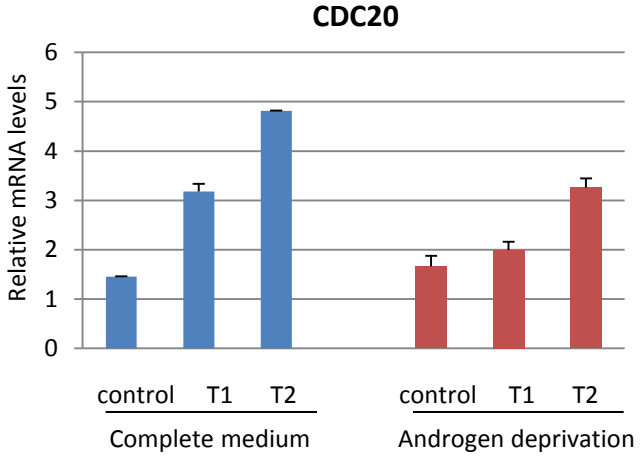
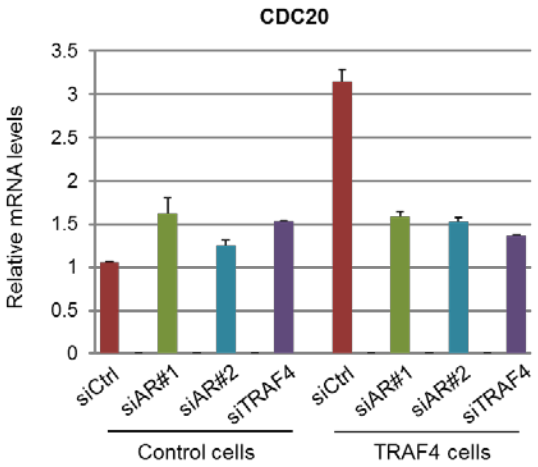


Fig S4

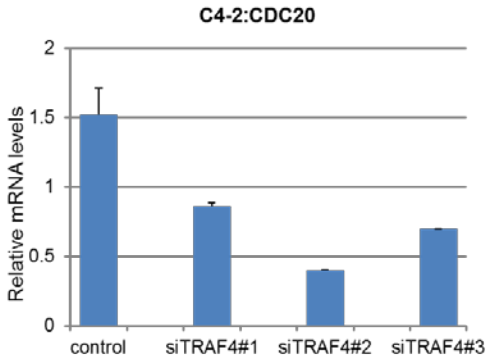
A


CDC20
Relative mRNA levels
6
5
4
3
2
1
0
control
T1
T2
control
T1
T2
Complete medium
Androgen deprivation

B


CDC20
Relative mRNA levels
3.5
3
2.5
2
1.5
1
0.5
0
siCtrl
siAR#1
siAR#2
siTRAF4
siCtrl
siAR#1
siAR#2
siTRAF4
Control cells
TRAF4 cells

C


C4-2:CDC20
Relative mRNA levels
2
1.5
1
0.5
0
control
siTRAF4#1
siTRAF4#2
siTRAF4#3

D

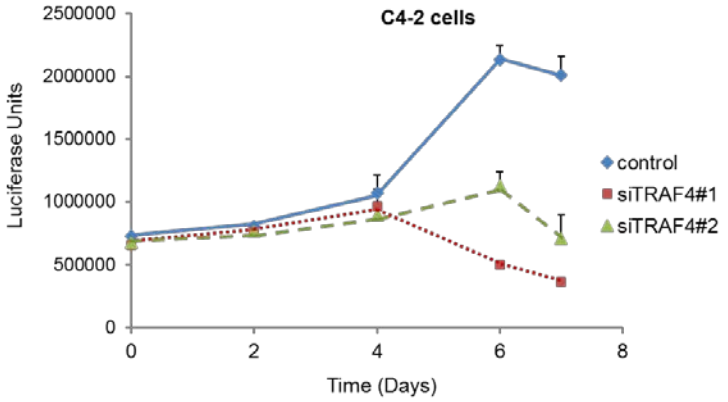
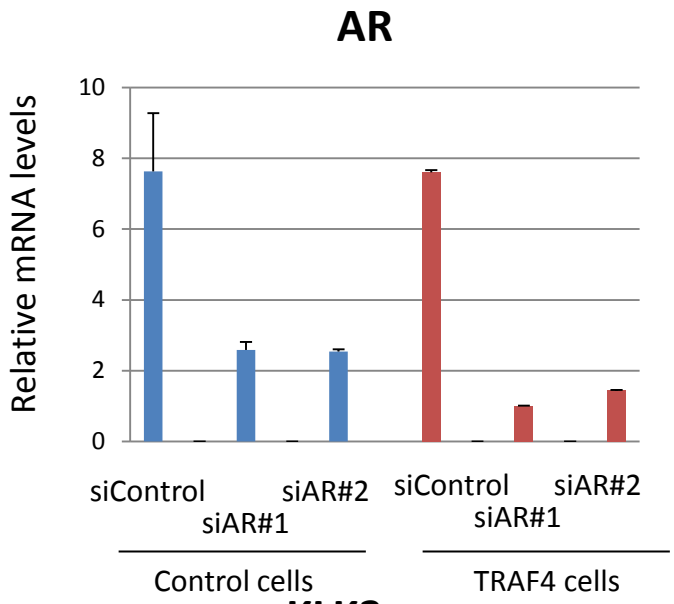
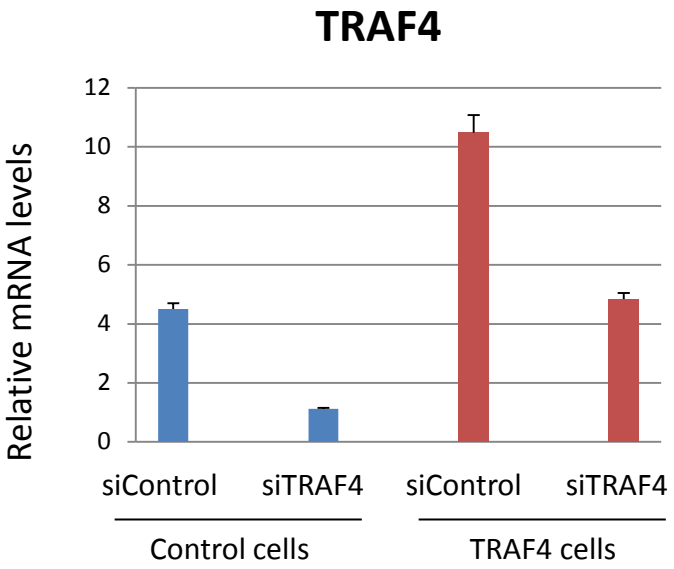

C4-2 cells
Luciferase Units
2500000
2000000
1500000
1000000
500000
0
0
2
4
6
8
Time (Days)
control
siTRAF4#1
siTRAF4#2

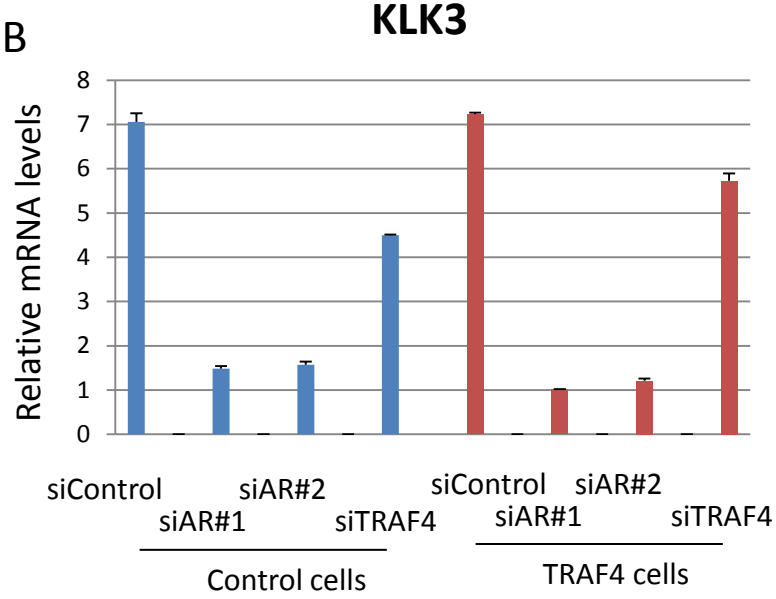
Fig S5

A


AR
Relative mRNA levels
10
8
6
4
2
0
siControl
siAR#1
siAR#2
siControl
siAR#1
siAR#2
Control cells
TRAF4 cells


TRAF4
Relative mRNA levels
12
10
8
6
4
2
0
siControl
siTRAF4
siControl
siTRAF4
Control cells
TRAF4 cells

B


KLK3
Relative mRNA levels
8
7
6
5
4
3
2
1
0
siControl
siAR#1
siAR#2
siTRAF4
siControl
siAR#1
siAR#2
siTRAF4
Control cells
TRAF4 cells

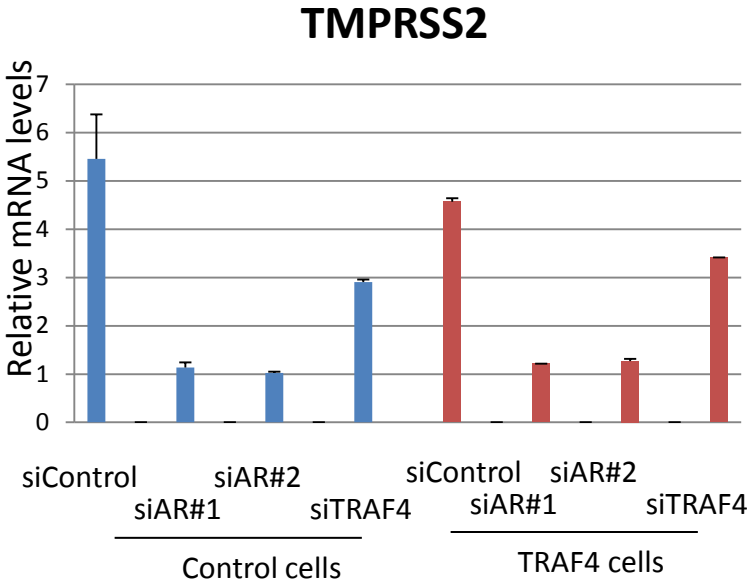
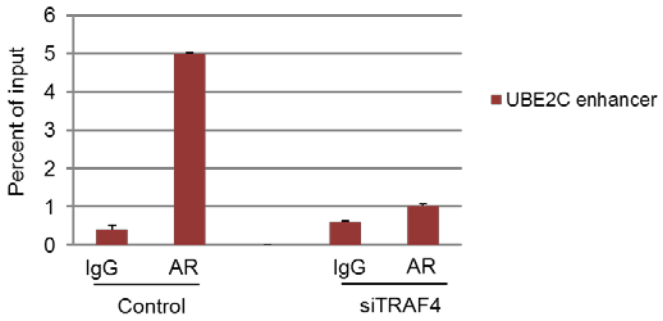

TMPRSS2
Relative mRNA levels
7
6
5
4
3
2
1
0
siControl
siAR#1
siAR#2
siTRAF4
siControl
siAR#1
siAR#2
siTRAF4
Control cells
TRAF4 cells

Fig S6

A.


Percent of input
6
5
4
3
2
1
0
IgG
AR
IgG
AR
Control
siTRAF4
UBE2C enhancer

B.

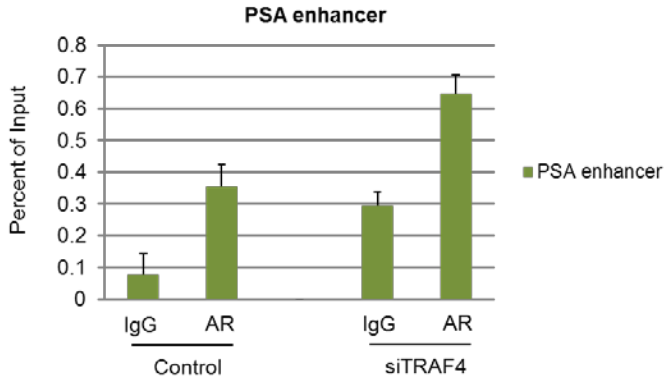
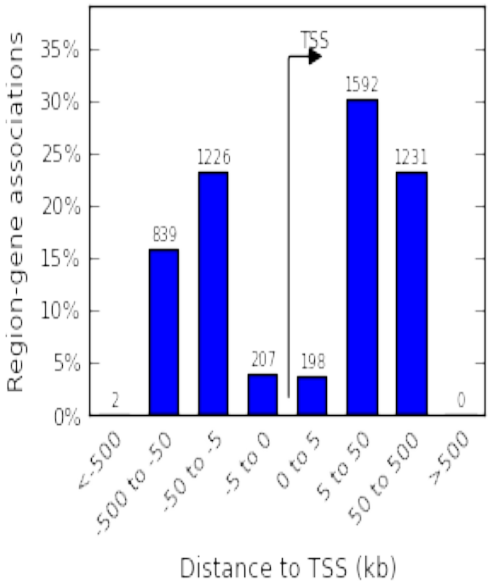

PSA enhancer
Percent of Input
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
IgG
AR
IgG
AR
Control
siTRAF4
PSA enhancer

Fig S7

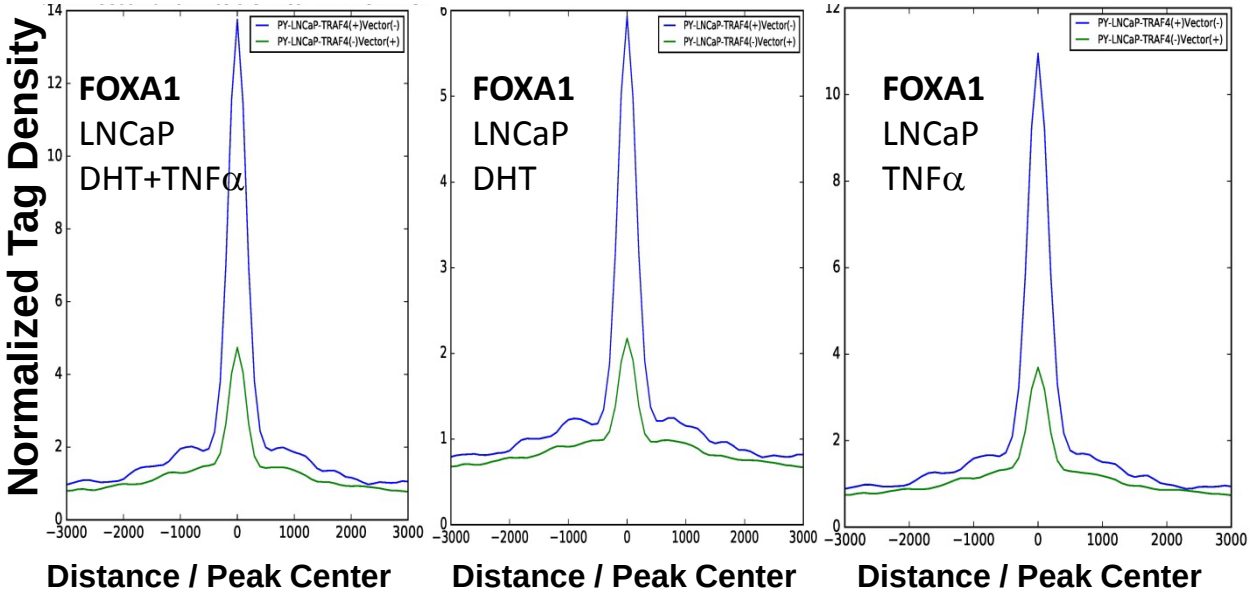
A

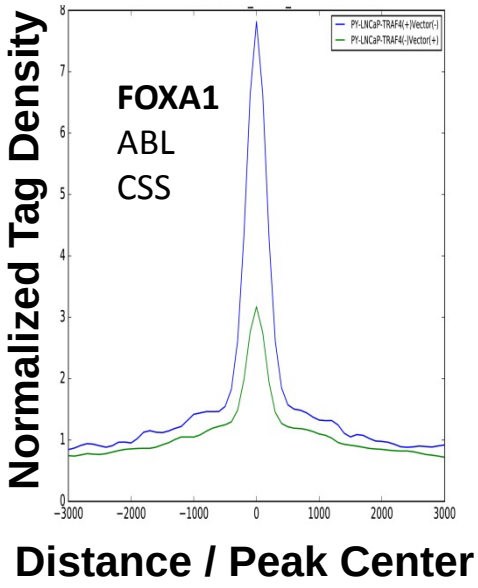


TRAF4(+)Vector(-) AR

TRAF4(-)Vector(+) AR

B





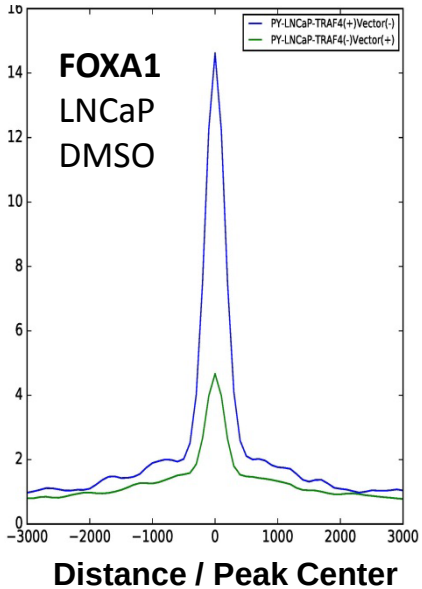
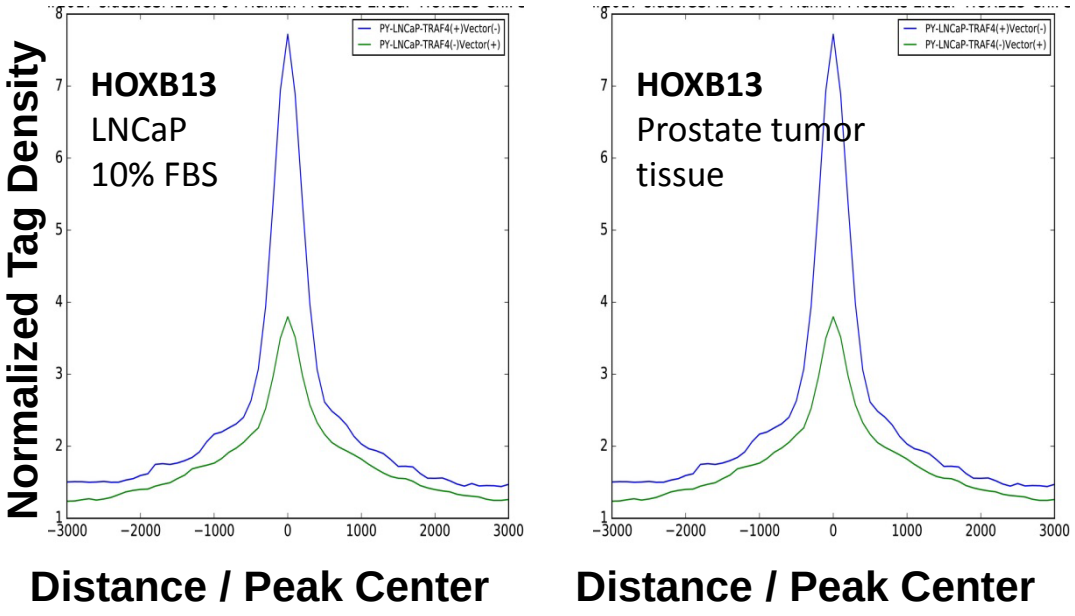


Fig S7

C


Normalized Tag Density
HOXB13
LNCaP
10% FBS
HOXB13
Prostate tumor
tissue
PY-LNCaP-TRAF4(+)Vector(-)
PY-LNCaP-TRAF4(-)Vector(+)
Distance / Peak Center
Distance / Peak Center
TRAF4(+)Vector(-) AR
TRAF4(-)Vector(+) AR

JCI The Journal of Clinical Investigation

TRAF4-mediated ubiquitination of NGF receptor TrkA regulates prostate cancer metastasis

Ramesh Singh, … , Bert W. O'Malley, Ping Yi

J Clin Invest. 2018;128(7):3129-3143. https://doi.org/10.1172/JCI96060.

Research Article | Cell biology | Genetics

Receptor tyrosine kinases (RTKs) are important drivers of cancers. In addition to genomic alterations, aberrant activation of WT RTKs plays an important role in driving cancer progression. However, the mechanisms underlying how RTKs drive prostate cancer remain incompletely characterized. Here we show that non-proteolytic ubiquitination of RTK regulates its kinase activity and contributes to RTK-mediated prostate cancer metastasis. TRAF4, an E3 ubiquitin ligase, is highly expressed in metastatic prostate cancer. We demonstrated here that it is a key player in regulating RTK-mediated prostate cancer metastasis. We further identified TrkA, a neurotrophin RTK, as a TRAF4-targeted ubiquitination substrate that promotes cancer cell invasion and found that inhibition of TrkA activity abolished TRAF4-dependent cell invasion. TRAF4 promoted K27- and K29-linked ubiquitination at the TrkA kinase domain and increased its kinase activity. Mutation of TRAF4-targeted ubiquitination sites abolished TrkA tyrosine autophosphorylation and its interaction with downstream proteins. TRAF4 knockdown also suppressed nerve growth factor (NGF) stimulated TrkA downstream p38 MAPK activation and invasion-associated gene expression. Furthermore, elevated TRAF4 levels significantly correlated with increased NGF-stimulated invasion–associated gene expression in prostate cancer patients, indicating that this signaling axis is significantly activated during oncogenesis. Our results revealed a posttranslational modification mechanism contributing to aberrant non-mutated RTK activation in cancer cells.

TRAF4-mediated ubiquitination of NGF receptor TrkA regulates prostate cancer metastasis

Ramesh Singh,[1] Dileep Karri,[1] Hong Shen,[1] Jiangyong Shao,[2] Subhamoy Dasgupta,[1] Shixia Huang,[1,3] Dean P. Edwards,[1,4] Michael M. Ittmann,[4,5] Bert W. O'Malley,[1] and Ping Yi[1]

[1]Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas, USA. [2]Diana Helis Henry Medical Research Foundation, New Orleans, Louisiana, USA. [3]Dan L. Duncan Comprehensive Cancer Center and [4]Department of Pathology and Immunology, Baylor College of Medicine, Houston, Texas, USA. [5]Michael E. DeBakey Department of Veterans Affairs Medical Center, Houston, Texas, USA.

Receptor tyrosine kinases (RTKs) are important drivers of cancers. In addition to genomic alterations, aberrant activation of WT RTKs plays an important role in driving cancer progression. However, the mechanisms underlying how RTKs drive prostate cancer remain incompletely characterized. Here we show that non-proteolytic ubiquitination of RTK regulates its kinase activity and contributes to RTK-mediated prostate cancer metastasis. TRAF4, an E3 ubiquitin ligase, is highly expressed in metastatic prostate cancer. We demonstrated here that it is a key player in regulating RTK-mediated prostate cancer metastasis. We further identified TrkA, a neurotrophin RTK, as a TRAF4-targeted ubiquitination substrate that promotes cancer cell invasion and found that inhibition of TrkA activity abolished TRAF4-dependent cell invasion. TRAF4 promoted K27- and K29-linked ubiquitination at the TrkA kinase domain and increased its kinase activity. Mutation of TRAF4-targeted ubiquitination sites abolished TrkA tyrosine autophosphorylation and its interaction with downstream proteins. TRAF4 knockdown also suppressed nerve growth factor (NGF) stimulated TrkA downstream p38 MAPK activation and invasion-associated gene expression. Furthermore, elevated TRAF4 levels significantly correlated with increased NGF-stimulated invasion–associated gene expression in prostate cancer patients, indicating that this signaling axis is significantly activated during oncogenesis. Our results revealed a posttranslational modification mechanism contributing to aberrant non-mutated RTK activation in cancer cells.

Introduction

Ubiquitination is an important posttranslational modification regulating protein degradation, trafficking, and activity, as well as protein-protein interaction. Dysregulation of the ubiquitin pathways has been implicated in a number of diseases including cancer (1–5). Targeting the ubiquitination machinery has been considered to be an effective therapeutic strategy (3, 6, 7).

The RING domain E3 ubiquitin ligase TRAF4 is emerging as a key regulator in cancer development, metastasis, and chemoresistance (8–15). It was originally identified as a gene upregulated in metastatic breast cancer (16). TRAF4 belongs to the TRAF family, which consists of 7 members. They are adaptor/scaffold proteins that couple TNF receptors and interleukin receptors to downstream signaling pathways. Unlike other TRAFs, TRAF4 weakly interacts with very few TNF receptor family members (17). It also does not substantially contribute to the development and normal function of the immune system, except for facilitating immune cell migration (18). The biological function of TRAF4 has remained elusive. It is expressed at basal levels in most adult tissues (17) but is overexpressed and amplified in a variety of human cancers (11). We found that TRAF4 has a significantly higher expression level in metastatic prostate cancer compared with primary tumor and plays an important role in prostate cancer cell invasion.

After screening TRAF4 ubiquitination substrates in prostate tumors, we identified tyrosine receptor kinase A (TrkA, also named NTRK1) as a prominent substrate for TRAF4-mediated ubiquitination. TrkA is a receptor tyrosine kinase (RTK) that binds to nerve growth factor (NGF) at the cell membrane. It activates Ras/MAPK, PI3K, and PLCγ signaling pathways to promote cell survival, proliferation, and invasion (19). In addition to the nervous system, NGF is also abundant in prostate cancer, and its receptor has been linked to prostate cancer proliferation and metastasis (19–25). Targeting genetically altered constitutively active protein kinases has led to dramatic clinical responses in several cancers. Although TrkA-activating mutations through genomic rearrangement and deletion have been documented in a number of cancers (26–31), its mutations were not identified in prostate cancer (32). The pathways leading to aberrant activation of non-mutated TrkA have remained poorly understood. Herein we present evidence that TRAF4 promotes TrkA ubiquitination at its kinase domain through atypical K27 and K29 ubiquitin linkages. This posttranslational modification hyperactivates TrkA kinase activity and alters its phosphorylation status. Our study deciphered that the TRAF4-regulated signaling cascade is an important driver for prostate cancer metastasis.

Results

TRAF4 plays a role in prostate cancer metastasis. To investigate the expression of TRAF4 in prostate tumor specimens, we used a prostate cancer tissue cDNA array consisting of 39 prostate cancer and 9 normal prostate tissue samples. TRAF4 mRNA was

Conflict of interest: The authors have declared that no conflict of interest exists.
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https://doi.org/10.1172/JCI96060.

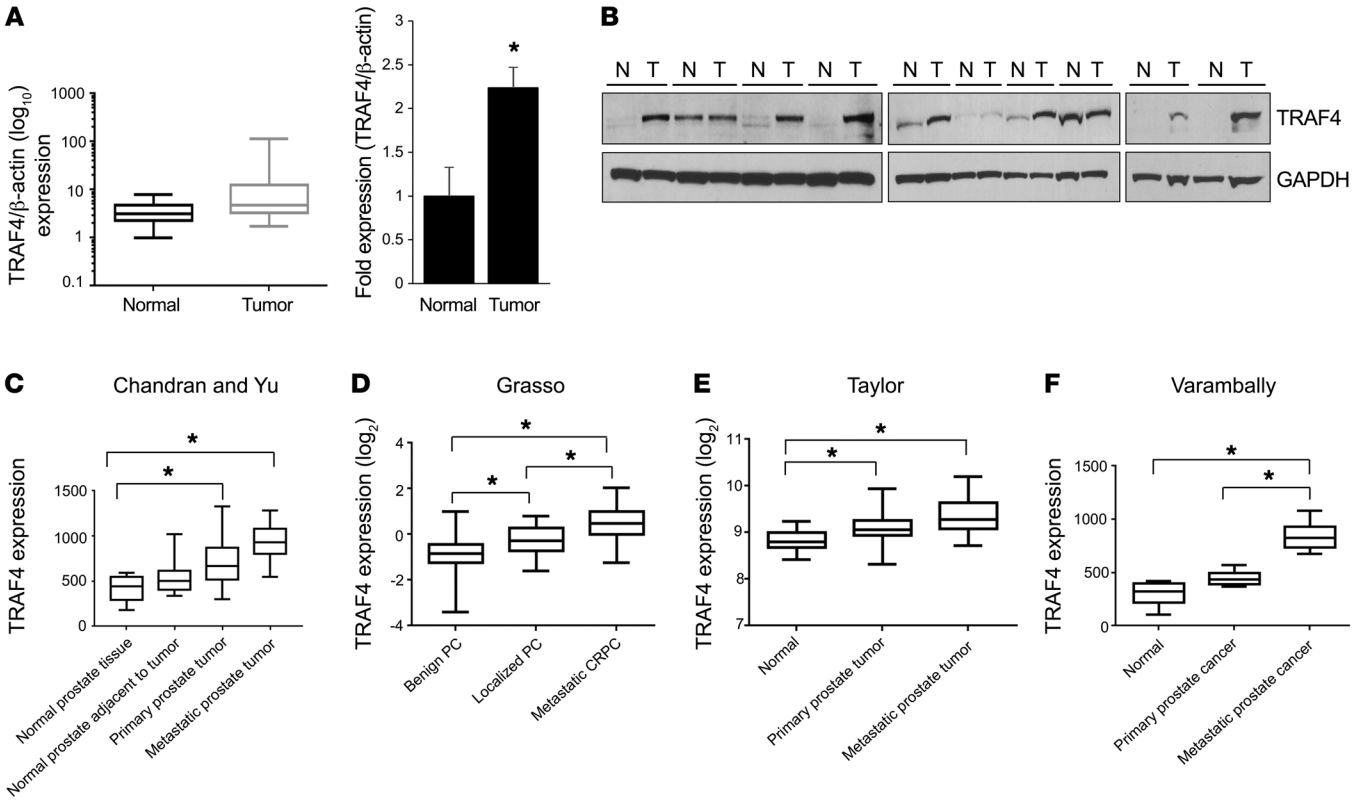

Figure 1. TRAF4 is overexpressed in prostate cancers. (**A**) TRAF4 mRNA is upregulated in prostate tumors (n = 39) as compared with normal tissue (n = 9) in a prostate cancer tissue scan array (OriGene) as analyzed by RT-qPCR. Left: Relative TRAF4 mRNA expression in normal and tumor samples as normalized against β-actin. *P < 0.05 by Mann-Whitney U test. Right: Average fold induction of TRAF4 expression in tumor samples. *P < 0.05 by 2-tailed Student's t test. (**B**) TRAF4 protein is upregulated in human prostate tumors (T) compared with matched benign tissue (N) samples (n = 10 each) as analyzed by Western blot. (**C**–**F**) TRAF4 is highly expressed in metastatic prostate cancers. The expression of TRAF4 was analyzed in 4 different prostate cancer datasets: Chandran and Yu, refs. 37, 38; Grasso, ref. 39; Taylor, ref. 35; Varambally, ref. 36 and presented as mean ± SEM. β-Actin was used as an internal control. *P < 0.05 by 1-way ANOVA with multiple comparisons test. PC, prostate cancer; CRPC, castration-resistant prostate cancer.

highly expressed in prostate tumors compared with normal tissues (Figure 1A). We also found that TRAF4 protein levels were significantly higher in 7 of 10 human prostate tumors compared with matched benign prostate tissues (Figure 1B). Since TRAF4 has been reported to be associated with cell migration and cancer metastasis (9, 15, 18, 33, 34), we also analyzed its expression in several publicly available prostate cancer datasets containing a substantial number of metastatic cancers (35–39). Consistent with our analysis of tumors (Figure 1, A and B), TRAF4 expression was significantly elevated in prostate tumors compared with adjoining prostate tissues (Figure 1, C–F). Interestingly, patients with metastatic disease all had enhanced levels of TRAF4 compared with those with localized tumors (Figure 1, C–F). These findings suggest the possibility of a critical role of TRAF4 in aggressive metastatic prostate cancers.

To determine whether TRAF4 plays a role in prostate cancer metastasis, we first analyzed the effect of TRAF4 knockdown in prostate cancer cell migration and invasion. TRAF4 knockdown was achieved using pooled siRNA or 2 different shRNAs. PC3 cells, a highly invasive prostate cancer line, were subjected to TRAF4 or control knockdown, followed by seeding at equal numbers in a Transwell invasion chamber either with (for invasion) or without Matrigel (for migration assays) in a serum-free medium for 12–16 hours. No significant difference in cell proliferation was observed under these conditions (data not shown). We found that the numbers of migratory and invasive PC3 cells were significantly decreased upon TRAF4 knockdown compared with non-targeting control (Figure 2, A and B, respectively). A similar observation was made in another metastatic prostate cancer line, DU145 (Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI96060DS1).

We next examined the role of TRAF4 in prostate cancer cell metastatic potential in an experimental lung metastatic mouse model by injecting prostate tumor cells via tail vein. PC3 luciferase-expressing cells selected for stable knockdown of TRAF4 or non-targeting shRNA control were injected via tail vein into 4- to 5-week-old male NOD/SCID mice. Numbers of injected tumor cells in the circulation in the control and shTRAF4 groups were compared at 5 minutes after tail vein injection by bioluminescence imaging (BLI) of the mouse lung (0 week) (Figure 2C). Although the same number of cells was injected, the shTRAF4 cells showed higher luminescence intensities compared with shControl cells due to greater expression levels of the luciferase gene in shTRAF4 cells (Supplemental Figure 1B). Both groups showed a gradual decrease in luminescence signals 1–3 weeks after injection, indicating that most of the injected cells did not survive in the lung.

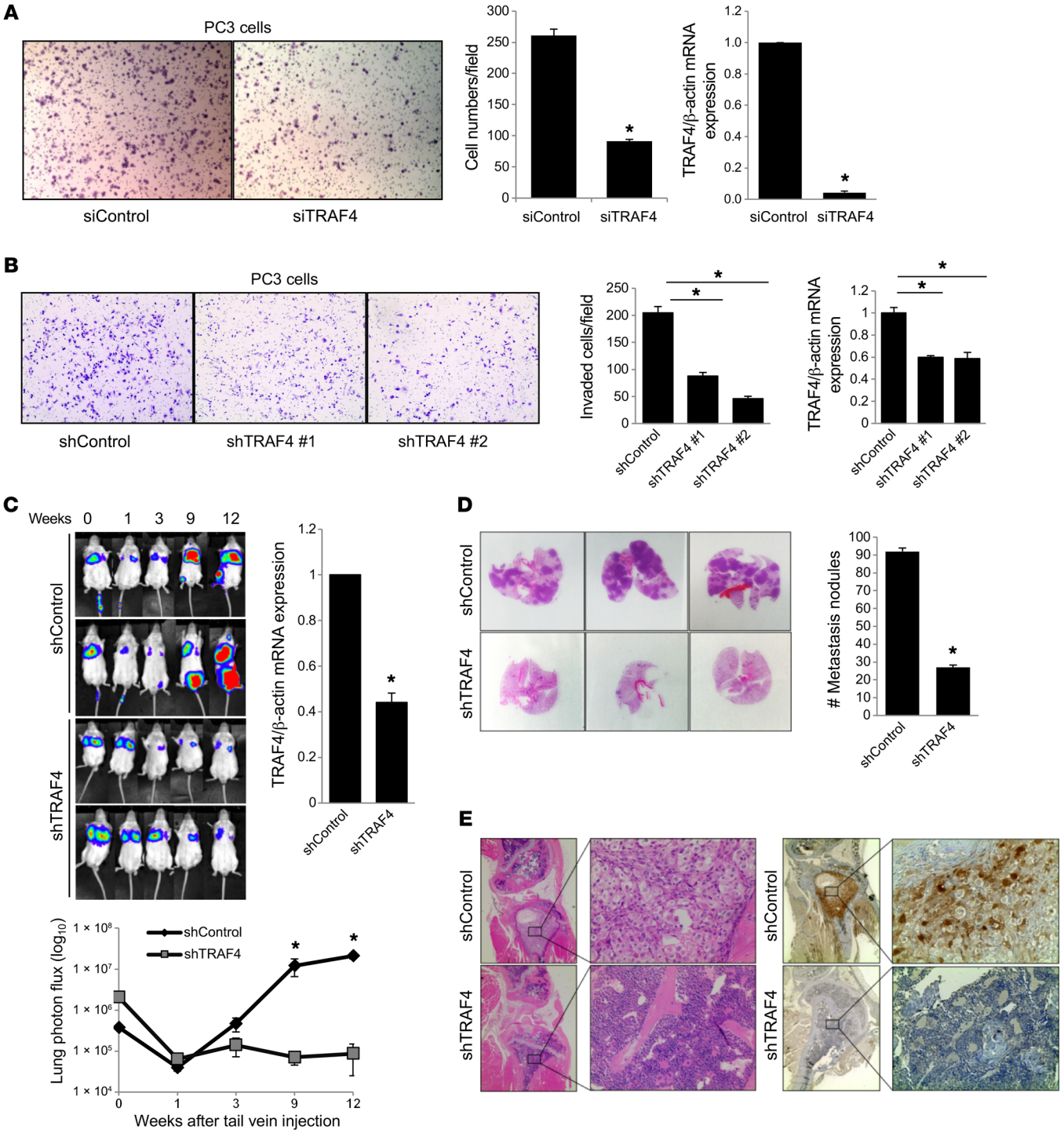

Figure 2. TRAF4 plays a role in prostate cancer cell migration, invasion, and metastasis. Knockdown of TRAF4 in PC3 cells inhibited cell migration (**A**) and invasion (**B**). Left panels: Cells migrated through migration chamber (**A**) or Matrigel (**B**) (n = 3). Images were obtained at ×100 magnification. Middle panels: Quantitation of cell numbers per field. Right panels: Levels of TRAF4 in control or TRAF4-knockdown cells as assessed by qRT-PCR. *P < 0.05 by Student's t test versus siControl or siTRAF4 (**A**) and *P < 0.05 by 1-way ANOVA with Dunnett's multiple comparisons test (**B**). Data are presented as mean ± SEM. (**C**) TRAF4 knockdown reduced prostate cancer cell colonization and metastasis in vivo. Top left: Representative bioluminescence images of SCID mice at different time periods after tail vein injection of PC3 shControl or shTRAF4 cells. Lower left: Quantitation of bioluminescence signals at the lung area in PC3 shControl- or shTRAF4-injected mice (n = 5). Right: Levels of TRAF4 in control or TRAF4-stable-knockdown cells as assessed by qRT-PCR. *P < 0.05 by 2-tailed Student's t test. (**D**) TRAF4 knockdown reduced lung metastatic nodules. H&E-stained lung sections and graph showing the number of lung metastasis nodules after tail vein injection (n = 10). Data are presented as mean ± SEM. *P < 0.05 by 2-tailed Student's t test. (**E**) H&E-stained bone sections (left) and immunohistochemistry using anti-luciferase antibody staining (right) confirming bone metastasis in the control group but not in the shTRAF4-knockdown group. Images were obtained at ×40 and ×100 (insets) magnification.

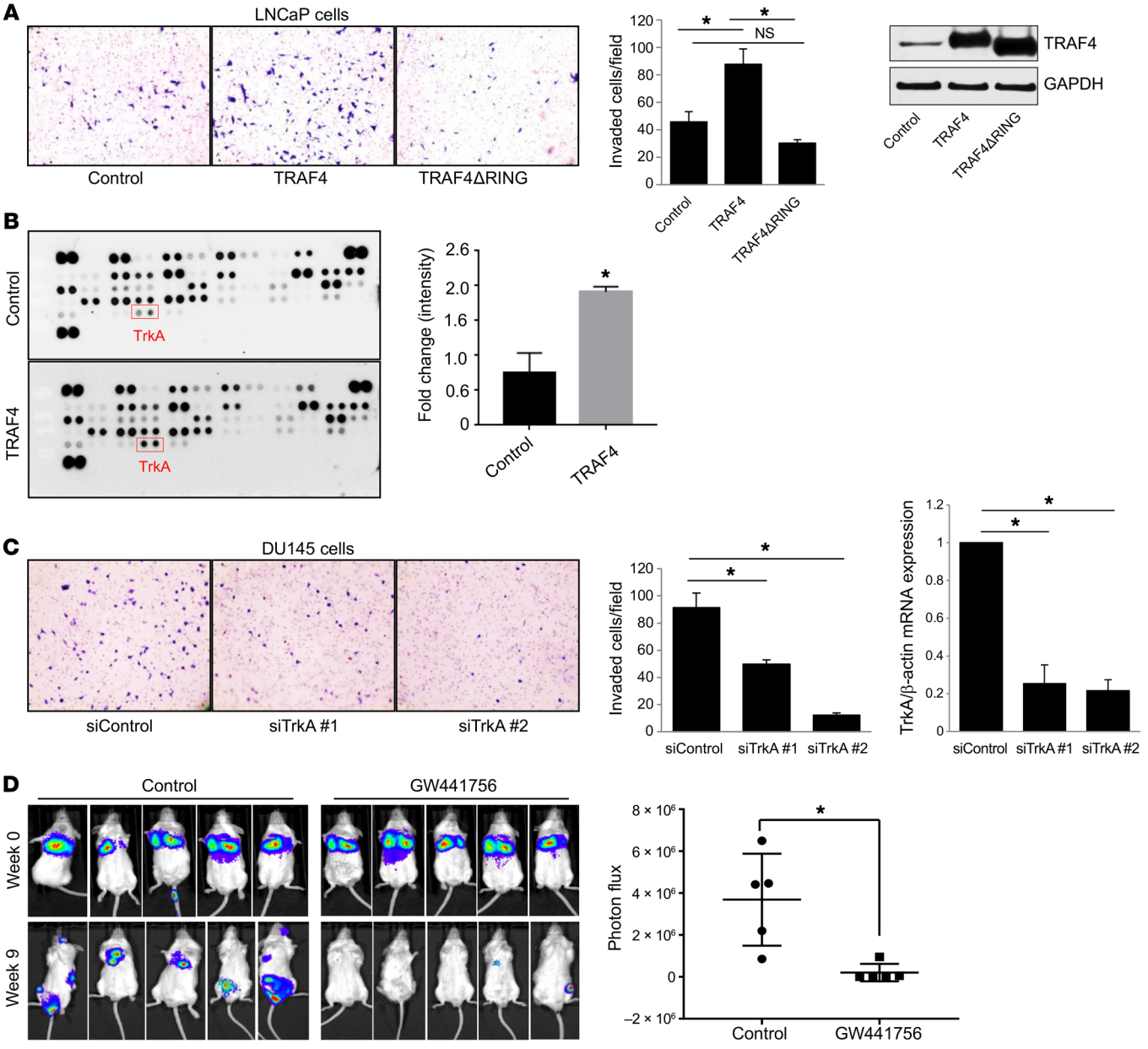

Figure 3. TRAF4-mediated TrkA ubiquitination is critical for cell invasion. (**A**) Overexpression of TRAF4 WT but not the RING domain deletion mutant promoted LNCaP cell invasion. Left: LNCaP cells invaded through Matrigel were stained with crystal violet (n = 3). Images were obtained at ×100 magnification. Middle: Quantitation of invaded cells per field. *P < 0.05 by 1-way ANOVA. Right: Western blot analysis of the expression of TRAF4 WT or its mutant in LNCaP stable cells using a TRAF4-specific antibody. (**B**) TRAF4 overexpression enhanced TrkA ubiquitination. The human ubiquitin array kit was used to identify TRAF4 ubiquitination targets. Cell lysate from GFP- (control) or TRAF4-expressing adenovirus–infected PC3 cells were used on each array. Each dot represents the ubiquitination level of a target protein recognized by an anti-ubiquitin antibody. *P < 0.05 by 2-tailed Student's t test. (**C**) Knockdown of TrkA using specific siRNAs decreased DU145 cell invasion (n = 3). Images were obtained at ×100 magnification. Right: Levels of TrkA in control or TrkA-knockdown cells as assessed by qRT-PCR. *P < 0.05 by 1-way ANOVA with Dunnett's multiple comparisons test. (**D**) TrkA inhibition by GW441756 reduced prostate cancer cell colonization and metastasis in vivo. Left: Bioluminescence images of SCID mice at 0 or 9 weeks after tail vein injection of PC3 luciferase cells with or without GW441756 treatment. Right: Quantitation of the bioluminescence signals at the metastasis area in control or GW441756-injected mice 9 weeks after injection (n = 5 per group). Data are presented as mean ± SEM. *P < 0.05 by 2-tailed Student's t test.

Through 9 weeks, the luminescence signals at the lung areas of control shRNA–injected mice steadily increased (Figure 2C), indicating the growth of successfully colonized tumor cells. In contrast, the signals from the shTRAF4 cell–injected mice continued to fade. This difference is unlikely due to a difference in proliferation rates of tumor cells, since comparable Ki-67 staining intensities were found in the luminescence signal–positive tumor areas of the 2 mouse groups (Supplemental Figure 1C). These results suggest that the injected shTRAF4 cells had reduced ability to colonize into the lung compared with control cells. H&E staining of the lungs also showed a reduction in size and number (*P < 0.05) of metastatic nodules in TRAF4-knockdown cells (Figure 2D). We

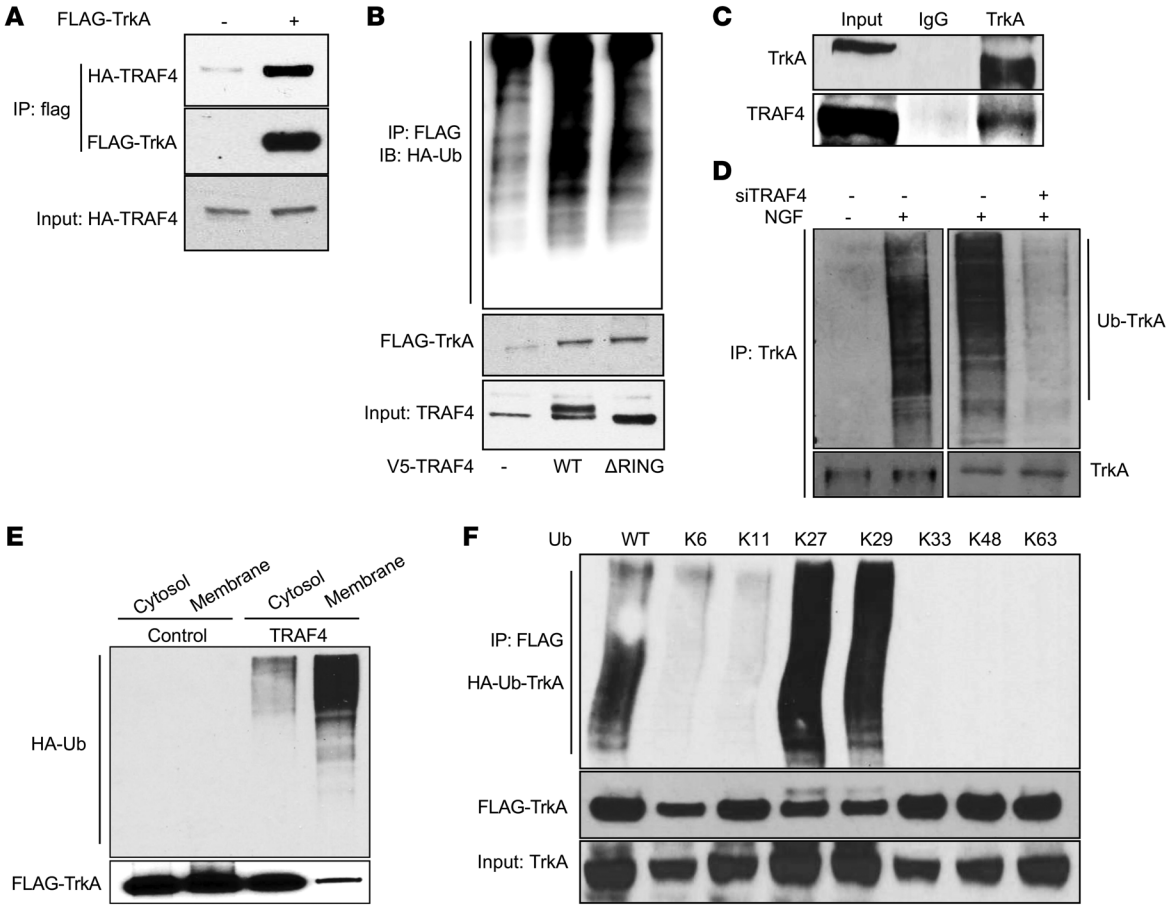

Figure 4. TRAF4 interacted with TrkA and promoted its ubiquitination. (**A**) HA-TRAF4 interacted with FLAG-TrkA in transiently transfected 293T cells. Shown is a co-IP experiment using an anti-FLAG antibody for immunoprecipitation. (**B**) WT TRAF4 but not the RING domain deletion mutant promoted TrkA ubiquitination. 293T cells were cotransfected with constructs as indicated. FLAG-TrkA was immunoprecipitated with an anti-FLAG antibody, and the ubiquitinated TrkA was visualized by Western blot analysis using an anti-HA antibody. (**C**) Endogenous TrkA interacted with endogenous TRAF4 in DU145 cells. Shown is a co-IP experiment using a TrkA-specific antibody or IgG control for immunoprecipitation. (**D**) TRAF4 knockdown abolished NGF-induced TrkA ubiquitination. DU145 cells were transfected with control siRNA or siTRAF4 and HA-ubiquitin. Cells were then treated with 50 ng/ml NGF for 15 minutes before harvest. Ubiquitinated TrkA was detected using an anti-ubiquitin antibody in a Western blot analysis from cell lysates immunoprecipitated with an anti-TrkA antibody. Ub, ubiquitin. (**E**) TRAF4 overexpression promoted TrkA ubiquitination at the cell membrane. 293T cells were cotransfected with TrkA and HA-Ub in the absence or presence of TRAF4 cotransfection. Cytosolic and membrane fraction were isolated and subjected to immunoprecipitation using an anti-FLAG antibody, and the ubiquitinated TrkA was visualized by Western blot analysis using an anti-HA antibody. (**F**) TRAF4-mediated TrkA polyubiquitination through K27- or K29-linked ubiquitin chain. K6–K63 represent the ubiquitin mutant with all lysine mutations except the indicated number of lysine.

found that some of the shRNA control cell– but not shTRAF4 cell–injected mice developed luminescence signals 8–9 weeks after injection at areas in addition to the lung, an indication of further metastasis. Tumor bone metastasis was confirmed via immunohistochemistry using an anti-luciferase antibody in shRNA control mice (Figure 2E). However, we did not find any bone metastasis in shTRAF4-injected mice. These results suggest that TRAF4 plays an important in vivo role in prostate cancer cell metastasis.

The RING domain is critical for TRAF4-mediated cell invasion in prostate cancer cell lines. Next we investigated the molecular mechanisms by which TRAF4 drives prostate cancer cell invasion and eventually systemic metastasis. TRAF4 is an E3 ubiquitin ligase containing a RING domain that plays an important role in TRAF4-mediated ubiquitination. To determine whether the E3 ubiquitin ligase activity of TRAF4 is important for its invasive function, we deleted the RING domain and compared the effect of this truncated protein on the invasion ability of a poorly invasive prostate cancer cell line, LNCaP, with that of the full-length TRAF4. LNCaP cell lines stably expressing vector control, TRAF4 WT, or TRAF4ΔRING mutant were used in an invasion assay. The expression levels of FLAG-TRAF4 WT and FLAG-TRAF4ΔRING mutant were comparable. As shown in Figure 3A, WT TRAF4 overexpression significantly increased cell invasion, confirming the role of TRAF4 in mediating cell invasion. In contrast, the RING domain deletion mutant of TRAF4 lost the ability to promote cell invasion (Figure 3A). These results suggest that the TRAF4 RING domain vital for its E3 ubiquitin ligase activity also is critical for driving prostate tumor cell invasion.

TRAF4 interacts with and ubiquitinates the neurotrophin receptor TrkA. To identify a TRAF4-targeted ubiquitination substrate that mediates TRAF4's ability to promote cell invasiveness, we performed an unbiased screen on a ubiquitin array, which measured

Table 1. TRAF4 regulates genes involved in cell migration/invasion

Protein	shTRAF4 vs. shControl	*P* value
p-Akt(S473)	0.121	1.197×10^{-8}
p-Akt(T308)	0.318	4.997×10^{-8}
p-p38(T180/Y182)	0.609	6.174×10^{-6}
p38/MAPK	0.901	0.000236
COX2	0.166	1.163×10^{-8}
Slug	0.439	7.875×10^{-14}
Beclin-1	0.662	1.696×10^{-7}
Integrin α4	0.461	1.397×10^{-9}
Integrin β4	0.221	1.612×10^{-20}
Integrin β1	0.605	7.499×10^{-16}
HIF-1α	0.303	2.528×10^{-13}
p-FAK(Y397)	0.441	6.392×10^{-12}

Representative cell migration/invasion-related proteins/protein phosphorylation are regulated by TRAF4 in an RPPA study comparing their levels in TRAF4-knockdown and non-targeting control PC3 cells ($n = 12$). The median of the triplicate experimental values (normalized signal intensity) was taken for each sample for statistical analysis using Student's *t* test (significant for $P < 0.05$).

the ubiquitination levels of 49 different proteins. Among them we found TrkA as one of the top-hit candidate proteins showing significantly enhanced ubiquitination upon TRAF4 overexpression in PC3 cells (Figure 3B). TrkA is a tyrosine kinase receptor that is activated upon binding to its ligand, NGF, a member of the neurotrophin family that regulates brain development and function. TrkA was previously reported to also regulate prostate cancer cell metastasis (19–25). To test the hypothesis that TrkA is an important downstream mediator of TRAF4-dependent prostate tumor cell invasion, we examined the invasion potential of DU145 or PC3 cells following TrkA depletion. Our data revealed that TrkA silencing significantly reduced cell invasion, similar to the effect of TRAF4 knockdown (Figure 3C and Supplemental Figure 2, A and B), indicating that TrkA likely is a potential TRAF4 ubiquitination substrate involved in cell invasion.

We further tested the role of TrkA in prostate cancer cell metastasis in vivo using a TrkA-specific inhibitor, GW441756 (40). PC3 luciferase-expressing cells were injected via tail vein into male NOD/SCID mice. The mice were then randomized into control and drug treatment groups. The drug treatment mice group received 10mg/kg GW441756 i.p. twice a week, while the control group received solvent only. We did not observe any significant change in mouse weight between the 2 groups (Supplemental Figure 2C). Nine weeks after injection, the TrkA inhibitor treatment group had significantly reduced tumor metastasis compared with the control group (Figure 3D). These results suggest that TrkA inhibition in PC3 cells has an inhibitory effect on prostate cancer metastasis.

To test whether TRAF4 can interact with TrkA to promote its ubiquitination in cells, we transiently transfected FLAG-tagged TrkA or empty vector along with HA-tagged TRAF4. Immunoprecipitation of FLAG-tagged TrkA revealed a direct association with HA-tagged TRAF4 compared with cells expressing only FLAG-vector as a control (Figure 4A). Next we carried out an ubiquitination assay in 293T cells by transiently transfecting HA-ubiquitin, FLAG-TrkA, and V5-TRAF4 or its RING domain deletion mutant. The levels of TrkA ubiquitination were detected through immunoprecipitation using a FLAG-specific antibody, followed by Western blot analysis using an HA-specific antibody. The results confirmed that the WT TRAF4 but not the RING domain deletion mutant promoted the ubiquitination of TrkA in cells (Figure 4B). To confirm that the TRAF4-TrkA interaction exists in prostate tumor cells, we immunoprecipitated endogenous TrkA from DU145 cells and identified a strong interaction with TRAF4 (Figure 4C). Since NGF stimulation triggers TrkA activation, we investigated whether TrkA ubiquitination is regulated by NGF induction and whether TRAF4 plays a role in the ubiquitination process of endogenous TrkA. As shown in Figure 4D, NGF treatment substantially increased TrkA ubiquitination, whereas TRAF4 knockdown dramatically reduced the levels of ubiquitinated TrkA. We also performed an in vitro ubiquitination assay using purified FLAG-TrkA from 293T cells, purified TRAF4, HA-ubiquitin, ubiquitin-activating enzyme UBE1, and ubiquitin-conjugating enzyme UbcH5a to demonstrate that TRAF4 can directly promote TrkA ubiquitination (Supplemental Figure 3A). These results suggest that TRAF4 plays an important role in NGF-induced TrkA ubiquitination in prostate cancer cells.

TrkA undergoes internalization following activation at the cell membrane. To determine where the TRAF4-mediated ubiquitination event happens, we isolated the cytosolic and membrane protein fractions from cells transfected with FLAG-TrkA with or without TRAF4 overexpression and then examined TrkA ubiquitination. Although TrkA was located at both cytoplasm and cell membrane, TRAF4-mediated TrkA ubiquitination mainly occurred at the cell membrane (Figure 4E).

Ubiquitination chain linkage involves one or more of 7 ubiquitin lysine residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63). The type of ubiquitin linkage determines the fate of proteins in the cell. The most common ubiquitin linkage is K48, and it is usually associated with protein degradation. Interestingly, we did not observe any substantial change in the TrkA protein level upon TRAF4 overexpression in the absence of proteasome inhibitors, suggesting the unlikelihood that TRAF4-mediated TrkA ubiquitination promotes its protein degradation (Supplemental Figure 4A, right panel). It has been reported previously that TRAF4 is capable of mediating K63-linked ubiquitination (8, 9), suggesting that TRAF4 may target TrkA ubiquitination through nonclassical ubiquitin linkage. To determine which lysine linkage is involved in TRAF4-mediated TrkA ubiquitination, we utilized a series of ubiquitin mutants that only contain one of the 7 lysine residues, while all other lysine residues are mutated into arginine residues. As shown in Figure 4F, the ubiquitin mutants containing only K27 or K29 residue were able to promote TrkA ubiquitination similar to the WT ubiquitin, suggesting that TRAF4-mediated TrkA ubiquitination occurs through the K27 and K29 ubiquitin linkages.

TrkA plays a role in TRAF4-promoted cell invasion. To understand how TRAF4 promoted cell invasion and how TrkA plays a role in this function, we carried out a reverse phase protein array (RPPA) study using cell lysates from PC3 cells expressing 2 different shTRAF4 or control shRNA plasmids. A total of 213 antibodies recognizing different proteins or protein phosphorylation forms were analyzed in the RPPA study. Among the proteins that were downregulated or had

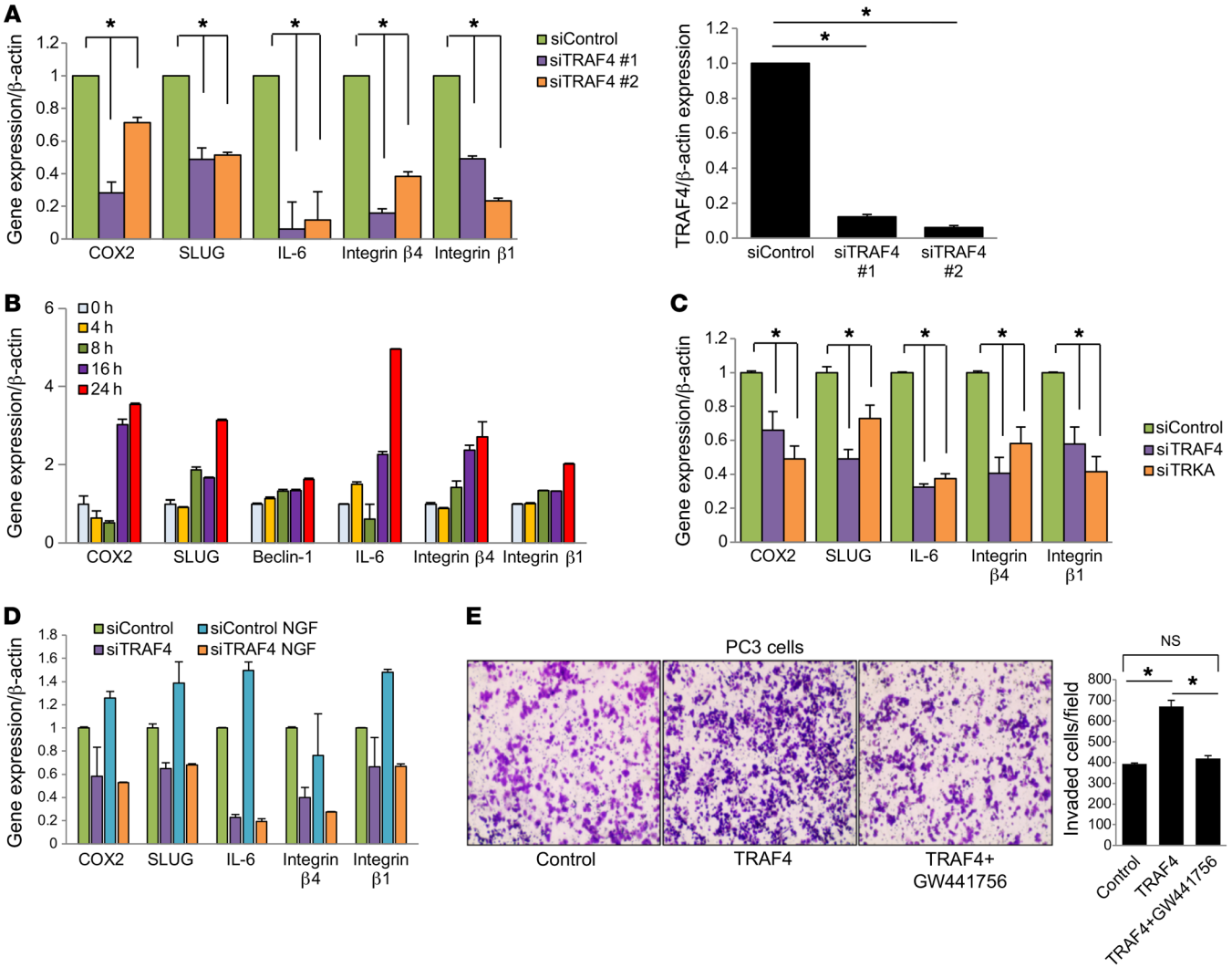

Figure 5. TrkA plays an important role in TRAF4-promoted cell invasion. (**A**) Validation of the RPPA data for some of the invasion-related genes after TRAF4 knockdown using qRT-PCR. Right: Levels of TRAF4 in control or TRAF4-knockdown cells as assessed by qRT-PCR. *$P < 0.05$ by 1-way ANOVA with Dunnett's multiple comparisons test. $n = 3$. (**B**) Expression of TRAF4-regulated genes at different time points following NGF stimulation as assessed by qRT-PCR. $n = 3$. (**C**) TrkA knockdown also downregulates TRAF4-regulated invasion-related genes. *$P < 0.05$ by 1-way ANOVA with Dunnett's multiple comparisons test. $n = 3$. (**D**) Knockdown of TRAF4 inhibited NGF-stimulated gene expression. $n = 3$. (**E**) TrkA-selective inhibitor treatment abolished TRAF4-stimulated cell invasion ($n = 3$). Images were obtained at ×100 magnification. PC3 cells were infected with GFP or TRAF4 adenovirus and then treated with or without 0.5 μM GW441756 for 2 days before seeding in an invasion chamber. *$P < 0.05$ by 1-way ANOVA with Tukey's multiple comparisons test. Data are presented as mean ± SEM.

reduced phosphorylation levels in TRAF4-knockdown cells compared with control cells, we found a number of epithelial-mesenchymal transition– (EMT-) and invasion-associated proteins (Table 1). We further validated the RPPA results using quantitative real-time PCR (qRT-PCR) to examine the effect of siTRAF4 on the expression of these target genes (Figure 5A). Interestingly, many of them, including COX2, Slug, IL-6, and integrin β1, were also upregulated upon NGF stimulation (Figure 5B). To determine whether TrkA plays a role in TRAF4-regulated expression of these target genes, we knocked down TrkA using siRNAs, followed by measurement of target gene expression. TrkA-knockdown cells showed a pattern of reduced gene expression of TRAF4 target genes, as was observed with siTRAF4 treatment (Figure 5C). We also found that knockdown of TRAF4 abolished NGF-stimulated gene upregulation (Figure 5D), confirming that TRAF4 is important for NGF-induced gene expression.

Since TrkA is a tyrosine kinase, we then determined whether inhibition of TrkA kinase activity affects TRAF4-mediated cell invasion. We first examined whether the TrkA-selective inhibitor GW441756 affects cell growth in our experimental condition. An MTT assay was performed on PC3 cells treated with a series of concentrations of GW441756 for 2 days. No significant effect of the inhibitor treatment on cell growth was found (Supplemental Figure 3B). We next chose a 0.5-μM concentration of GW441756 to examine its effect on TRAF4-promoted cell invasion. As shown in Figure 5E, overexpression of TRAF4 significantly increased PC3 cell invasion. Treatment with GW441756 for 2 days abolished the TRAF4-mediated effects. These results substantiate the hypothesis that the interplay of TRAF4 and TrkA regulates cell invasion–associated gene expression and cell invasion.

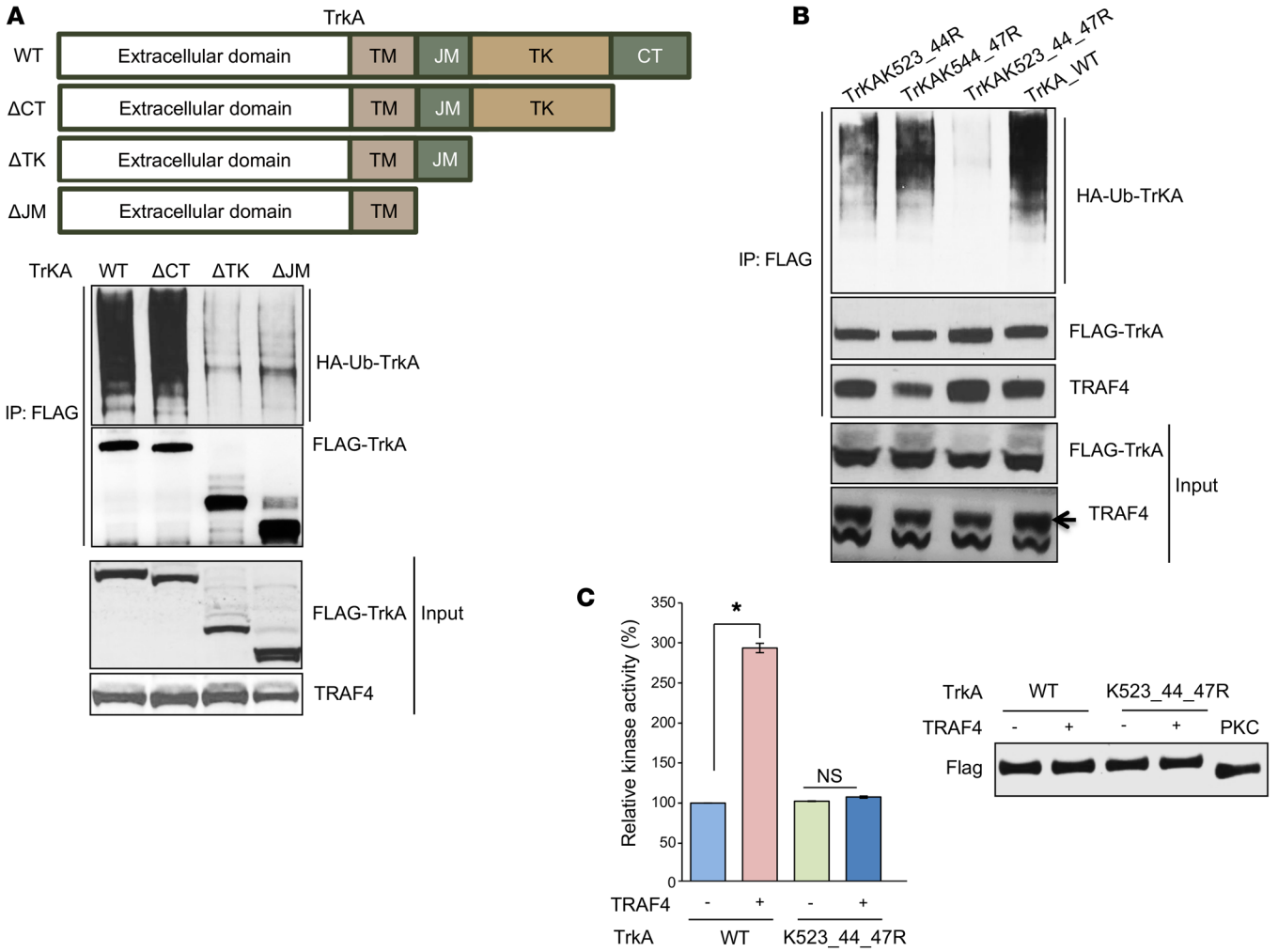

Figure 6. TRAF4 ubiquitinated 3 lysine residues present in the kinase domain of TrkA. (**A**) Deletion of the tyrosine kinase domain (TK) of TrkA abolished its ubiquitination. Upper panel: Schematic representation of TrkA and its deletion mutants. Lower panels: Ubiquitination levels of different TrkA deletion mutants. FLAG-TrkA and the mutants were cotransfected with TRAF4 and HA-Ub into 293T cells. The ubiquitinated TrkA was immunoprecipitated using a FLAG antibody and then detected using an anti-HA antibody in the Western blot. (**B**) Mutation of K523, K544, and K547 residues at the TK domain abolished TrkA ubiquitination. (**C**) TRAF4 hyperactivated TrkA WT but not the K523_544_547R mutant in an in vitro kinase assay. Left: Purified FLAG-TrkA in vitro kinase activity using a poly(Glu4, Tyr1) synthetic peptide as a substrate. The activity was measured through an ADP-Glo Kinase assay. Right: Protein levels of purified TrkA, its mutant, and PKCδ used in the kinase assay with or without TRAF4 overexpression as demonstrated by Western blotting using an anti-FLAG antibody. Data are presented as mean ± SEM. $n = 3$. $^*P < 0.05$ by 1-way ANOVA.

Lysine residues at the TrkA kinase domain are responsible for TRAF4-mediated TrkA ubiquitination. Since TRAF4 regulates TrkA ubiquitination, we next investigated the ubiquitination target sites in TrkA to understand the importance of this posttranslational modification on its function. TrkA is a 140-kDa transmembrane receptor containing extracellular domains involved in NGF binding, a transmembrane domain (TM), a juxtamembrane domain (JM), a tyrosine kinase domain (TK), and a short C-terminal domain (CT). A schematic representation of these domains is shown in Figure 6A. To determine which domain is targeted by TRAF4 for ubiquitination, we generated different deletion mutants of TrkA and then tested their relative ubiquitination levels in TRAF4-overexpressing cells (Figure 6A). TrkA ubiquitination was abolished upon deletion of the TK domain (ΔTK and ΔJM vs. WT and ΔCT). These results suggested that the TK domain is likely the region targeted by TrkA ubiquitination.

Next we determined the ubiquitination sites in the TK domain. Ubiquitin ligases often target multiple neighboring lysine residues for ubiquitination (41–43). There are 10 lysine residues present in the TK domain. Three of them (K523, K544, and K547) are located close to each other to form a lysine cluster in the crystal structure (Supplemental Figure 3C and ref. 44). To test whether this lysine cluster is the TRAF4-targeted ubiquitination site, we used site-directed mutagenesis to generate lysine-to-argine mutants of TrkA. Mutation of 2 of these 3 lysine residues to arginine (K523_44R or K544_47R) markedly reduced TRAF4-mediated ubiquitination, while mutation of all 3 lysine residues completely abolished the ubiquitination (Figure 6B). These results suggested that TRAF4 targets TrkA ubiquitination at a lysine cluster containing K523, K544, and K547 residues.

Interestingly, the 3 lysine residues we identified are located in proximity to the kinase activation loop (Supplemental Figure 3C). It is likely that posttranslational modification at the lysine

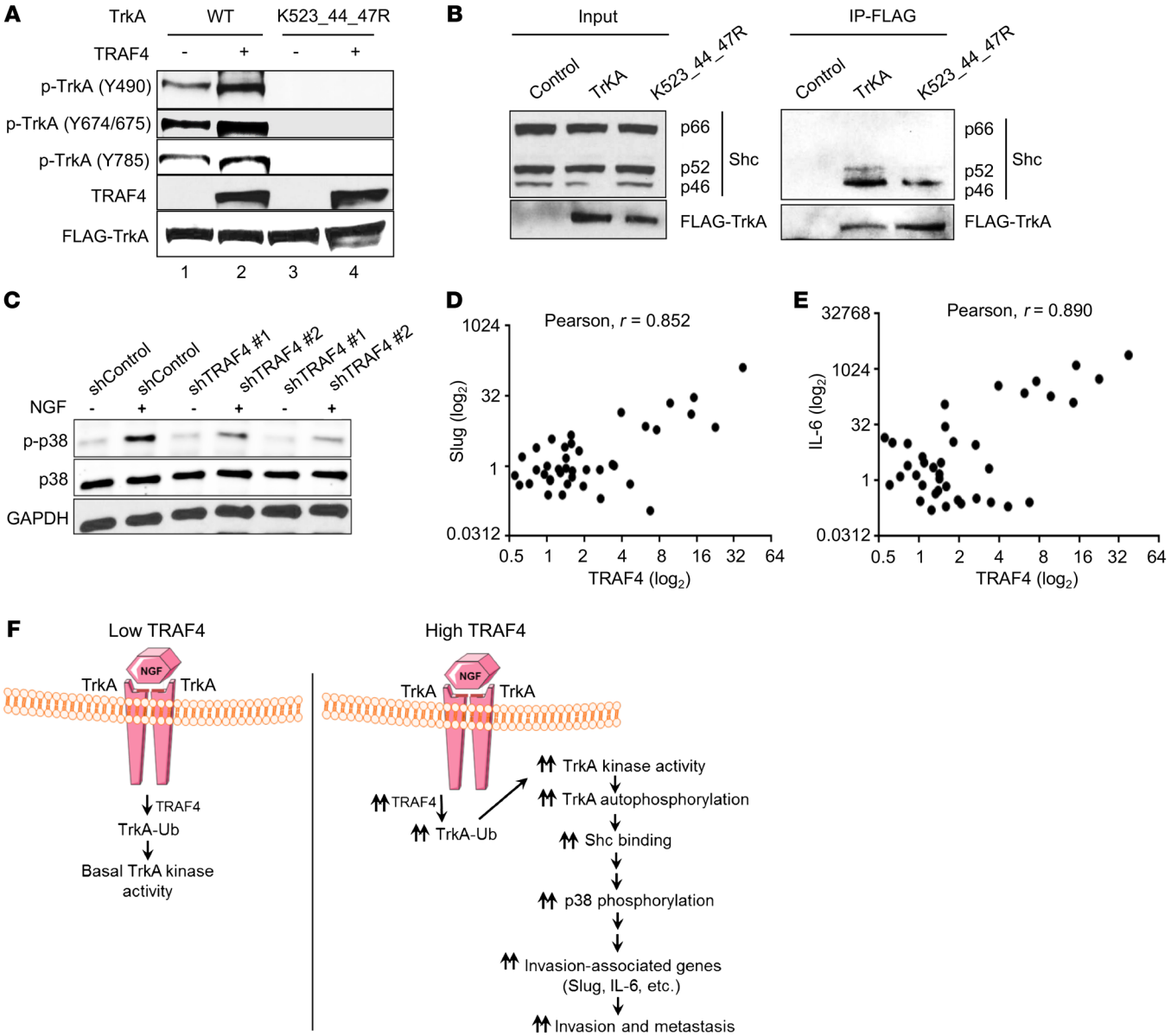

Figure 7. TRAF4-mediated TrkA ubiquitination regulated NGF-stimulated TrkA signaling cascade. (**A**) TRAF4 overexpression increased the phosphorylation level of WT TrkA but not the K523_544_547R mutant in the presence of NGF. Specific tyrosine phosphorylation antibodies were used in the Western blot analysis in cells treated with 50 ng/ml NGF for 15 minutes. (**B**) Mutation of the K523, K544, and K547 residues reduced the association of Shc protein with TrkA. FLAG-TrkA WT or its mutant was transfected into DU145 cells, and the interaction between Shc protein and TrkA was determined in a co-IP experiment using a FLAG antibody for immunoprecipitation. (**C**) TRAF4 knockdown substantially reduced NGF-induced p38 MAPK phosphorylation (pT180/Y182). PC3 cells were treated with and without NGF (50 ng/ml) for 10 minutes after 18 hours of serum starvation. (**D**) The level of TRAF4 expression correlated with Slug gene expression in a prostate cancer patient cDNA array. (**E**) The level of TRAF4 expression correlated with the IL-6 gene expression in a prostate cancer patient cDNA array. Pearson's correlations for **D** and **E** for fold change ($\log_2$) in gene expression as determined by qRT-PCR ($r = 0.852$, $P < 0.0001$ and $r = 0.890$, $P = 2.53 \times 10^{-14}$, respectively). (**F**) Working model of the role of TRAF4 in TrkA signaling and prostate cancer cell invasion. In low-TRAF4-expressing cells, NGF induce limited TrkA ubiquitination, resulting in low levels of TrkA kinase signaling. In TRAF4-overexpressing cells, high levels of TRAF4 significantly increase TrkA K27- and K29-linked ubiquitination at K523, K544, and K547 in the kinase domain upon NGF stimulation. This ubiquitination enhances TrkA kinase activity, its tyrosine phosphorylation levels, and the recruitment of downstream adaptor proteins, resulting in a hyperactivated TrkA signaling cascade. Consequently, NGF-responsive invasion-associated targeted gene transcription is upregulated to promote cell migration and invasion.

cluster affects the kinase activity. We next determined whether TRAF4 directly regulates TrkA kinase activity through an in vitro kinase assay. FLAG-tagged WT TrkA or the K523_44_47R mutant was purified from TRAF4-overexpressing cells or vector control cells using a FLAG antibody. A FLAG-tagged serine kinase, PKCδ, was purified following the same procedure to serve as a negative control. Comparable levels of TrkA, its ubiquitin mutant, and PKCδ were then used in an in vitro luminescence kinase assay utilizing a poly(Glu4, Tyr1) synthetic peptide as a substrate (Figure 6C). The luminescence value from the PKCδ kinase reaction was used as a background reading, and subsequent kinase assay readings were adjusted according to

it. We found that TRAF4 overexpression significantly enhanced the in vitro kinase activity of WT TrkA, whereas TRAF4 had no effect on the kinase activity of the TrkA ubiquitination–deficient mutant (Figure 6C). The K547 residue was previously reported to be an important site for ATP binding, and the K547A mutant of TrkA was considered as a kinase-dead mutant (45). It is not clear whether or not K547R mutation could behave similarly to K547A mutation to inactivate the kinase activity. To avoid the potential complication caused by K547R mutation, we also tested the in vitro kinase activity of the K523_44R double mutant, which had substantially reduced ubiquitination compared with the WT TrkA (Figure 6B). Similar to the triple mutation, the double mutation abolished TRAF4-mediated enhancement of TrkA in vitro kinase activity (Supplemental Figure 3D). These results suggest that TRAF4-mediated TrkA ubiquitination at the lysine cluster (K523_44_47) adjacent to the kinase domain hyperactivates TrkA kinase activity.

TRAF4-mediated ubiquitination affects TrkA tyrosine phosphorylation. Next we assessed how TRAF4-mediated ubiquitination affects the NGF-induced signal transduction cascade. The ubiquitination mainly occurs at the cell membrane (Figure 4E). It was reported previously that TrkA ubiquitination at a different site regulates the receptor internalization (46). We did not observe a major change in TrkA subcellular localization when TRAF4 was overexpressed (Supplemental Figure 4A), suggesting that TRAF4-mediated ubiquitination does not have a marked effect on receptor trafficking. When TrkA is activated, several of its tyrosine residues are phosphorylated. The tyrosine phosphorylation could either release autoinhibition that is important for kinase activity (Y674/675) or serve as a docking site for binding to adaptor proteins to activate downstream signaling cascades (Y490 or Y785). Y674/675 phosphorylation correlates positively with the TrkA kinase activity, and it precedes the phosphorylation of other Tyr residues (47). To test whether TRAF4-mediated ubiquitination affects TrkA tyrosine phosphorylation, we analyzed the levels of TrkA tyrosine phosphorylation in the absence or presence of TRAF4 overexpression. As shown in Figure 7A, TRAF4 overexpression increased the levels of TrkA tyrosine phosphorylation at Y674/675, Y490, and Y785 sites in the presence of NGF, consistent with its ability to enhance TrkA kinase activity (Figure 6C). More importantly, mutation of the 3 TRAF4 targeted ubiquitination sites (K523_544_547R) abolished the tyrosine phosphorylation of TrkA. We observed a higher basal level of TrkA WT phosphorylation in the absence of TRAF4 overexpression compared with phosphorylation of the mutant (Figure 7A, lane 1 vs. lane 3). This is probably due to the presence of endogenous TRAF4. A similar result was observed for the K523_544R mutant (Supplemental Figure 4B). Interestingly, K547R single mutation appears to have had minor effects on TrkA phosphorylation levels, suggesting that K547R mutation by itself did not abolish its kinase activity (Supplemental Figure 4C) in contrast to the K547A mutation, as previously reported (48). These results suggest that TRAF4-mediated TrkA ubiquitination regulates TrkA tyrosine phosphorylation.

Upon NGF stimulation, the binding of phosphatases, such as SHP-1, to TrkA is transiently induced, which counterbalances TrkA activation (49). This could affect the phosphorylation levels of TrkA. To determine whether TRAF4-mediated ubiquitination affects phosphatase recruitment, we performed a co-IP experiment using a TrkA-specific antibody. As shown in Supplemental Figure 4D, no substantial difference in the association of SHP-1 with TrkA was found whether in the absence and presence of TRAF4 overexpression. Next, we asked whether mutation of the ubiquitination sites affects the ability of TrkA to recruit adaptor proteins. Y490 phosphorylation serves as a docking site for recruiting the Shc adaptor protein and is necessary for subsequent activation of downstream Ras/MAPK and PI3K pathways (49–51). A co-IP experiment was performed to examine the interaction between Shc and TrkA or its mutant in the presence of NGF. There are 3 Shc isoforms, p66, p52, and p46. P52 and p46 but not p66 play a role in mediating growth factor signaling. As shown in Figure 7B, p52 and p46 but not p66 were indeed associated with FLAG-TrkA WT. The K523_544_547R mutant, however, had a substantially reduced ability to interact with p52/p46 Shc. TrkA dimerizes upon NGF stimulation. The reason we did not observe complete loss of the interaction between Shc and the TrkA mutant is likely due to heterodimerization between the mutant TrkA and endogenous WT receptor.

In the RPPA analysis (Table 1), we found that p38 MAPK phosphorylation levels were downregulated when TRAF4 was knocked down. p38 MAPK plays an important role in EMT, invasion, extravasation, and organ colonization during cancer metastasis (11, 44). Since TrkA ubiquitination mutant has reduced interaction with the Shc adaptor protein, we examined whether TRAF4 regulates Shc downstream p38 kinase activation upon NGF induction. As shown in Figure 7C, the level of p38 phosphorylation (T180/Y182) increased when cells were stimulated with NGF. TRAF4 knockdown substantially reduced NGF-induced p38 phosphorylation. This result confirms that TRAF4 plays a role in regulating NGF-induced TrkA signaling pathways.

The levels of TrkA phosphorylation and its downstream p38 phosphorylation were also substantially reduced in metastatic mouse tumors derived from tail vein–injected shTRAF4-PC3 cells compared with controls (Supplemental Figure 4E), suggesting that TRAF4 expression levels regulate TrkA phosphorylation and its downstream signaling in vivo.

Taken together, our results suggested that TRAF4-mediated TrkA ubiquitination is important for TrkA function.

Correlation of TRAF4 expression and TrkA-regulated gene transcription in human prostate cancer samples. To determine whether the regulation of TRAF4 on TrkA signaling also exists in human prostate cancer patients, we analyzed the expression of TRAF4 and 2 of the TrkA-regulated genes involved in cancer metastasis, Slug and IL-6, in a prostate cancer cDNA array. As shown in Figure 7, D and E, a significant correlation between expression levels of TRAF4 and Slug and of TRAF4 and IL-6 was observed ($r = 0.852$, $P < 0.0001$ and $r = 0.890$, $P = 2.53 \times 10^{-14}$, respectively). These results suggest that TRAF4 also can regulate TrkA signaling in human prostate cancers.

Altogether, our results demonstrate that TRAF4 promoted TrkA ubiquitination through K27 and K29 linkages at the tyrosine kinase domain. This posttranslational modification enhanced TrkA kinase activity, its tyrosine phosphorylation levels, and subsequent downstream signaling activation to promote cancer metastasis.

Discussion

Malfunction of the ubiquitination system can contribute substantially to cancer development and metastasis. In addition to the well-studied proteasome-dependent protein degradation, non-proteolytic ubiquitination is emerging as a pivotal player in cancers. Herein we demonstrated that the RING domain E3 ubiquitin ligase TRAF4 is highly expressed in metastatic prostate cancers and plays an important role in regulating prostate cancer invasion and metastasis. Its E3 ubiquitin ligase activity is essential for promoting cell invasion. We further revealed that TrkA, a member of the receptor tyrosine kinase superfamily, is a ubiquitination substrate of TRAF4 that mediates the effect of TRAF4 on prostate cancer cell invasion.

Receptor tyrosine kinases are important signaling molecules that regulate cell proliferation, survival, differentiation, apoptosis, and migration. Aberrant activation of tyrosine kinases has been linked to a variety of cancers. In addition to genomic alterations, the activation of non-mutated kinases can contribute to cancer development and metastasis, especially in cancers with a low mutation rates such as prostate cancer (52–55). Global tyrosine phosphorylation levels are significantly increased in advanced prostate cancers even in the absence of kinase somatic mutations (56, 57). It was reported that several WT tyrosine kinases, including Trk family members TrkB (NTRK2) and TrkC (NTRK3), can drive prostate cancer bone and visceral metastasis in vivo (58). Thus, mechanisms other than genomic alterations are important for aberrantly activating tyrosine kinases in prostate cancers. Our study here underscores a role for non-proteolytic ubiquitination in aberrant activation of WT TrkA. Emerging evidence has documented the function of neurotrophins and their receptors in prostate cancer development and metastasis (21, 22, 24, 58, 59). Our present results provide strong evidence supporting the functional role of the TRAF4/TrkA axis in prostate cancer cell invasion.

Ubiquitination of several receptor tyrosine kinases has been reported previously, but the ubiquitination (mainly K48- or K63-linked) was associated with receptor protein turnover or receptor trafficking (46, 60–65). Similar to other RTKs, TrkA was found to be ubiquitinated by the RING-type E3 ubiquitin ligase c-Cbl (64) and the HECT-type ligase Nedd4-2, which promote its degradation through the proteasome (62). TRAF6 promotes K63-linked TrkA ubiquitination at the juxtamembrane domain and subsequent receptor internalization (46). Our results demonstrate that TRAF4-mediated ubiquitination occurs at the tyrosine kinase domain and it hyperactivates TrkA kinase activity, suggesting that TRAF4-targeted ubiquitination regulates TrkA function through a novel mechanism apart from other E3 ubiquitin ligases.

Interestingly, TRAF4 promoted TrkA ubiquitination through atypical K27- and K29-linked ubiquitin chains (Figure 4F). Unlike the well-studied K48 and K63 polyubiquitin chain, the functional roles of K27 and K29 ubiquitin linkages are less clear. It appears that they are nonproteolytic, and they recently have been implicated in protein-protein association/dissociation, negative regulation of protein degradation, and protein aggregation (66–70). It is not clear how the ubiquitination precisely affects TrkA kinase activity. The cellular localization of TrkA and its interaction with phosphatase SHP-1 were not substantially changed upon TRAF4 overexpression (Supplemental Figure 4, A and D). We found that TrkA is ubiquitinated at the K523, K544, and K547 residues (Figure 6B). These lysine residues are located adjacent to each other in the N-lobe of the kinase domain, which is close to the center of the kinase active site: the DFG motif at the activation loop and the ATP-binding site (Supplemental Figure 3C and ref. 44). Conformational change of the activation loop, especially the position of the DFG motif, upon ligand binding is essential for tyrosine kinase activation (71). Protein-protein interaction is one of the mechanisms to stabilize the active conformation of the activation loop of RTK or serine/threonine kinases during activation. Binding of cyclins to CDKs induces conformational change of the CDK kinase domain and stabilizes the active activation loop conformation (71). Similarly, one of the EGFR molecules in the ligand-bound EGFR dimer serves as an activator kinase to stabilize the active conformation of its partner through dimerization (71). It is possible that the K27- and K29-linked ubiquitin chain conjugation at the K523, K544, and K547 sites stabilizes the active conformation of the TrkA activation loop, functioning in a similar manner to cyclin/CDK and EGFR. The second possibility is that the positive charges introduced by the ubiquitin modification at these positions affect the position of the Asp residue at the DFG motif and stabilize the active conformation of the activation loop, or stabilize ATP binding, to sustain the kinase activation. Consistent with these speculations, we found that tyrosine phosphorylation (Y674/675) at the kinase domain, which is associated with kinase activation, was abolished when these lysine residues were mutated (Figure 7A).

It is known that NGF binds and activates TrkA, which autophosphorylates tyrosine residues in TrkA (Figure 7F). This activates several important signaling pathways, such as PI3K/Akt, PLC-γ, and MAPK, which regulate migration, invasion, and metastasis. We found that overexpression of TRAF4 increased TrkA tyrosine phosphorylation in the presence of NGF. The TrkA ubiquitination mutant not only was defective in tyrosine phosphorylation, but also had reduced ability to interact with downstream adaptor proteins. Consequently, we found that NGF-induced p38 MAPK phosphorylation and the expression of downstream invasion-associated genes were suppressed in TRAF4-knockdown cells. All of these results support the notion that TRAF4-mediated TrkA ubiquitination is required for TrkA phosphorylation and subsequent pathway activation (Figure 7F).

We also investigated whether other RTKs may be regulated by TRAF4 in a manner similar to TrkA, which might indicate that regulation of kinase activity by ubiquitination could be more generally applicable. We explored this concept using an array containing multiple receptor tyrosine kinases and found that TRAF4 overexpression increased ubiquitination of several receptor tyrosine kinases in addition to TrkA (Supplemental Figure 4F). These new substrate kinases need to be further explored as additional therapeutic targets in prostate cancer.

Taken together, our study not only expands knowledge of the role of TRAF4 in prostate cancer metastasis, but also provides a potential novel drug target for treating aggressive prostate cancers. Unlike genomic alteration or protein overexpression, WT tyrosine kinase activation other than kinase overexpression is not easily diagnosed in patients. The study we present here reveals a potential biomarker that could help in the prediction of TrkA activation in cancer patients.

Methods

Animal and human studies. All animal experiments were performed in accordance with the Institutional Animal Care and Use Committees (IACUCs) at Baylor College of Medicine. For in vivo studies, 5- to 6-week-old male SCID mice (The Jackson Laboratory) were used for experimental lung metastasis assays. Human tissue samples were obtained from the Human Tissue Acquisition and Pathology Core of the Dan L. Duncan Comprehensive Cancer Center and were collected from fresh radical prostatectomy specimens after informed consent was obtained under an Institutional Review Board–approved protocol. Cancer samples contained a minimum of 70% cancer, and benign tissues were free of cancer on pathologic examination.

Cell culture. The human prostate cancer cell lines LNCaP, PC3, and DU145 and HEK293T cells were obtained from ATCC. PC3, DU145, and LNCaP cells were maintained in RPMI 1640 medium containing 10% FBS, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin at 37°C and 5% CO_2. The human embryonic kidney epithelial cell line HEK293T was maintained in DMEM supplemented with 10% FBS. For NGF treatment experiments, cells were maintained in serum-free culture medium for the stated periods of time supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin and then treated with NGF (50 ng/ml) for the specified periods of time.

Reagents and antibodies. Human NGF-β (catalog 300-174P) was obtained from Gemini Bio-Products. Primary antibodies were as follows: anti-pTrkA(Y785) (catalog 4168), anti-pTrkA(Y674/675) (catalog 4621), anti-p38 (catalog 9212), anti–p-p38 (catalog 9211), anti-Shc (catalog 2432), anti–SHP-1 (catalog 3759), anti–Na,K-ATPase (catalog 3010) from Cell Signaling Technology; anti-TRAF4 (catalog sc-10776), anti–HA-probe (catalog sc-805), anti-Ub (catalog sc-8017), anti-GAPDH (catalog sc-32233) from Santa Cruz Biotechnology Inc.; anti-TrkA (catalog 06-574), anti-Shc (catalog 06-203) from EMD Millipore; anti-pTrkA(Y490) (catalog ab85130), anti–Ki-67 (catalog 66155) from Abcam. HRP-conjugated secondary anti-mouse (catalog 1706516) or anti-rabbit (catalog 1706515) antibodies were obtained from Bio-Rad. Monoclonal ANTI-FLAG M2-peroxidase (HRP) antibody (catalog 8592A), EZview Red ANTI-FLAG M2 Affinity Gel (catalog F2426) were obtained from Sigma-Aldrich. TRAF4 adenovirus (catalog VH819961) was obtained from Vigene Biosciences. GFP adenovirus was produced in the Gene Vector Core at Baylor College of Medicine. GW441756 (catalog 141051) was purchased from Abcam.

Reverse transcription and quantitative real-time PCR. Total RNA was extracted from the indicated cells by using an RNeasy Mini Kit (QIAGEN). RNA concentration and purity were measured by a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific). 2 μg total RNA was used to generate cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Real-time PCR was performed using SYBR green PCR master mix (Life Technologies, Thermo Fisher Scientific). Primers used are listed in Supplemental Tables 1 and 2. For all RT-qPCR analysis, β-actin was used to normalize RNA input, and expression levels were calculated according to the comparative Ct method (ΔΔCT).

Construction of expression vectors and TrkA mutants. The TRAF4 TrkA cDNA was cloned into FLAG-tagged pSG5 expression vector. All TrkA deletion as well as lysine mutants were also cloned into FLAG-tagged pSG5 expression vector. In addition, TRAF4 was cloned into HA-tagged pCM5 expression vector. SHP-1 (catalog 8572), WT ubiquitin, and its mutant constructs were obtained from Addgene. TRAF4 shRNA was cloned into pLenti6/TR vector (Thermo Fisher Scientific). Primers used for cloning are listed in Supplemental Tables 1 and 2.

Transfection and lentivirus infection. Cells were transfected with plasmid DNA using Lipofectamine 3000 and siRNA using Lipofectamine RNAiMAX transfection reagent (both from Thermo Fisher Scientific) following the manufacturer's protocol. Virus packaging was performed in HEK293T cells after cotransfection of plasmid with the packaging plasmid psPAX2 and envelope plasmid pMD2.G using Lipofectamine 3000. Viruses were harvested 48 hours after transfection, and viral titers were determined. Target cells were infected with recombinant lentivirus-transducing units in the presence of 8 μg/ml Polybrene (Sigma-Aldrich).

Immunoblotting. Cells were harvested and protein was extracted from cells as previously described (72). The protein concentration was determined using a protein assay kit (Bio-Rad), and samples were separated in SDS polyacrylamide gels, with various concentrations depending on the molecular weight of the protein under investigation. After probing with a primary antibody, the membrane was incubated with a secondary antibody conjugated with HRP. Finally, signal intensity was determined using the enhanced chemiluminescence reagents. Endogenous GAPDH was used as the internal control.

MTS assay. The CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) reagent (catalog G358A) was obtained from Promega, and the assay was performed according to the manufacturer's instructions. Briefly, cells were seeded in a 96-well plate and treated with or without the TrkA inhibitor GW441756. The plate was incubated at 37°C in a humidified, 5% CO_2 atmosphere. 20 μl CellTiter 96 AQueous One Solution Reagent was added to each well containing 100 μl media and again incubated for 3 hours. Absorbance was measured at 490 nm using a microplate reader.

Cell migration and invasion assay. Cell invasion activity was determined in vitro using a BD BioCoat tumor invasion system (catalog 354483; BD Biosciences), which contains an 8-μm polyethylene terephthalate membrane with a thin layer of reconstituted Matrigel basement membrane matrix per the manufacturer's protocol. In brief, cells were harvested, resuspended in serum-free medium, and then transferred to the hydrated Matrigel chambers (~25,000 cells per well). The chambers were then incubated for 16 hours in culture medium with 10% FBS in the bottom chambers before examination. The cells on the upper surface were scraped and washed away, whereas the invaded cells on the lower surface were fixed and stained with 0.05% crystal violet for 2 hours. Finally, invaded cells were counted under a microscope, and the relative number was determined. Cell migration assay was performed by following the same procedures as in the cell invasion assay, except that a modified 2-chamber Transwell system was used in the migration assay (catalog 354578; BD Biosciences).

Ubiquitination screen assay. Targets of TRAF4-mediated ubiquitination were determined using an R&D Systems Proteome Profiler Human Ubiquitin Array Kit (catalog ARY027), which consists of 49 different protein samples, and a Proteome Profiler Human Phospho-RTK Array Kit (catalog ARY001B) consisting of 49 human receptor tyrosine kinases. Relative expression levels of ubiquitination of human proteins in samples were determined as per the manufacturer's protocol. Briefly, the human ubiquitin array nitrocellulose membranes spotted with 49 different antibodies to human ubiquitin target proteins were incubated with prepared cell lysates for 1 hour on a rocking platform

shaker. After thorough washing to remove unbound proteins, the membrane was incubated with biotinylated pan–anti-ubiquitin detection antibody cocktail at 4°C overnight. The next day the membrane was washed thoroughly, followed by addition of streptavidin-HRP. The signal produced at each capture spot corresponding to the relative amount of ubiquitinated protein bound was exposed to autoradiography film and analyzed.

In vitro ubiquitination assay. FLAG-TrkA was transiently transfected into 293T cells. The protein was then purified from 293T cell lysates using anti-FLAG M2 beads and eluted from the beads using 3X FLAG peptide (Sigma-Aldrich). The purified protein was incubated with 100 ng UBE1, 150 ng UbcH5a, and 5 μg HA-ubiquitin (Boston Biochem) in the absence or presence of 500 ng TRAF4 (Novus Biologicals) with ubiqutination buffer (50 mM Tris-Cl, pH 7.4, 2 mM ATP, 5 mM $MgCl_2$, 2 mM DTT) at 30°C for 90 minutes. The incubation mixture was then subjected to immunoprecipitation using an anti-FLAG antibody, followed by Western blot analysis using an anti-HA antibody.

Tail vein injection and IVIS imaging. The animal studies were conducted in accordance with NIH animal use guidelines, and the experimental protocol was approved by the Baylor College of Medicine Animal Care Research Committee. To evaluate the role of TRAF4 in tumor metastasis, luciferase-positive PC3 cells (control and TRAF4-knockdown by shRNA) were injected into SCID mice through the tail vein (1×10^6 cells per mouse). To study the effect of TrkA inhibition in tumor metastasis, we used a TrkA-specific inhibitor, GW441756. Luciferase-positive PC3 cells were injected into SCID mice through the tail vein (1×10^6 cells per mouse). Thereafter, mice were treated with i.p. injection twice a week with solvent control or GW441756 (10 mg/kg). Tumor metastasis in mice was assessed via in vivo bioluminescence measurement using the IVIS Imaging System (PerkinElmer). For the luciferase detection imaging, 200 μl of 15 mg/ml D-luciferin (Caliper Life Sciences) in PBS was injected i.p. before imaging. The photometry of the tumor was calculated by Living Image 3.1.0 software (Caliper Life Sciences), and the results were used to generate the tumor metastasis progression. Nine weeks after injection, mice were sacrificed, lungs were collected and fixed in Bouin's solution, and images were captured.

RPPA analysis. RPPA assays were carried out as described previously with minor modifications (73). Protein lysates were prepared from cultured cells with modified Tissue Protein Extraction Reagent (TPER; Pierce) and a cocktail of protease and phosphatase inhibitors (Roche Life Science) (73). The lysates were diluted into 0.5 mg/ml total protein in SDS sample buffer and denatured on the same day. The Aushon 2470 Arrayer (Aushon BioSystems) with a 40-pin (185 μm) configuration was used to spot samples and control lysates onto nitrocellulose-coated slides (Grace Bio-Labs) using an array format of 960 lysates/slide (2,880 spots/slide). The slides were processed as described (73) and probed with a set of 213 antibodies against total and phosphoproteins using an automated slide stainer (Autolink 48, Dako). Each slide was incubated with one specific primary antibody, and a negative control slide was incubated with antibody diluent instead of primary antibody. Primary antibody binding was detected using a biotinylated secondary antibody, followed by streptavidin-conjugated IRDye 680 fluorophore (LI-COR Biosciences). Total protein content of each spotted lysate was assessed by fluorescence staining with Sypro Ruby Protein Blot Stain according to the manufacturer's instructions (Molecular Probes).

Fluorescence-labeled slides were scanned on a GenePix AL4200 scanner, each slide, along with its accompanying negative control slide, was scanned at an appropriate photomultiplier (PMT) to obtain optimal signal for this specific set of samples. The images were analyzed with GenePix Pro 7.0 (Molecular Devices). Total fluorescence signal intensities of each spot were obtained after subtraction of the local background signal for each slide and were then normalized for variation in total protein, background, and nonspecific labeling using a group-based normalization method as described previously (Chang et al., 2015). For each spot on the array, the background-subtracted foreground signal intensity was subtracted by the corresponding signal intensity of the negative control slide (omission of primary antibody) and then normalized to the corresponding signal intensity of total protein for that spot. Each image, along with its normalized data, was carefully evaluated for quality through manual inspection and control samples. Antibody slides that failed the quality inspection were either repeated at the end of the staining runs or removed before data reporting. A total of 213 antibodies remained in the list. A complete list of validated antibodies can be found in Supplemental Table 3.

The median of the triplicate experimental values (normalized signal intensity) was taken for each sample for subsequent statistical analysis. We determined significantly changed proteins between experimental groups by employing Student's *t* test (significant for $P < 0.05$).

Kinase assay. The TrkA kinase assay was performed using the TrkA kinase enzyme system (catalog V2931) and ADP-Glo Kinase Assay kit (catalog V6930; Promega) as per the manufacturer's protocol. To compare the kinase activity of TrkA with and without TRAF4 overexpression and TrkA ubiquitin mutant with WT TrkA, 293T cells were transfected with different plasmids using Lipofectamine 3000. Cells were grown in complete media, followed by serum starvation for 4 hours. Thereafter, cells were briefly induced with NGF before harvesting. Cell lysis was performed using M-PER mammalian protein extraction reagent (Thermo Fisher Scientific). Overexpressed FLAG-tagged proteins were immunoprecipitated using EZview Red ANTI-FLAG M2 Affinity Gel. After thoroughly washing the beads with wash buffer (1× PBS, 1 mM DTT, 1× protease inhibitor), protein was eluted by incubating the beads at 4°C for 30 minutes with 100 μl of 3X FLAG peptide at a 100-ng/ml working concentration. An equal amount of protein was used to compare the kinase activity using the TrkA kinase enzyme system and ADP-Glo Kinase Assay kit. The TrkA reaction utilizes ATP and generates ADP. Then the ADP-Glo reagent terminates the kinase reaction and depletes the remaining ATP. Finally, the kinase detection reagent converts ADP to ATP, and the newly synthesized ATP emits light using the luciferase/luciferin reaction. The light generated correlates to the amount of ADP generated in the kinase or ATPase assay, which is indicative of kinase activity.

Statistics. Unless otherwise indicated, all results represent mean ± SEM, and statistical comparisons between different groups were performed using the 2-tailed Student's *t* test or 1-way ANOVA with multiple comparisons corrections. For all statistical analyses, differences of $P < 0.05$ were considered statistically significant, and experiments were repeated at least 3 times. GraphPad Prism software version 4.0/7.0 (GraphPad Software) was used for data analysis.

Study approval. All animal experiments were approved by the Institutional Animal Care and Use Committees (IACUCs) at Baylor College of Medicine. Human tissue samples were obtained from the Human Tissue Acquisition and Pathology Core of the Dan L. Duncan

Comprehensive Cancer Center and were collected from fresh radical prostatectomy specimens after obtaining written informed consent under an Institutional Review Board–approved protocol.

Author contributions

RS, BWO, and PY conceived and designed the experiments. RS and DK performed cell studies. RS, JS, SD, and HS performed animal studies. SH and DPE performed RPPA analysis. MMI provided human prostate tumor samples. RS, BWO, and PY interpreted the data and wrote the manuscript. All the authors discussed the results and commented on the manuscript.

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Address correspondence to: Ping Yi, Department of Molecular and Cellular Biology, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030, USA. Phone: 713.798.6247; Email: pyi@bcm.edu.

DK's present address is: University of Texas Southwestern Medical School, Dallas, Texas, USA.

SD's present address is: Department of Cell Stress Biology, Roswell Park Comprehensive Cancer Center, Buffalo, New York, USA.

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