PNAS

Supporting Information for

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

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Other supporting materials for this manuscript include the following:

Datasets S1 to S3

Supplemental 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 phenol red-free RPMI 1640 containing 5% charcoal stripped serum 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 a 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 Tables S1 and Dataset S3. 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 cDNA was cloned into pLenti6/TR vector (Thermo Fisher Scientific). The E2F-responsive plasmid pE2F-TA-Luc was from CLONTECH Laboratories, Inc. (Palo Alto, CA). Flag-tagged AR wild type or

K913R mutant was also cloned into pINDUCER vector for doxycycline-induced overexpression experiments.

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. Silencer Select negative control siRNA or specific siRNAs were obtained from ThermoFisher. 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 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. For Abl cell growth and some LNCaP cell growth experiments, equal number of cells were seeded on 6-well plates and cell were counted in triplicates at indicated days through Countess automated cell counter (Invitrogen). For C4-2 cell growth assay, CellTiter-Glo luminescence assay was used following the manufacturer's instructions.

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. For mass spectrometry identification of AR ubiquitination sites, the same in vitro ubiquitination assay was performed using purified recombinant AR protein, followed by 4-15% SDS-PAGE separation. Different regions of the gel were then cut and subjected to chymotrypsin/trypsin digestion followed by mass spectrometry analysis.

RNA-Seq The RNA samples from LNCaP control or TRAF4 overexpressing cells (n=5), or tumor tissues (n=2) were isolated with the miRNeasy Mini Kit (Qiagen) per the manufacturer's instructions. The quantity and integrity of the RNA was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and 1% agarose gel electrophoresis. 500ng to 1ug of total RNA from the individual cell lines or tumor tissues were converted into mRNA-Seq libraries with the non-directional, Poly-A tail selection and library preparation kit (Illumina) per the manufacturer's protocol. Only the RNA samples with RIN values ≥8 were used for constructing the cDNA libraries. The cDNA libraries were sequenced using the Illumina Platform PE150 Q30≥80% with a paired-end read length of 150 bp for an average of 30 million paired-end reads per sample. The library construction and sequencing were performed by the Novogene Corporation (Beijing, China). The Fastq files containing raw RNA-Seq reads were aligned to the human genome (GRCh38/hg38) using HISAT2 (2.1.0). The paired-end reads below MAPQ quality score 15 were removed. The RNA-Seq quantification was performed with featureCounts (1.6.4, default parameters). Protein-coding genes were quantified by UCSC RefSeq gene annotation. GSEA with default phenotype permutation option was used for all the UCSC genes mapped to hg38 (in total 26485 RefSeq genes) with normalized counts at counts-per-million (CPM). GSEA Hallmarks gene sets and oncogenic signatures (v2022.1.Hs) were utilized for the unbiased GSEA enrichment analysis. Differential expression analyses were performed using DeSeq2 and limma-voom. For volcano, heatmap and other differential expression plots, a threshold at an adjusted P value < 0.005 and TRAF4/Control fold change (FC) > 1.5 was utilized. At this cutoff we identified 3491 differentially expressed genes, 1723 upregulated and 1768 downregulated genes, respectively (TRAF4 overexpression/Control).

ChIP assay ChIP assays were performed using the ChIP-IT Express Kit (Active Motif) following the manufacturer's instructions. Briefly, cells 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.

ChIP seq Around 20 million LNCaP control or TRAF4 stable T2 cells were grown in RPMI1640 supplemented with 10% FBS and streptomycin. The culture medium was changed to phenol red-free RMPI1640 supplemented with 10% charcoal-stripped FBS and streptomycin for two days before harvesting. Cells were fixed in 1% formaldehyde at room temperature for 15 minutes before harvesting. The frozen cell pellets were then submitted to Active Motif for ChIP seq service using an AR-specific antibody.

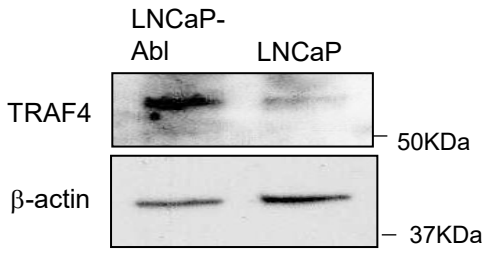
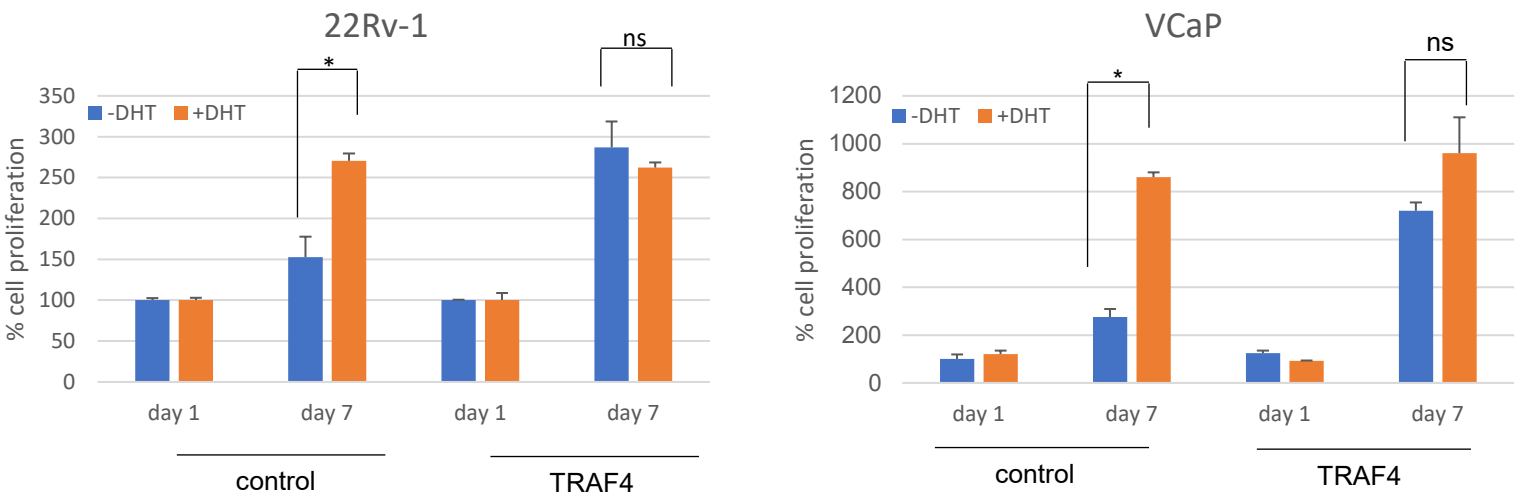


Fig S1: TRAF4 protein level is higher in androgen-insensitive compared to androgen-sensitive prostate cancer cells. Shown is a Western blot analysis using TRAF4-specific antibody. LNCaP-Abl is a LNCaP derivative grown in androgen-deprived culture medium.

A



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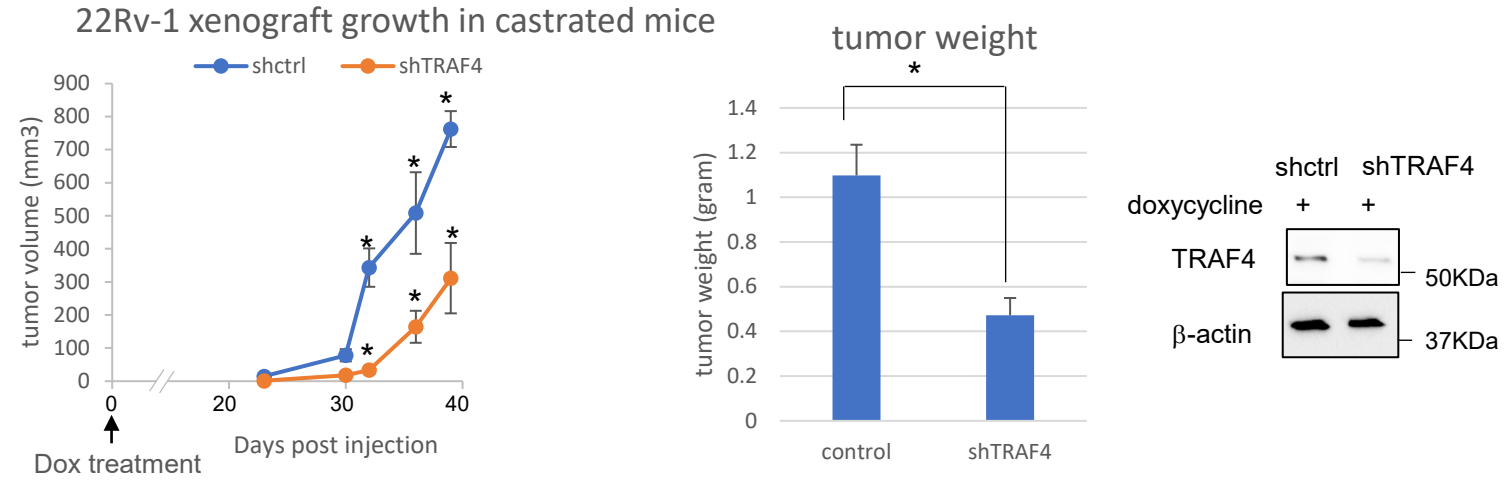
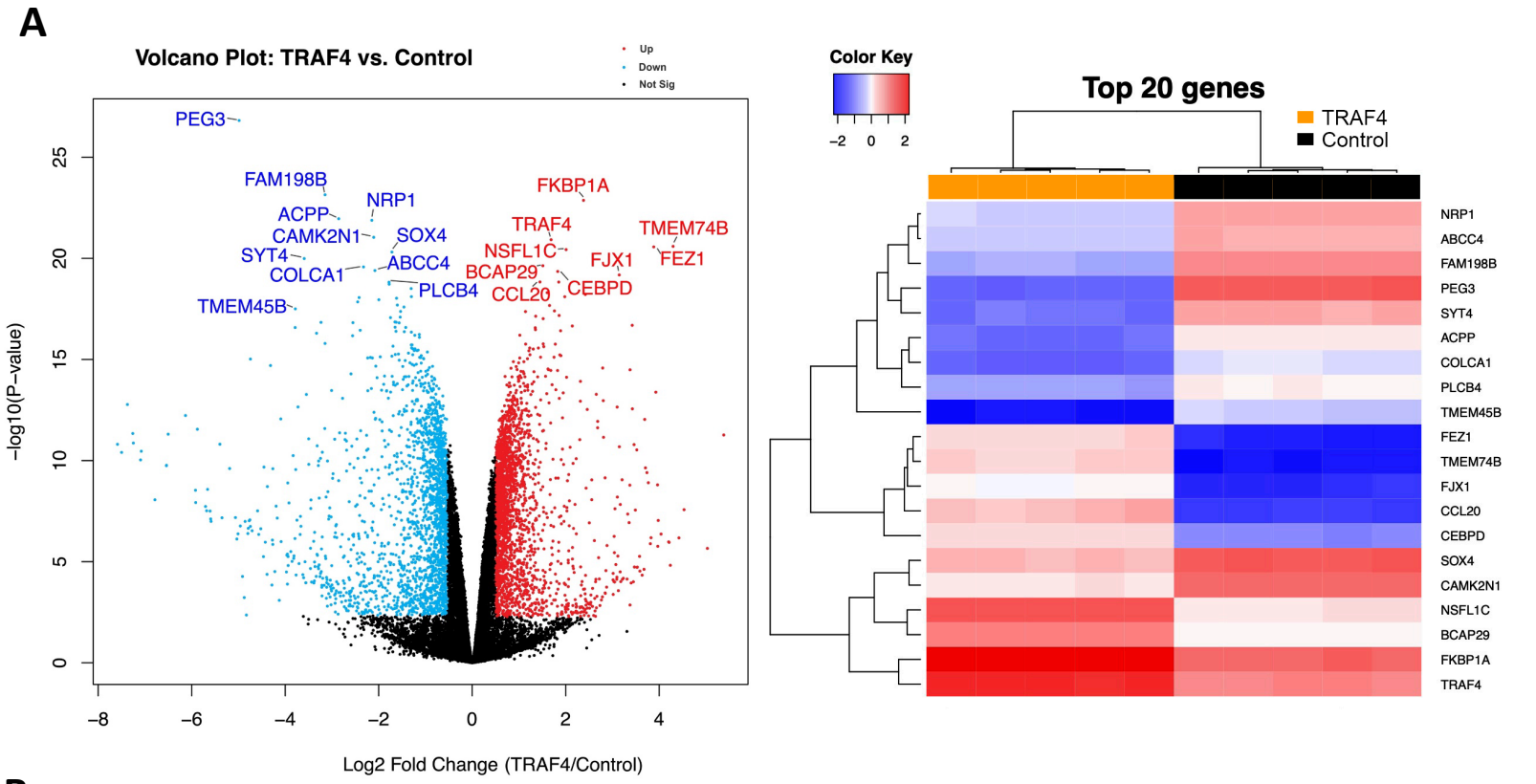


Fig S2: TRAF4 overexpression promotes 22Rv1 and VCaP cell androgen-independent cell growth. (A) MTS cell proliferation assay was performed in TRAF4 overexpressing 22Rv1 and VCaP cells or control cells grown in culture media with and without 1nM androgen (DHT). (B) TRAF4 knockdown reduced 22Rv-1 xenograft (subcutaneously injected) tumor growth (left panel) in castrated mice (n=6). Mice received 2mg/ml doxycycline containing water to induce TRAF4 knockdown starting at the time of cell injection. Middle panel: the tumor weights of control or TRAF4 knockdown tumors. Right panel: Western blot analysis of the protein levels of TRAF4 in shTRAF4 vs. shctrl 22Rv-1 cells. * represents $P<0.05$.

Fig S3


A
Volcano Plot: TRAF4 vs. Control
Up
Down
Not Sig
PEG3
FAM198B
NRP1
ACPP
CAMK2N1
SOX4
SYT4
ABCC4
COLCA1
PLCB4
TMEM45B
FKBP1A
TRAF4
NSFL1C
BCAP29
CCL20
CEBPD
FJX1
TMEM74B
FEZ1
−log10(P−value)
Log2 Fold Change (TRAF4/Control)
Color Key
−2 0 2
Top 20 genes
TRAF4
Control
NRP1
ABCC4
FAM198B
PEG3
SYT4
ACPP
COLCA1
PLCB4
TMEM45B
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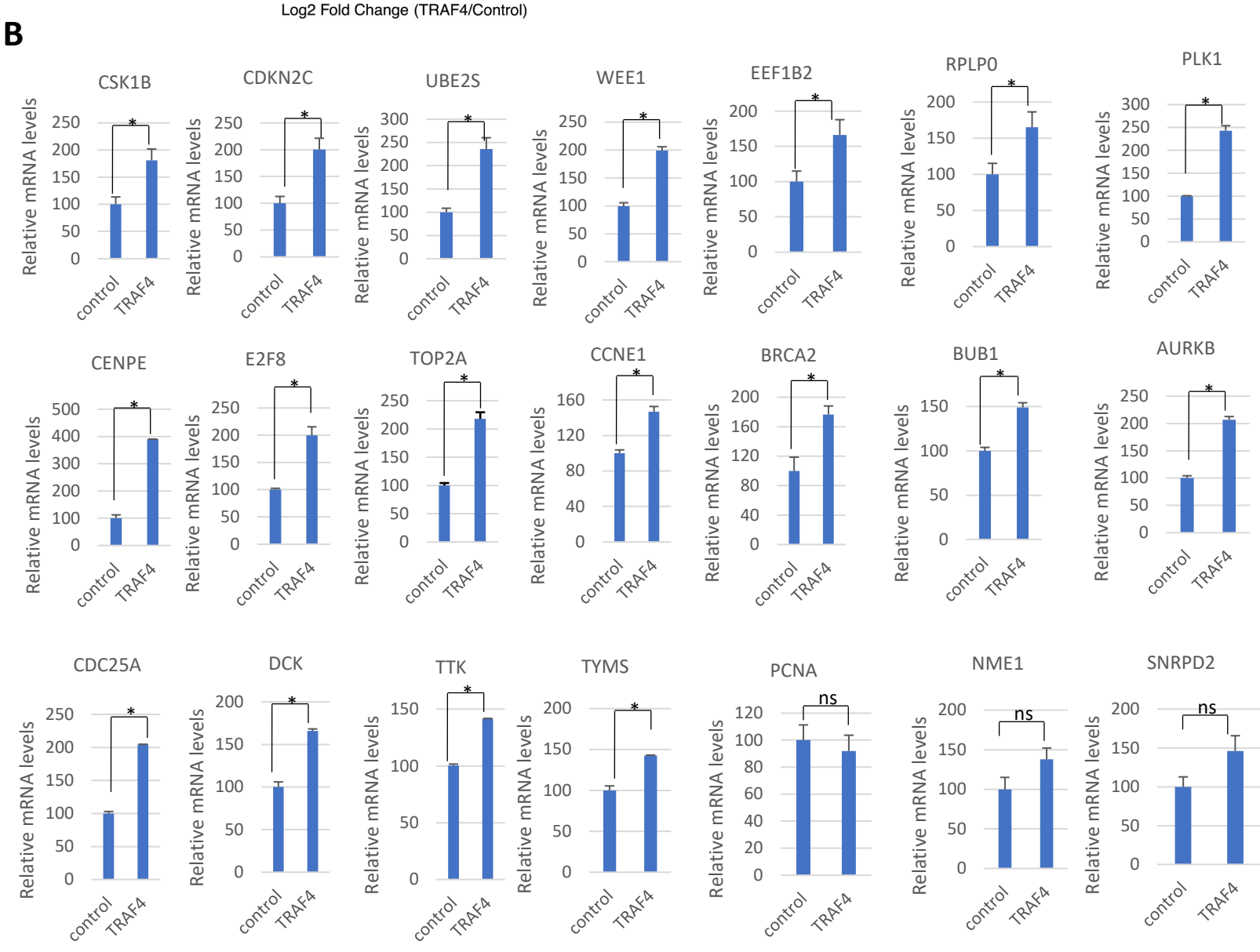
TRAF4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CDKN2C
UBE2S
WEE1
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E2F8
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CCNE1
BRCA2
BUB1
AURKB
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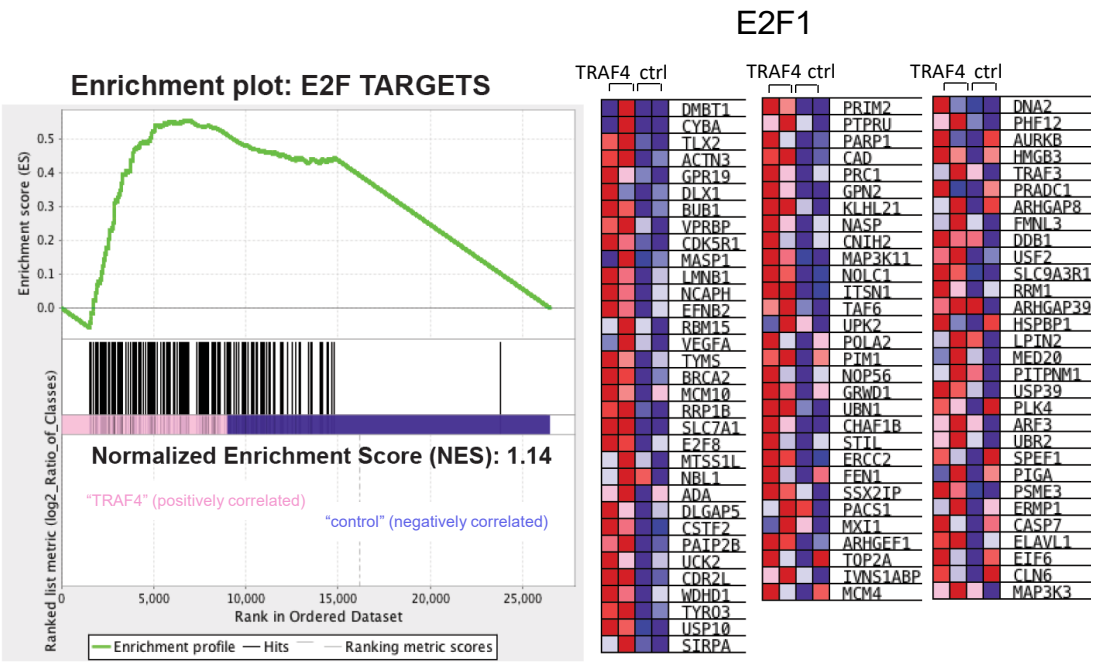
DCK
TTK
TYMS
PCNA
NME1
SNRPD2
Relative mRNA levels
control
TRAF4
*
ns

C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Enrichment score (ES)
Ranked list metric (log2_Ratio_of_Classes)
Normalized Enrichment Score (NES): 1.14
"TRAF4" (positively correlated)
"control" (negatively correlated)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores
TRAF4 ctrl
DMBT1
CYBA
TLX2
ACTN3
GPR19
DLX1
BUB1
VPRBP
CDK5R1
MASP1
LMNB1
NCAPH
EFNB2
RBM15
VEGFA
TYMS
BRCA2
MCM10
RRP1B
SLC7A1
E2F8
MTSS1L
NBL1
ADA
DLGAP5
CSTF2
PAIP2B
UCK2
CDR2L
WDHD1
TYRO3
USP10
SIRPA
PRIM2
PTPRU
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GPN2
KLHL21
NASP
CNIH2
MAP3K11
NOLC1
ITSN1
TAF6
UPK2
POLA2
PIM1
NOP56
GRWD1
UBN1
CHAF1B
STIL
ERCC2
FEN1
SSX2IP
PACS1
MXI1
ARHGEF1
TOP2A
IVNS1ABP
MCM4
DNA2
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MED20
PITPNM1
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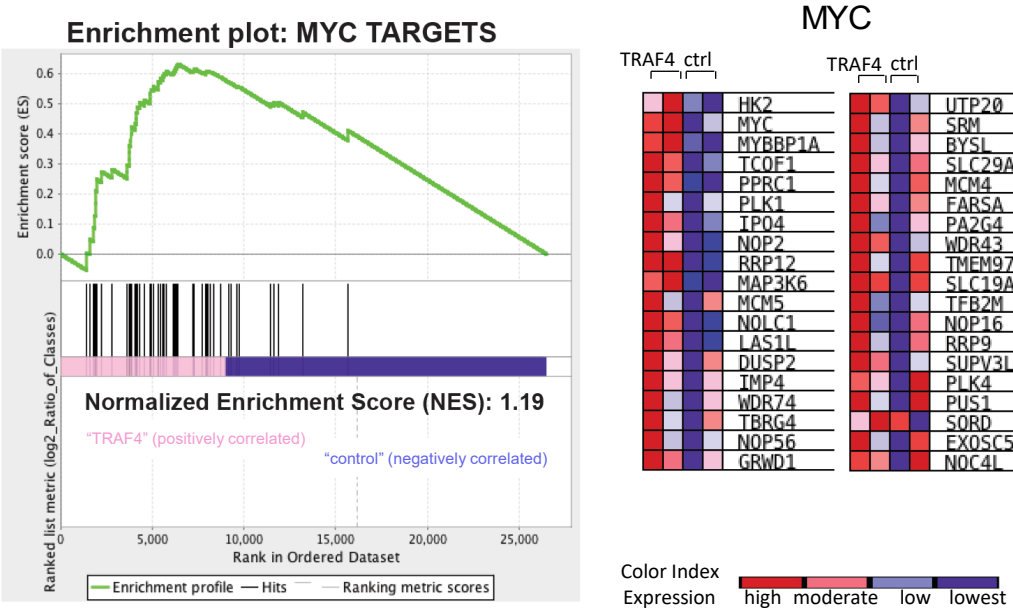
MYC
Enrichment plot: MYC TARGETS
Enrichment score (ES)
Ranked list metric (log2_Ratio_of_Classes)
Normalized Enrichment Score (NES): 1.19
"TRAF4" (positively correlated)
"control" (negatively correlated)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores
TRAF4 ctrl
HK2
MYC
MYBBP1A
TCOF1
PPRC1
PLK1
IPO4
NOP2
RRP12
MAP3K6
MCM5
NOLC1
LAS1L
DUSP2
IMP4
WDR74
TBRG4
NOP56
GRWD1
UTP20
SRM
BYSL
SLC29A2
MCM4
FARSA
PA2G4
WDR43
TMEM97
SLC19A1
TFB2M
NOP16
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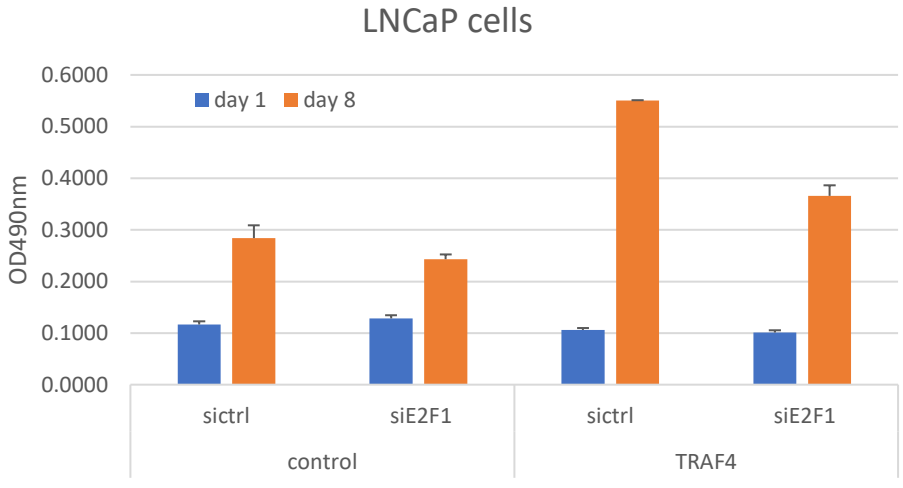
PLK4
PUS1
SORD
EXOSC5
NOC4L
Color Index
Expression
high
moderate
low
lowest

D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OD490nm
day 1
day 8
sictrl
siE2F1
sictrl
siE2F1
control
TRAF4

E

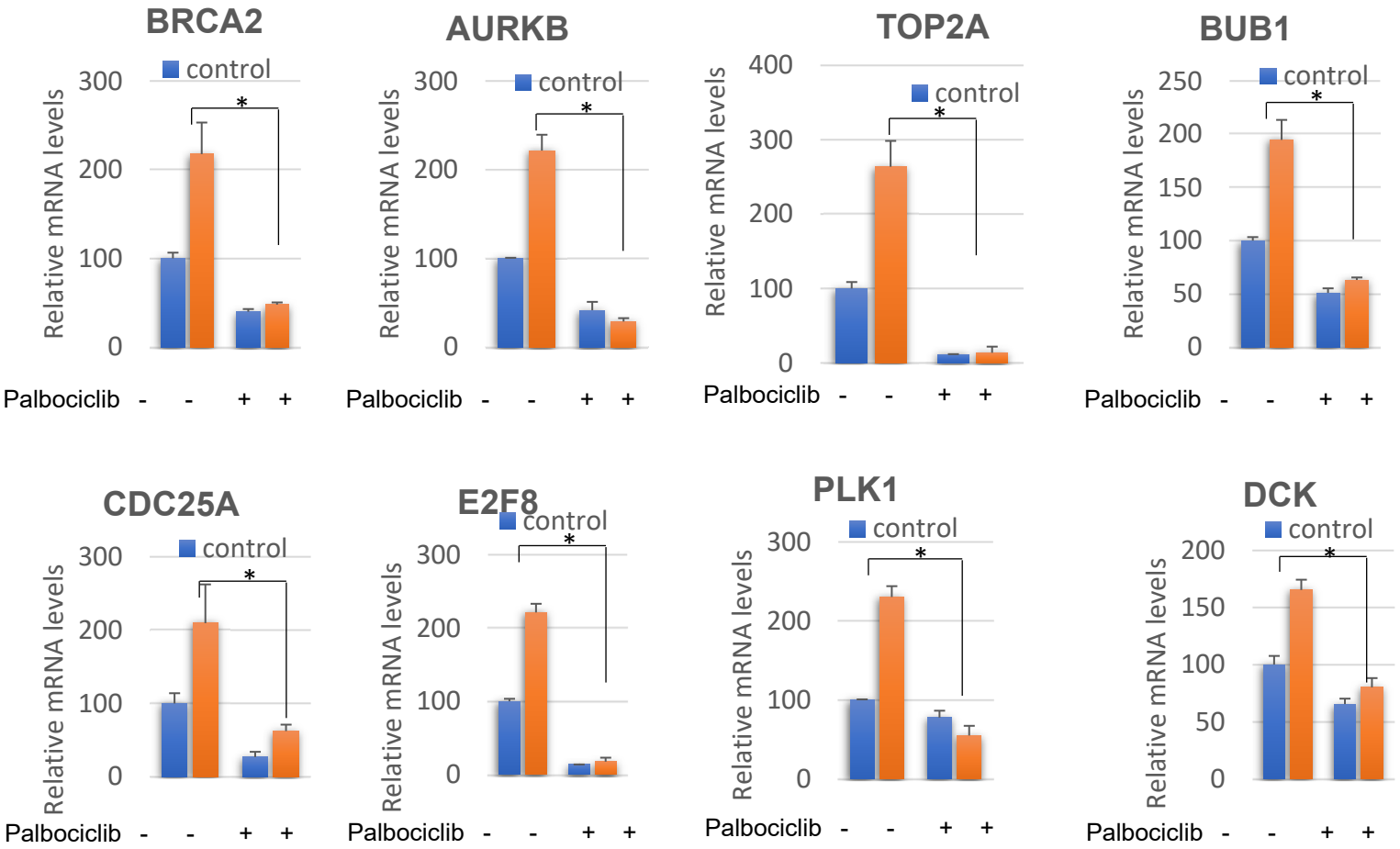


Fig. S3: TRAF4 overexpression up-regulates the E2F-regulated gene pathway. (A) Volcano plot (left) and heat map of top regulated genes (right) of TRAF4 overexpressing vs. control LNCaP RNA seq data. The cut-off at an adjusted P value < 0.005 and TRAF4/Control fold change (FC) > 1.5 were utilized. At this cut-off we identified 3491 differentially expressed genes, 1723 upregulated and 1768 downregulated genes, respectively. The differential genes in the volcano plot and the representative top 20 genes were labeled in red (upregulated), blue (downregulated) or black (not significant) as indicated. The Euclidean distance and complete clustering methods were used for computing the hierarchical clusters in the heat map (color key in log2 value). See the supplementary file-2 for the full gene list with normalized counts and statistics. (B) qPCR validation of TRAF4-upregulated E2F or Myc-regulated genes identified in RNA seq. (C) Gene set enrichment analysis (GSEA) of the genes associated with TRAF4 overexpression in xenograft tumors grown in castrated mice. Genes enriched in E2F1, and MYC pathways are shown. The genes associated with the leading edge of the enrichment score are shown in the heat map (right panel). The color gradient matches the expression status of individual genes, as shown in the color index. (D) E2F1 knockdown inhibited TRAF4-promoted cell growth under androgen-deprived conditions. Shown are MTS assay results of LNCaP control or TRAF4 overexpressing cells cultured in the absence of androgen at day 1 and day 8. The OD490nm is the colorimetric absorbance at 490nm determining the number of viable cells. (E) CDK4/6 inhibitor Palbociclib treatment abolished TRAF4-promoted E2F target gene upregulation. LNCaP control or TRAF4 overexpressing cells were treated with 1mM Palbociclib for one day before harvesting and RNA extraction. Shown are qPCR results of E2F target gene expressions.

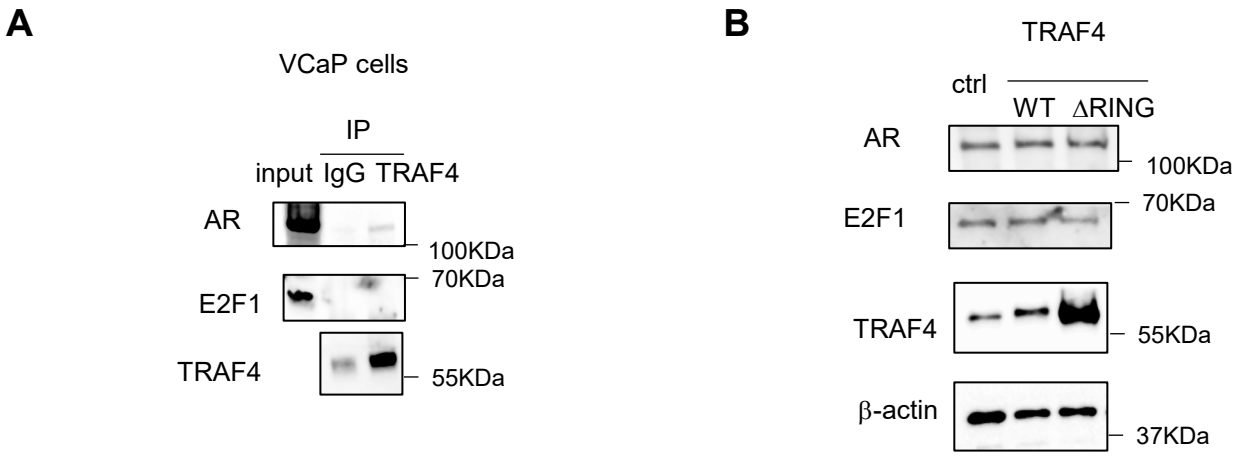


Fig S4:TRAF4 specifically interacts with AR (A) The association between endogenous AR and an endogenous TRAF4 in VCaP cells. Shown is a co-IP experiment using a TRAF4-specific antibody or IgG control. A specific binding between AR but not E2F1 with TRAF4 was detected. (B) AR or E2F1 protein levels are not significantly changed in TRAF4 overexpressing cells. Shown are Western blot results of AR and E2F1 in control, TRAF4 wild type or DRING overexpressing cells.

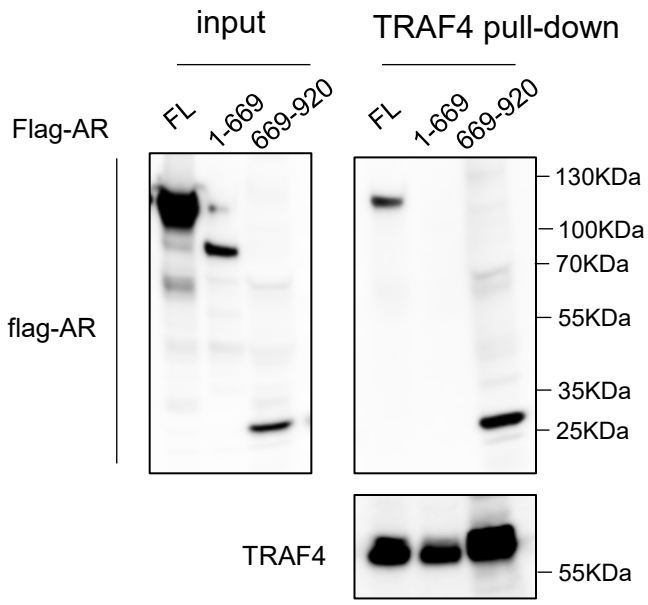
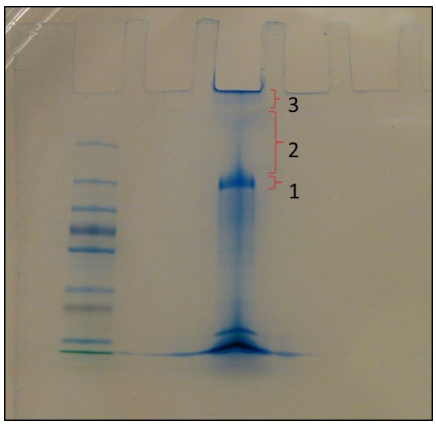


Fig. S5: TRAF4 interacts with the LBD in vitro. Ni beads-bound purified His-tagged recombinant TRAF4 protein was incubated with flag-tagged AR full-length or two different AR deletion mutants transfected 293T cell lysates. Shown are the Western blot results of associated AR proteins pulled-down by Ni-bound TRAF4 and the inputs of AR proteins.



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

Fig S6: K913 is a 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 of AR after in vitro TRAF4-mediated ubiquitination, and corresponding ubiquitination sites identified from each band.

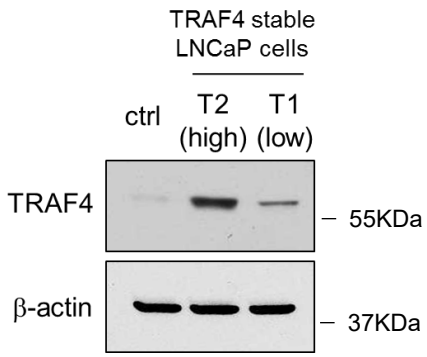
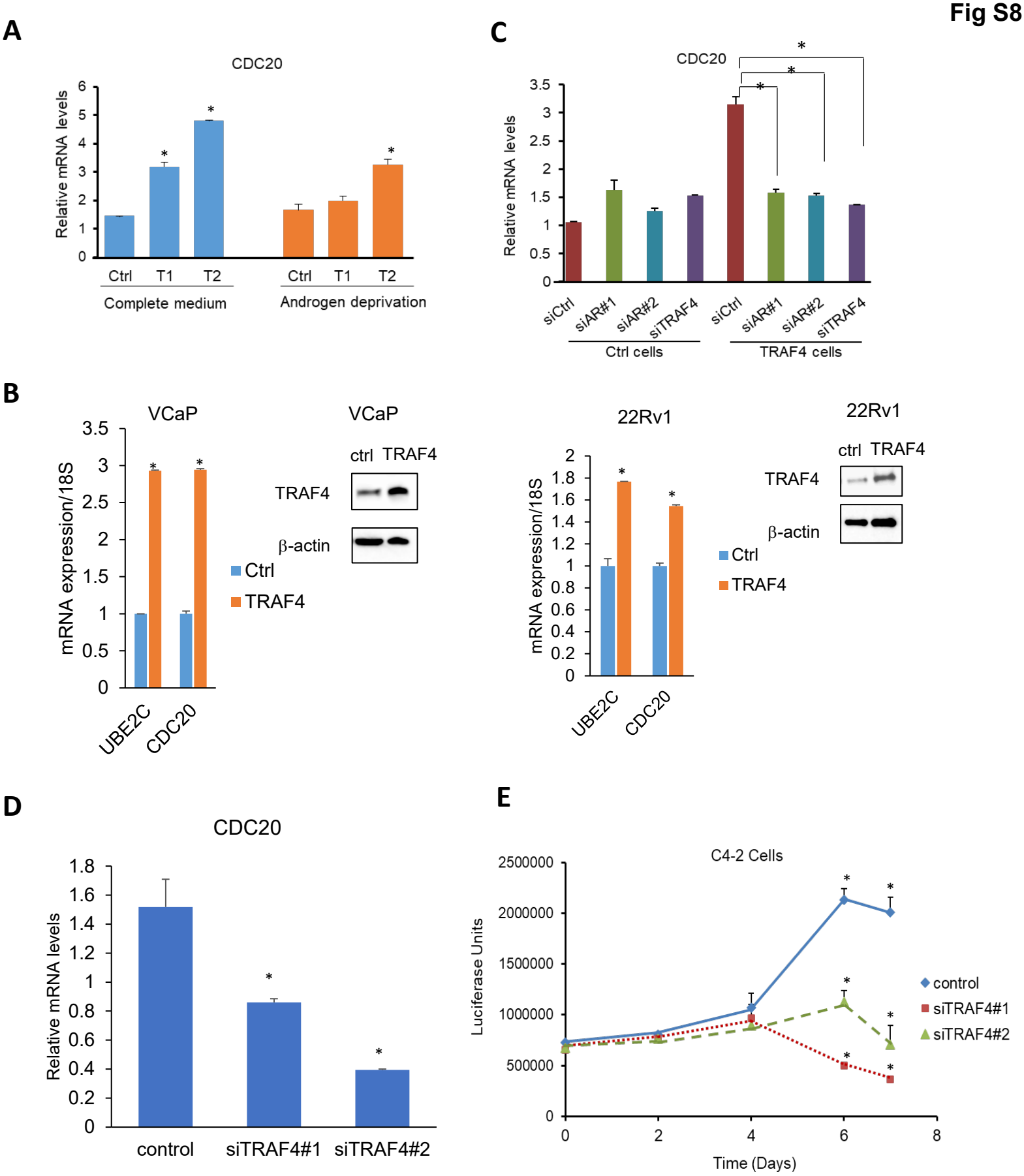


Fig S7: TRAF4 expression levels in TRAF4 high (T2) or low (T1) expressing LNCaP cells.

Fig S8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CDC20
Relative mRNA levels
Ctrl
T1
T2
Complete medium
Androgen deprivation
C
CDC20
Relative mRNA levels
siCtrl
siAR#1
siAR#2
siTRAF4
Ctrl cells
TRAF4 cells
B
VCaP
mRNA expression/18S
UBE2C
CDC20
ctrl
TRAF4
β-actin
Ctrl
TRAF4
22Rv1
D
CDC20
Relative mRNA levels
control
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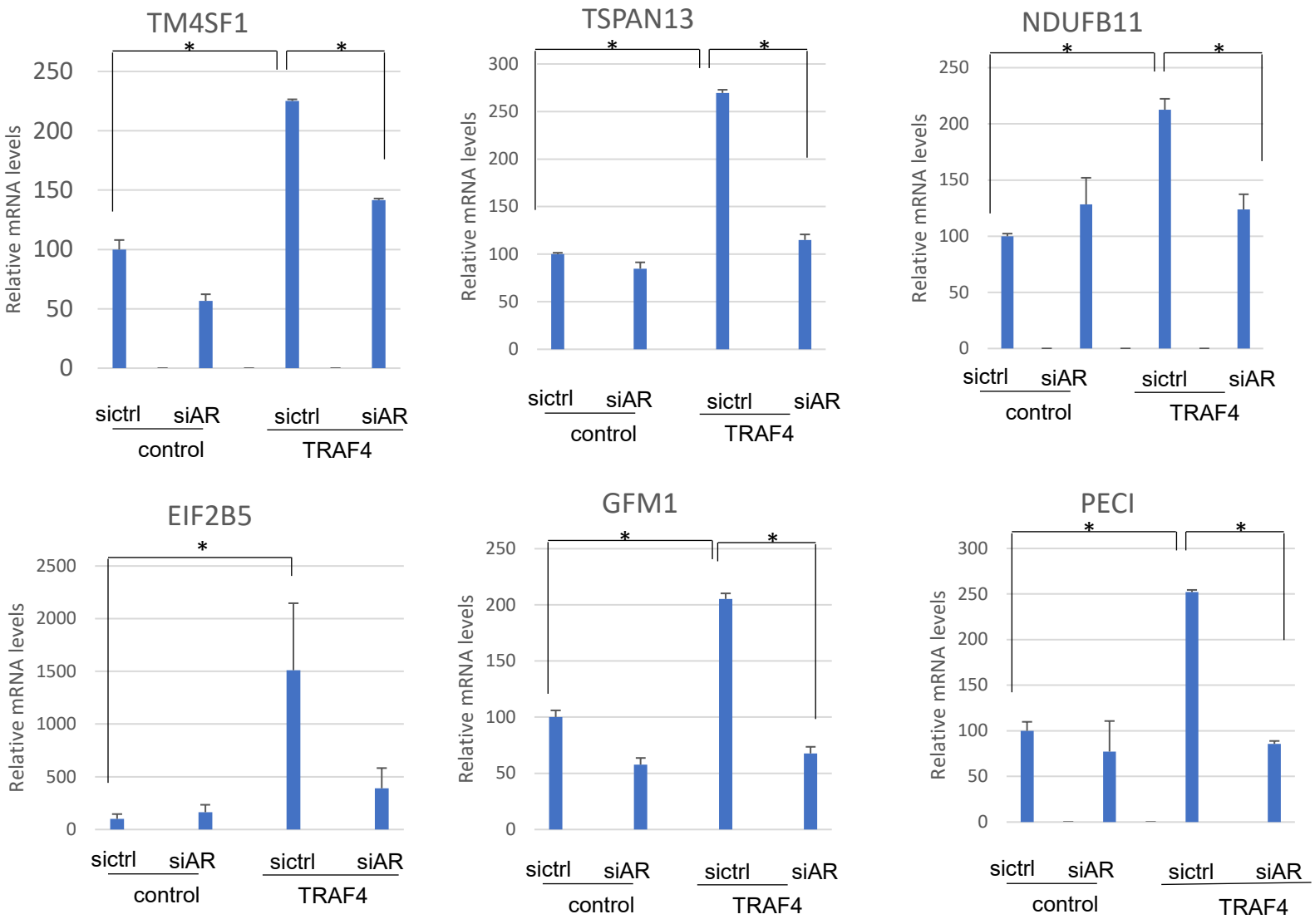
Luciferase Units
Time (Days)
control
siTRAF4#1
siTRAF4#2

F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Relative mRNA levels
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200
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100
50
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*
sictrl
siAR
sictrl
siAR
control
TRAF4
TSPAN13
Relative mRNA levels
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250
200
150
100
50
0
*
*
sictrl
siAR
sictrl
siAR
control
TRAF4
NDUFB11
Relative mRNA levels
250
200
150
100
50
0
*
*
sictrl
siAR
sictrl
siAR
control
TRAF4
EIF2B5
Relative mRNA levels
2500
2000
1500
1000
500
0
*
sictrl
siAR
sictrl
siAR
control
TRAF4
GFM1
Relative mRNA levels
250
200
150
100
50
0
*
*
sictrl
siAR
sictrl
siAR
control
TRAF4
PECI
Relative mRNA levels
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200
150
100
50
0
*
*
sictrl
siAR
sictrl
siAR
control
TRAF4

G

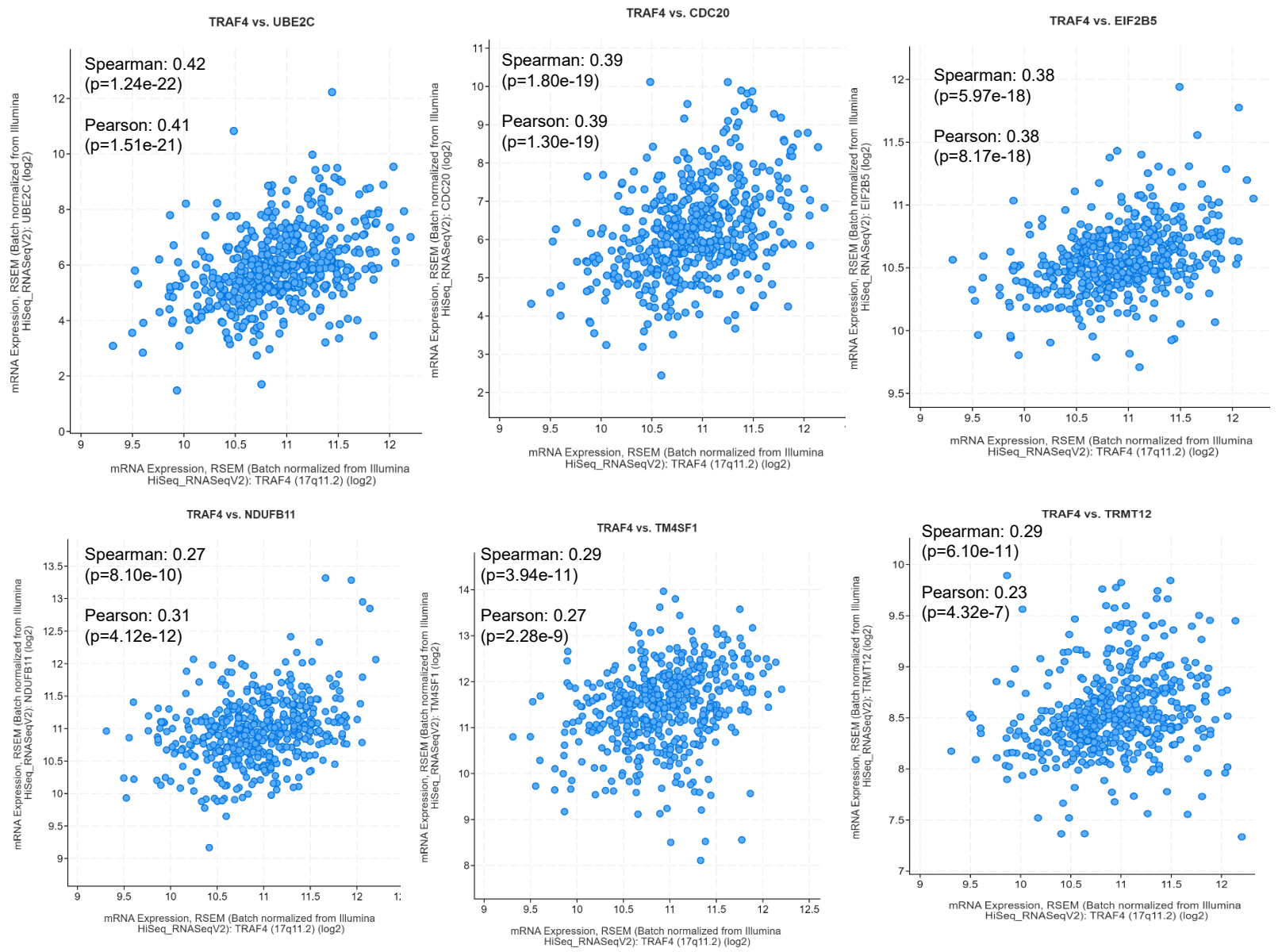


Fig S8: TRAF4 upregulates CDC20 gene expression through AR. (A) TRAF4 overexpression up-regulates CDC20 gene expression. Shown are qRT-PCR results of CDC20 mRNA levels in control or TRAF4 stable cells (T1 or T2). (B) TRAF4 overexpression upregulates UBE2C and CDC20 gene expressions in VCaP and 22Rv1 cell. The expression levels of TRAF4 in control and TRAF4 overexpressing cells were shown through Western blot analysis. (C) AR depletion abolishes TRAF4-upregulated CDC20 expression. (D) TRAF4 depletion in C4-2 CRPC cells decreases CDC20 expression. (E) Depletion of TRAF4 inhibits CRPC LNCaP-C4-2 cell growth as measured by CellTiter-Glo luminescent Cell viability assay. (F) TRAF4 upregulates several CRPC signature genes through AR. Shown are qRT-PCR results of several CRPC-associated genes in control and TRAF4 overexpressing cells treated with control siRNA or siAR. (G) TRAF4 expression levels are significantly correlated with AR-regulated CRPC signature genes in prostate adenocarcinoma (TCGA PanCancer Atlas) (n=488, from cBioPortal).

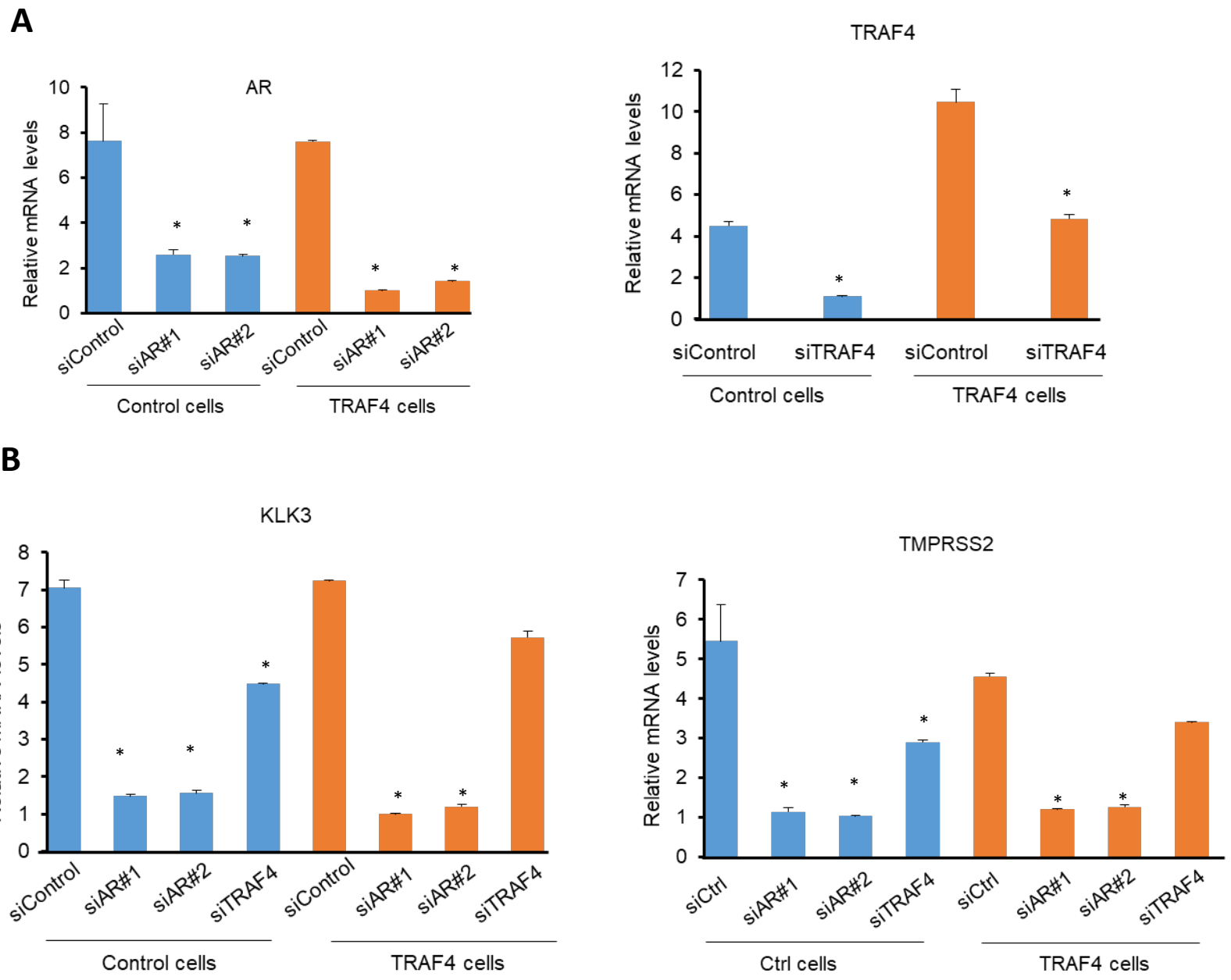
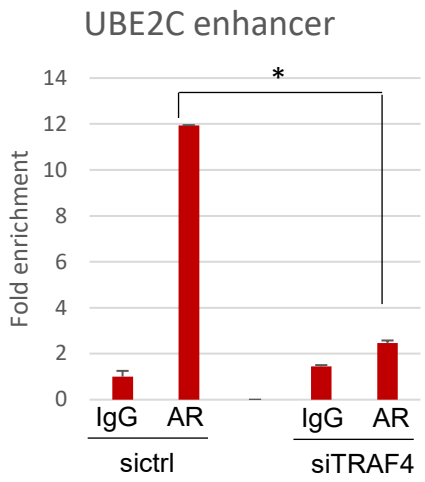


Fig S9: TRAF4 depletion does not dramatically reduce classical AR target expression. (A) The levels of AR (left panel) or TRAF4 (right panel) after siRNA treatment. (B) AR depletion but not TRAF4 depletion significantly reduced classical AR target gene (KLK3 and TMPRSS2) expression levels.

A



B

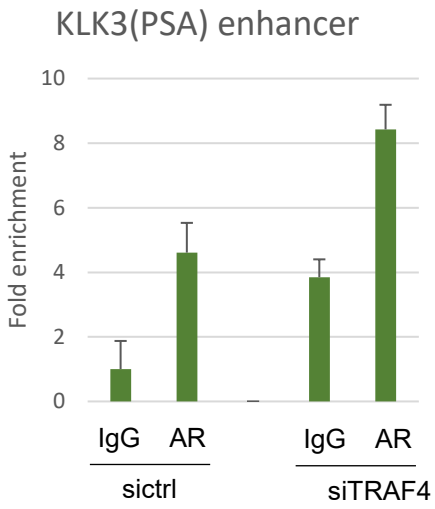


Fig S10: TRAF4 depletion abolished AR binding to UBE2C enhancer (A) but not the PSA enhancer (B) in LNCaP-Abl cells. Shown is a ChIP experiment using an AR-specific antibody or an IgG control.

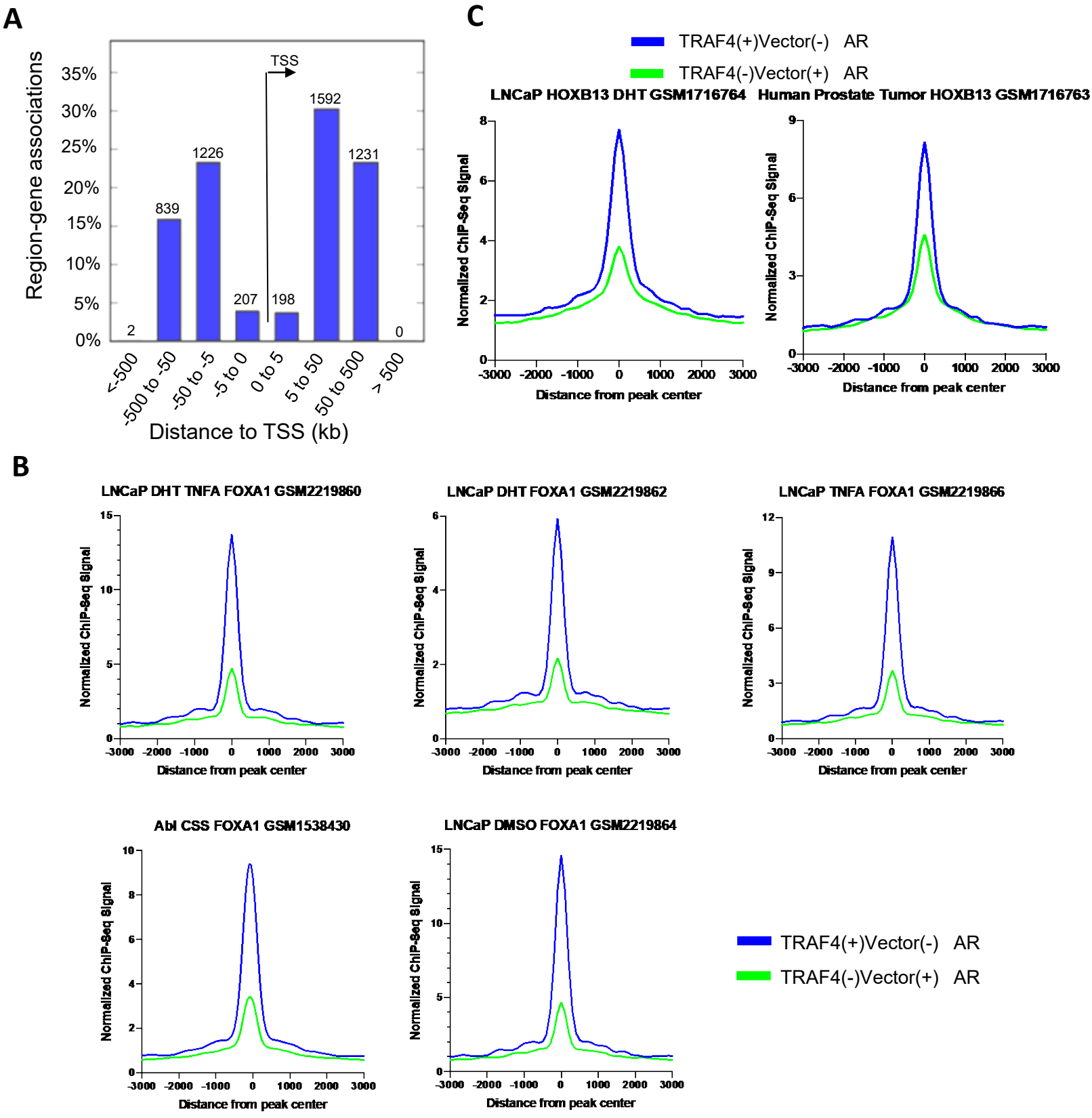


Fig S11: AR gained stronger FOXA1 and HOXB13 binding sites in TRAF4 overexpressing cells. (A) Distribution of gained AR binding peaks from transcription start site (TSS). (B) AR gained stronger FOXA1 binding sites in TRAF4 overexpressing cells in multiple datasets. (C) AR gained stronger HOXB13 binding sites in TRAF4 overexpressing cells.

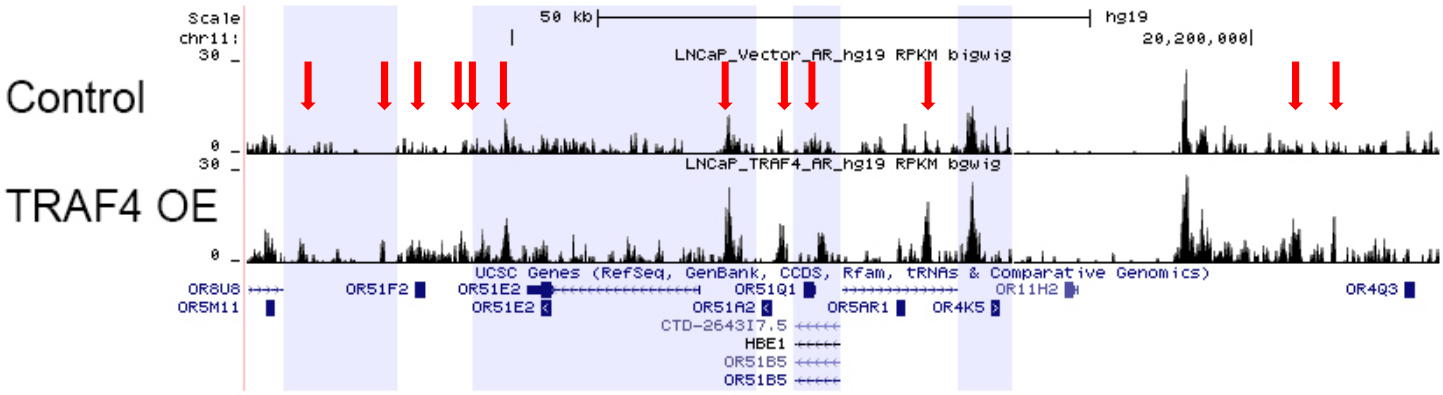


Fig S12: UCSC genome browser view of AR binding to OR gene chromatin locations in LNCaP control or TRAF4 overexpressing cells. Blue and white color separate different regions. Red arrows indicate some examples of significantly upregulated AR binding in TRAF4 overexpressing (OE) cells compared to in control cells.

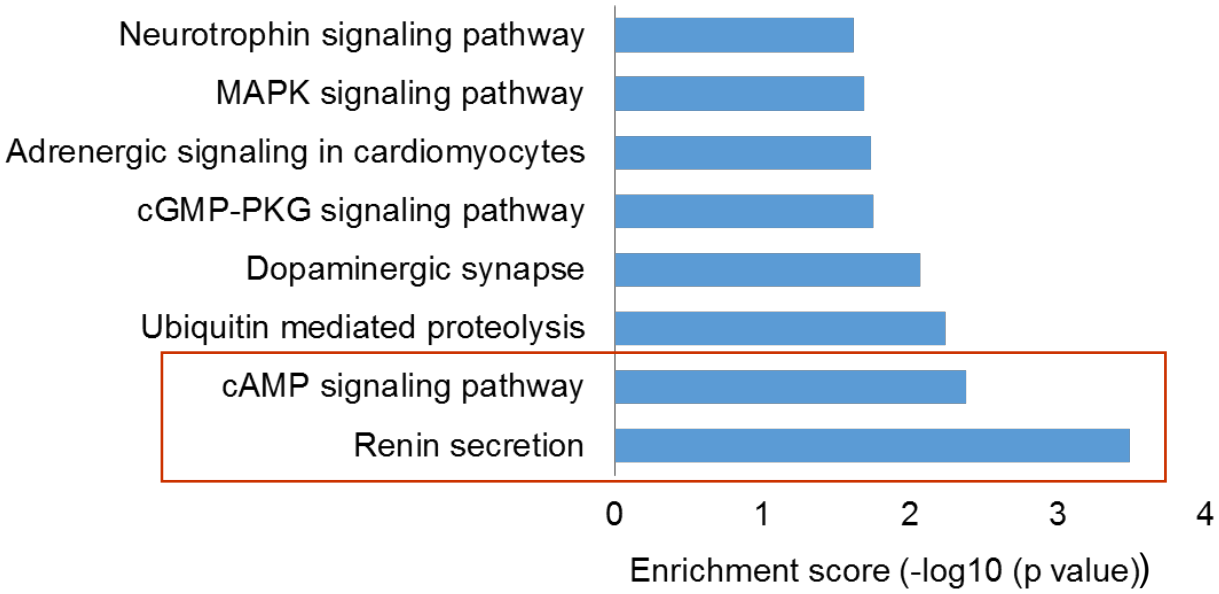


Fig S13: List of top enriched pathway in AR gained genomic binding loci upon TRAF4 overexpression. Shown is the KEGG pathway analysis in top AR gained peaks (log_2 TRAF4/vector >1).

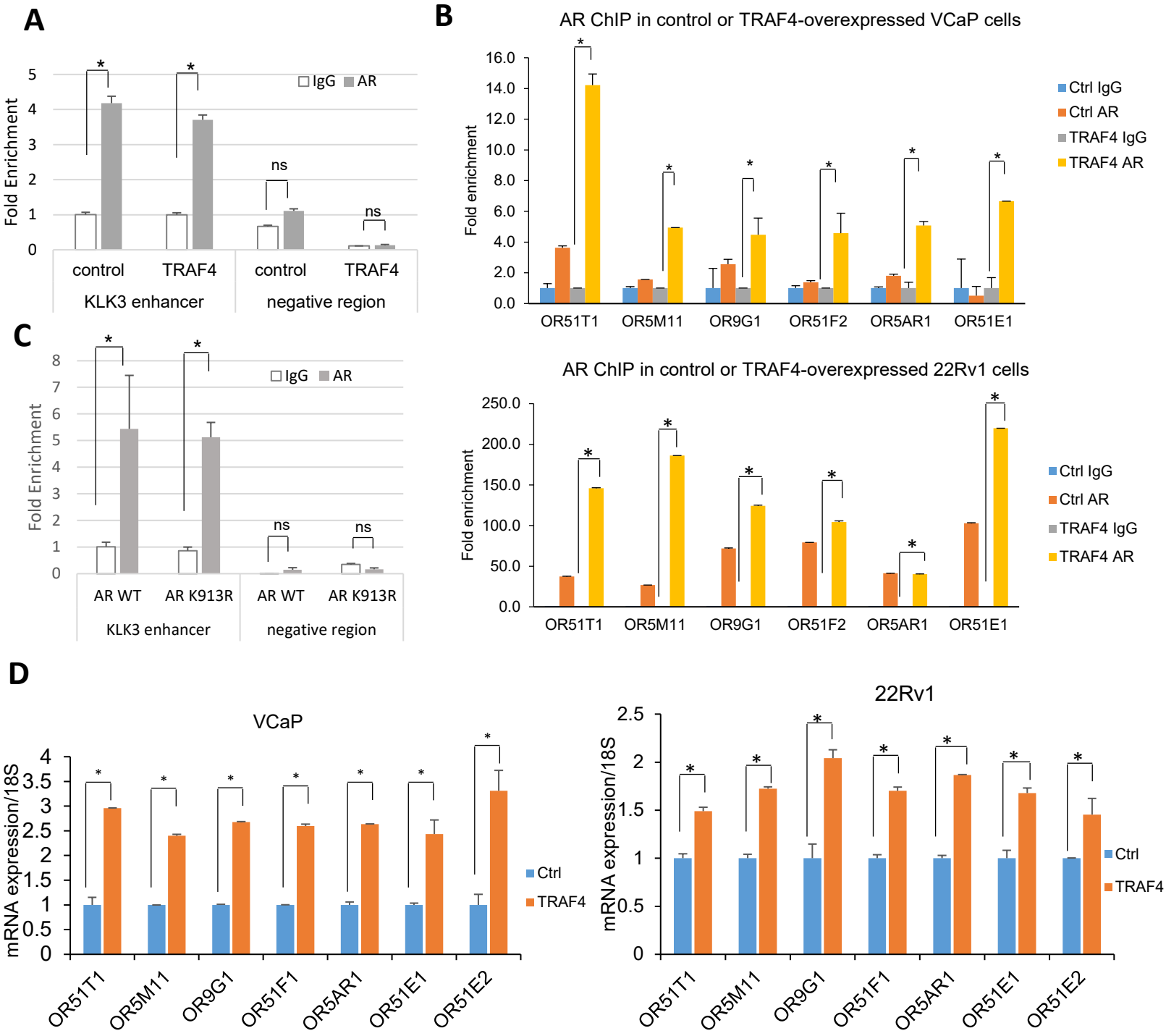


Fig S14: TRAF4 overexpression increased AR binding to OR family gene enhancers and upregulates OR gene expression. (A) ChIP experiment showing no significant changes in AR binding to KLK3 (PSA) enhancer or a negative binding region in control or TRAF4 overexpressing cells. (B) AR binding to OR gene enhancers in control or TRAF4 overexpressing VCaP (upper panel) and 22Rv1 cells (lower panel) as assessed through the ChIP experiment. (C) ChIP experiment showing no significant changes in AR binding to KLK3 (PSA) enhancer or a negative binding region in TRAF4 overexpressing cells treated with siAR followed by AR wild type or K913R overexpression. (D) TRAF4 increased OR gene expression in VCaP (left panel) and 22Rv1 cells (right panel) as assessed by qRT-PCR.

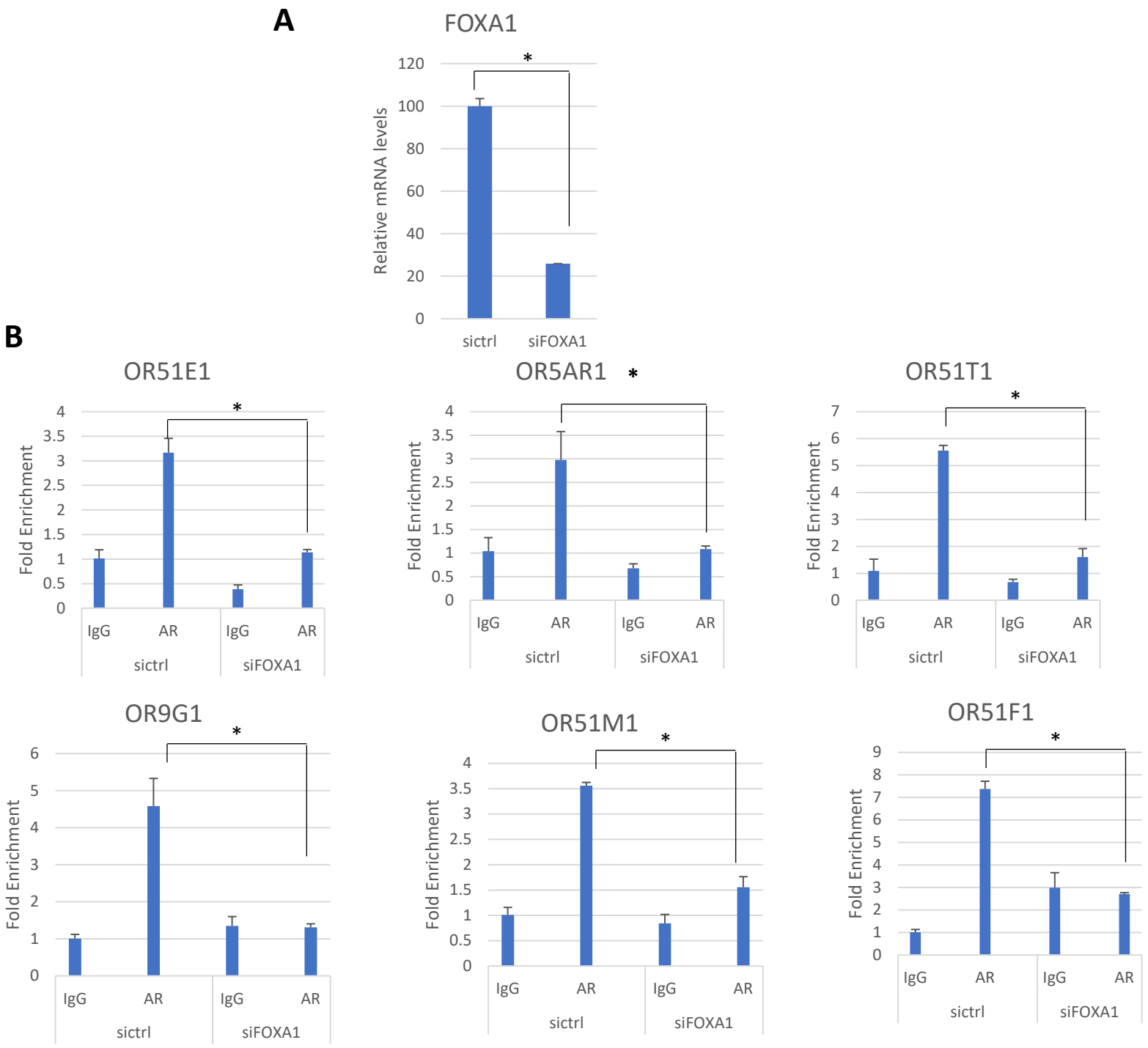
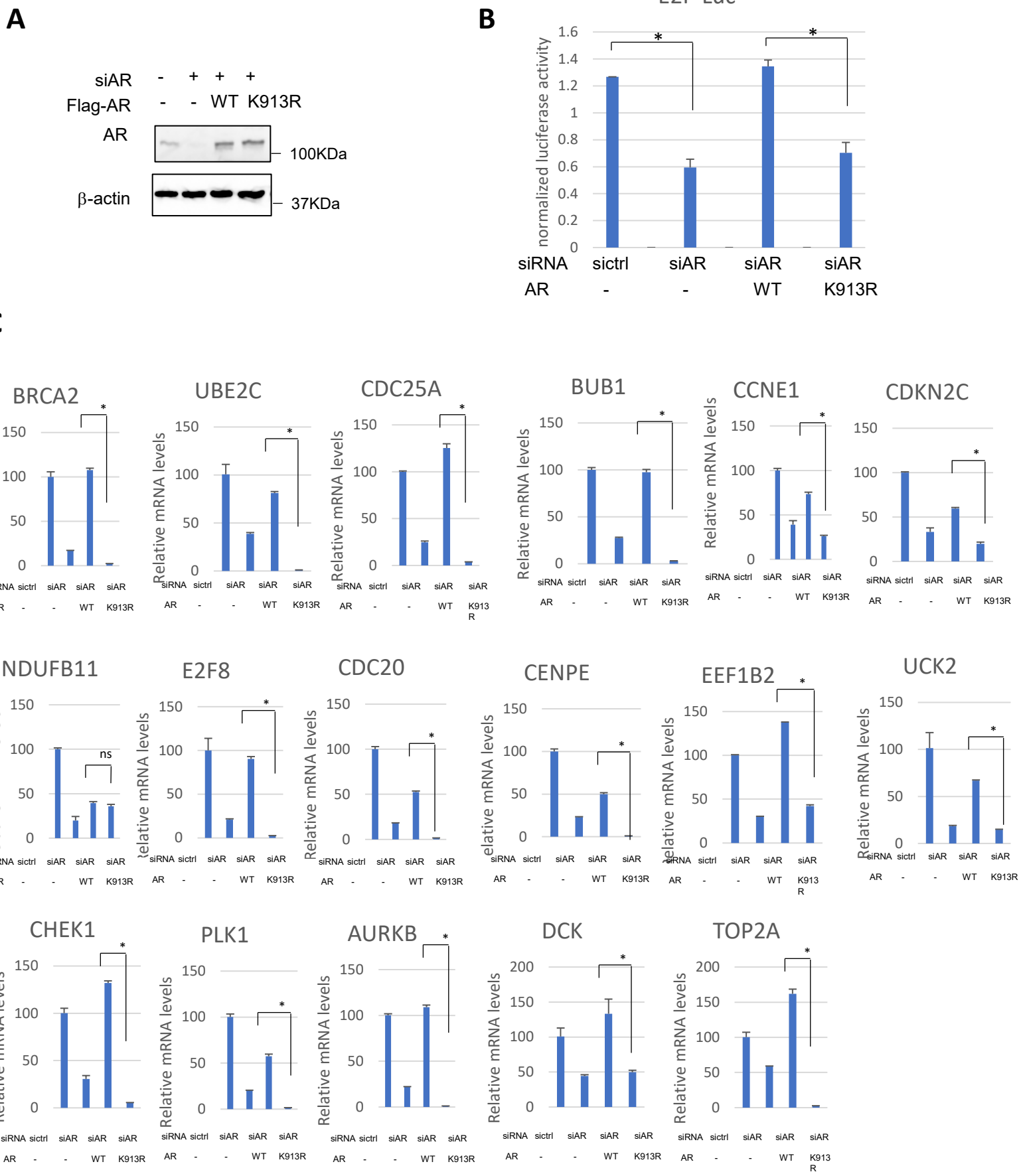


Fig S15: Knockdown of FOXA1 significantly reduced AR binding to OR gene enhancers in TRAF4 overexpressing cells. (A) FOXA1 knockdown reduced FOXA1 mRNA levels. (B) ChIP results Of AR binding in TRAF4 overexpressing LNCaP cells treated with control siRNA or siFOXA1.


A
siAR - + + +
Flag-AR - - WT K913R
AR
100KDa
β-actin
37KDa
B
E2F-Luc
normalized luciferase activity
siRNA sictrl siAR siAR siAR
AR - - WT K913R
C
BRCA2
UBE2C
CDC25A
BUB1
CCNE1
CDKN2C
NDUFB11
E2F8
CDC20
CENPE
EEF1B2
UCK2
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PLK1
AURKB
DCK
TOP2A
Relative mRNA levels
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D

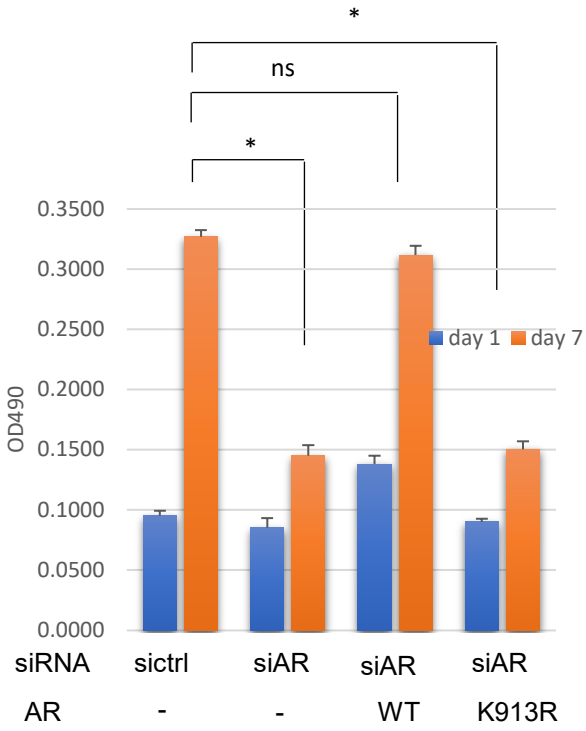

Fig S16: AR ubiquitination is important for TRAF4-promoted E2F activity and androgen-independent growth. (A) Western blot analysis demonstrating expression levels of AR WT or K913R in TRAF4 overexpressing LNCaP cells treated with siAR. (B) AR WT but not K913R restored the E2F-luciferase reporter activity after siAR treatment in LNCaP TRAF4 cells. (C) AR WT but not K913R rescued the effect of AR knockdown on TRAF4-promoted target gene expression. Shown are qRT-PCR results in LNCaP TRAF4 overexpressing cells. (D) AR WT but not K913R rescued the effect of AR knockdown on TRAF4-promoted androgen-independent cell growth. LNCaP TRAF4 overexpressing cells were treated with 40nM control siRNA or siAR Cells were grown in androgen-deprived medium and treated with 0.5μg/ml doxycycline to induce AR WT or K913R expression. MTS assays were carried out to monitor the cell growth at day 1 and day 7 through measuring the absorbance at 490nm.

Table S1 List of RT-qPCR primers

S NO	NAME (FORWARD F/REVERSE R)	SEQUENCE
	AR F	5'-GCCTTGCTCTCTAGCCTCAA-3'
	AR R	5'-GGTCGTCCACGTGTAAGTTG-3'
	KLK3 F	5'-CACAGCCTGTTTCATCCTGA-3'
	KLK3 R	5'-ATATCGTAGAGCGGGTGTGG-3'
	GAPDH F	5'-AGCCACATCGCTCAGACAC-3'
	GAPDH R	5'-GCCCAATACGACCAAATCC-3'
	ACTB F	5'-CCAACCGCGAGAAGATGA-3'
	ACTB R	5'-CCAGAGGCGTACAGGGATAG-3'
	OR51T1 F	5'-AATGTTCTTTGTGCATGCTTTC-3'
	OR51T1 R	5'-GTCAAAGGCCATGGCTACC-3'
	OR5M11 F	5'-TCATATCTGCTGGCTTCAACC-3'
	OR5M11 R	5'-GAATGAAGGCATAGGACACCA-3'
	OR9G1 F	5'-CCCTCATCGTGTTGATCTGTAA-3'
	OR9G1 R	5'-CCAGAAACGACAGATTTCCAGT-3'
	OR51F1 F	5'-AATTTCCAACCTTCTTGTTGACC-3'
	OR51F1 R	5'-AGATCCAGACATGGGCAGAC-3'
	OR5AR1 F	5'-TCAGCTTCACACACCCATGT-3'
	OR5AR1 R	5'-GGAGTAGCCCAGGTCAACAA-3'
	OR51E2 F	5'-CCTCCCCTGGAATCTAAAGC-3'
	OR51E2 R	5'-TGACTGGAGAGGGTGAGGTC-3'
	OR51E1 F	5'-CAGCCTGCCAGACCTCTT-3'
	OR51E1 R	5'-CAGCACCAGGCAGGTAGAG-3'
	TMPRSS2 F	5’-CGCTGGCCTACTCTGGAA-3’
	TMPRSS2 R	5’-CTGAGGAGTCGCACTCTATCC-3”
	SGK1 F	5’-CTCCTATGCATGCAAACACC-3’
	SGK1 R	5’-CCAAGGTTGATTTGCTGAGAA-3'
	UBE2C F	5’-CATGATGTCTGGCGATAAAGG-3'
	UBE2C R	5’-CGAGAGCTTATACCTCAGGTCTTC-3'
	TRAF4 F	5’-GGACCAGCTTCCTCTGGACT-3'
	TRAF4 R	5’-GGATAGGCAGGCCCAATACT-3'
	CDC20 R	5’-CTGTCTGAGTGCCGTGGAT-3'
	CDC 20 F	5’-TCCTTGTAATGGGGAGACCA-3'

Table S2 List of ChIP RT-qPCR primers

S NO	NAME (FORWARD F/REVERSE R)	SEQUENCE
	OR51T1 F	5’-TTTAAGCTCCTAACACTTTATTGCTCT-3'
	OR51T1 R	5’-AGAACCTGGGAAGGTAGAATTTATTA-3'
	OR5M11 F	5’-TTGGCAATTCATTCGATTTTT-3'
	OR5M11 R	5’-CCCACTAGCTCAAGCCTACAG-3'
	OR9G1 F	5’-GGTGCTGGGAAATGAATGTGGAAT-3'
	OR9G1 R	5’-AACCCCGACTCAGAAGACAAACAA-3'
	OR51F2 F	5’-AGGCCTTCCGTAAGAAGGTG-3'
	OR51F2 R	5’-ATCAGCAGTTTCCCTTGCAC-3'
	OR5AR1 F	5’-CATTTTGGGGAAAGAACCACCTGC-3'
	OR5AR1 R	5’-AAACTGAAGGCTAGAGCTGGACTG-3'
	OR51E2 F	5’-TCCTCAGGGAACCATTCATT-3'
	OR51E2 R	5’-CAAATGAAGGCCACATCAAA-3'
	OR51E1 F	5'-ACAAACAATTTCCCGTTTGC-3'
	OR51E1 R	5'-CAAATTCTTAGAAACAGAAAGTGGAAT-3'
	UBE2C ENHANCER F	5’-CCGTATACTCCCTGACCCTACA-3'
	UBE2C ENHANCER R	5’-CTGGTGCCTGATGTCGATAA-3'
	KLK3 ENHANCER F	5’-GCCTGGATCTGAGAGAGATATCATC-3'
	KLK3 ENHANCER R	5’-ACACCTTTTTTTTTCTGGATTGTTG-3'

Dataset S1 (separate file): A full gene list with the normalized counts and statistics in TRAF4 overexpressing and control LNCaP cells from RNA Seq analysis.

Dataset S2 (separate file): AR ubiquitination sites identified from Mass spectrometry

Dataset S3 (separate file): Additional qPCR primers