**Supplementary information**

**Supplementary tables**

Supplementary Table S1. Oligonucleotides

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| Cloning  ChIP  CLIP | AR-V7-Forward: ATAGGTACCCTGGCGGCATGGTGAG  AR-V7-Reverse: ATATCTAGATCAGGGTCTGGTCATTTTGAGATGCTTGCA  AdTR3-Forward: CGTGAATTCCCCTGTATCCAAGCCCAATAT  AdTR3-Reverse: ATAGATATCTCAGAAGGGCAGCGTGTCCAT  PForward: CAGAGATGAAATAAATGGGCAGATG  P Reverse: TCCTCTGCAACTTCAAGAGGA  A Forward: GACATGATGAATGTGAACATCCTTGA  A Reverse: CCAAGTTACTTAGGGTAAAAGCCATC  B Forward: ACACTTTCGAAAACATGGGTATAGAC  B Reverse: GATGGTCTGCTTTTGATCATTAATGC  C Forward: GATCGAATCAGCTACTGAAGCTTG  C Reverse: CCATCGCAAAGAAGTTAAAAACCTTG  Ctrl Forward: TCCTCCTCTTCCTCAATCTCG  Ctrl Reverse:AAGGCAACTTTCGGAACGG  Reg1 Forward: AAAGAAAGTGGTCTCTGGGTGCTGA  Reg1 Reverse: GTGAGTGCAAATCCTGTGAAGTCTTTAC  Reg2 Foward: CAACCCACTGTGTATTGCAGAATGTTTTA  Reg2 Reverse: TTTCCCTGGTTCCCTGTACAAAGTC  Reg3 Foward: AGAGATGGGCATATTCCTTGTTTGAATG  Reg3 Reverse: CTCCCCTTACTGCCTTCCTTATATG  Reg4 Foward: CTGCAGTTGGAATTTACATTTCCAAAGC  Reg4 Reverse: CAACCCATTTTGACTAGAGACCTGAG  Reg5 Foward: ATTGTGGTATCTGTATGTGGACCCTG  Reg5 Reverse: GATACTGCAGTTTTGAACACTGCAAATC  Reg6 Foward: GAGCTGGAAAAGAttTTAATGACTTTCCAG  Reg6 Reverse: AATCCCAGATGAGAGAAAGAGCAGTG  Reg7 Forward: GACATGATGAATGTGAACATCCTTGA  Reg7 Reverse: CCAAGTTACTTAGGGTAAAAGCCATC  Reg8 Forward: ACACTTTCGAAAACATGGGTATAGAC  Reg8 Reverse: GATGGTCTGCTTTTGATCATTAATGC  Reg9 Forward: GATCGAATCAGCTACTGAAGCTTG  Reg9 Reverse: CCATCGCAAAGAAGTTAAAAACCTT |

Supplementary Table S1. Oligonucleotides (*Continue*)

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| --- | --- |
| RT-PCR and qPCR  siRNA oligo  single strand RNA | e1-AR Forward: GAAATGGGCCCCTGGATG  e2-AR Reverse: CATCTCCACAGATCAGGCAGG  e3-AR Forward: TGCACTATTGATAAATTCCGAAGG  CE1-AR Reverse: CAAACACCCTCAAGATTCTTTCAG  CE3-AR Reverse: GTCATTTTGAGATGCTTGCAATTG  e4-AR Reverse: TTCTGGGTTGTCTCCTCAGT  1b-AR Forward: ATGATACTCTGGCTTCACAG  e2-AR Reverse: CAGATCAGGCAGGTCTTCTG  NOVA2 Forward: CCAAGCAGGCCAAGCTGATCGT  NOVA2 Reverse: ttaGGCCTTGTGCACCTGCTCG  HUB/HUR Forward: ATCGTCAACTACCTCCCTCAGAACATG  HUB/HUR Reverse: TGTTGATCGCTCTCTCTGCATCCT  hnRNP E1/E2 Forward: GGTCACCCTGAGGCTGGTGG  hnRNP E1/E2 Reverse: ATATCCCCTGCCACCTGGACCT  hnRNP A2B1 Forward: GAACATCACCTTAGAGATTACTTTGAGGA  hnRNP A2B1 Reverse: CTAGACAAAGCCTTTCTTACTTCTGCA  Gapdh Forward: ATCACCATCTTCCAGGAGCGAG  Gapdh Reverse: GAGATGATGACCCTTTTGGCTCC  PSA Forward: GGCCAGGTATTTCAGGTCAG  PSA Reverse: TCGTGGCTGGAGTCATCAC  siTR3: CAGUCCAGCCAUGCUCCUC(dTdT)  siCtrl: ACCCCGGAGAUGCUACCCGAA(dTdT)  ssRNA oligo: [Biotin]GAUUUGAAAAGAUUUAAUUUCCUCCCUUCU |

Supplementary Table S2. Antibodies and sources

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| Antibodies | Sources |
| Anti-AR (PG-21)  Anti-AR (C-19), Anti-FLAG; Anti-GAPDH, Anti-GFP; Anti-hnRNP A2B1 (B-7), Anti-hnRNP E1/E2; Anti-HUR (3A2); Anti-TR3, Rabbit-anti-goat  Anti-NOVA-2  Goat anti-mouse Alexa Fluor568, Goat anti-mouse Goat anti-rabbit. | Millipore  Santa Cruz  Aviva Systems Biology  Thermo Fisher |

**Supplementary figures**

** Supplementary Figure S1**. TR3 regulates AR and AR-V expression. **A,** RT-PCR analysis showing mRNA levels of TR3, AR, and AR-target gene PSA in prostate cancer cell lines treated with 10 nM DHT or vehicle. *β*-actin was used as a loading control. **B-C**, TR3 overexpression increases the expression levels of AR and AR-Vs. Western blot (**B**) RT-PCR (**C**) analysis showing the protein and mRNA levels of AR, AR-V7, and TR3 in LNCaP and C4-2 cells infected with AdTR3 or AdCtrl in the presence or absence of DHT. **D**, TR3 silencing causes a decrease in AR and AR-V mRNA levels. qPCR analysis showing AR, AR-V7, and TR3 mRNA levels in LNCaP and C4-2 cells transfected with siTR3 or siCtrl. Data are shown as mean ± SEM. \*, p<0.001; one-way ANOVA with Tukey’s post hoc test. **E,** Western blot analysis showing AR and TR3 protein levels in AR-positive prostate cancer cells (LNCaP, C4-2, and CWR22rv) treated with 20 *µ*M of TR3-specific antagonist (DIM-C-pPhOH) in the presence or absence of 10 nM DHT. **F**, The decreased AR and AR-V protein levels in TR3-silenced CWR22rv cells were not restored following treatment with MG-132 or chloroquine for 8 h. GAPDH was used as a loading control.

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**Supplementary Figure S2**. TR3 overexpression alters the expression level of AR-Vs. RT-PCR analysis showing the levels of mRNA containing alternative exons found in AR-Vs in C4-2 (top) and LNCaP (bottom) cells infected with AdTR3 or AdCtrl.

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**Supplementary Figure S3**. Western blot analysis showing the protein levels of several splicing factors (hnRNP A2B1, hnRNP E1/E2, HUB/HUR, and NOVA-2) in CWR22rv cells infected with AdTR3 or AdCtrl.

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**Supplementary Figure S4.** TR3 overexpression enhances AR transactivation. **A-C**, TR3 overexpression enhances the transactivation of ARs in a dose-dependent manner. PPC1 cells were transfected with full-length AR (AR-FL) (**A**), AR N-terminal domain (AR-NTD) (**B**), or AR-V7 (**C**) together with different amounts of FLAG-TR3 expression construct and pARE2-TATA-luc and incubated with or without 1 nM DHT. Luciferase activity was normalized to that of *β*-galactosidase. Data are shown as mean ± SEM. \*, p<0.05; \*\*, p<0.01; \*\*\*, p<0.001; one-way ANOVA with Tukey’s post hoc test. **D,** TR3 physically interacts with AR-NTD. PPC1 cells were co-transfected with AR-NTD and TR3 expression constructs. Physical interaction between TR3 and AR-NTD was examined through coimmunoprecipitation performed using anti-TR3 or anti-AR antibodies. Proteins were detected using western blot analysis.

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**Supplementary Figure S5.** TR3 is involved in the regulation of prostate cancer cell proliferation. TR3 expression was overexpressed (**A**) or silenced (**B**) in LNCaP cells. Cells were treated with 1 nM DHT, 50 ng/ml IL-6, 50 *µ*M FSK, or vehicle. Cell growth was assessed using MTS assays. Data are shown as mean ± SEM. \*, p<0.05; \*\*, p<0.01; \*\*\*, p<0.001; ns, not significant; two-tailed t-test. **C-D,** Xenograft mouse model and establishment of a stable cell line overexpressing TR3.Schematic presentation of the experimental set-up for CWR22rv xenograft mouse models (**C**). RT-PCR analysis confirmed TR3 expression in the inducible TR3-overexpressing CWR22rv cell sublines (#1, #2, and #3) and the control (EV) treated with 2 *µ*g/ml doxycycline (DOX) or vehicle (**D**).

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**Supplementary Figure S6.** TR3 overexpression impairs the immune system and activates the IL23R/STAT3/RORC axis involved in prostate cancer progression. **A-B,** Gene enrichment analysis (DAVID) of RNA-Seq data showing the upregulated expression of immune tolerance genes (**A**) and the downregulated expression of inflammatory genes (**B**) when TR3 was overexpressed in CWR22rv cells. **C-F,** Heatmaps (MeV) presenting the downregulated expression of inflammatory genes (**C**) and the upregulated expression of IL23R (**D**), STAT3 (**E**), and RORC (**F**) in TR3-overexpressing CWR22rv cells compared with the control.

**Additional methodology details**

Reagents

Forskolin (FSK), G418 disulfate salt (Geneticin), doxycyclin and chloroquine diphosphate salt (CQ) were purchased from Sigma-Aldrich (St Louis, MO, USA). The proteasome inhibitor carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG-132) was purchased from A.G. Scienctific, Inc. (San Diego, CA, USA). Recombinant human IL-6 was purchased from R&D systems Inc. (Minneapolis, Minnesota, USA), 5*α*-dihydrotestosterone (DHT) was purchased from Sigma (Poole, UK). Enzalutamide (MDV-3100) and Bicalutamide (BIC) were purchased from Sequoia Research Products Ltd. (St James Close, Pangbourne, UK). 1,1-Bis(3’-indolyl)-1-(ρ-hydroxyphenyl)methane (DIM-c-pPhOH) was purchased from TOCRIS Bioscience (Bristol, UK).

Plasmids

pARE2-TATA-Luc, GAL4.AR-LBD658-919, VP16.AR1-660, 5xGAL4-luc3, pcR3.1 SRC-1, pcDNA3.AR, pcDNA3.AR-NTD, pEGFP-AR, and pEGFP-AR-NTD have been previously described [1]. pcDNA3.AR-V7 was constructed to mimic AR-Vs in CWR22rv cells. Exons 2–8 were excised from pcDNA3.AR using *Kpn*I and *Xba*I and then exons 2 and 3 and CE3 amplified from CWR22rv cDNA library were inserted. pcDNA3.FLAG-TR3 was constructed by inserting TR3 PCR fragment into the pcDNA3.FLAG expression vector within *Eco*RI and *Eco*RV sites. The primers for PCR to construct the expression vectors are listed in Supplementary Table S1.

Cell Transfection and Reporter Assays

Cells were transiently transfected with expression constructs or siRNAs, and a luciferase reporter construct together with pCMV-LacZ or pRSV-LacZ (Clontech) using lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) with minor modifications of manufacturer’s instruction. Transfected cells were starved in media containing 5% cFBS for 24 h before stimulating with androgen, growth factors, or antagonists. Cells were then lysed in luciferase lysis buffer [0.2M Tris-Cl (pH 8.0), 0.2% Triton X100, and 1% NP-40] at RT for 15min. Luciferase activity was then analyzed in Beetle Luciferin (Promega Co., Madison, Wisconsin, USA) using a Centro XS3 LB960 Luminometer (Berthold Technologies GmbH & Co. KG, 75323 Bad Wildbad, Germany) and normalized to β-galactosidase activity read by Versa Max microplate reader (Molecular Devices, LLC., San Jose, CA). The duplet siRNA sequences for silencing study are listed in Supplementary Table S1.

RNA Isolation, RT-PCR and qPCR

Total RNA was isolated from prostate cancer cell samples by using TRI reagent® [Molecular Research Center (MRC), Inc. Cincinnati, OH]. Reverse transcription was performed using Oligo d(T)15 (ELPiS, Taejeon, Korea) and M-MLV Reverse transcriptase kit (Promega,  [Madison, Wisconsin, USA](https://www.google.com/search?sxsrf=ALeKk003Tb5rEARHYsaWF5RkyWvVdk0mdw:1616406675104&q=Madison,+Wisconsin&stick=H4sIAAAAAAAAAOPgE-LUz9U3MKswKilR4gAx08qNKrW0spOt9POL0hPzMqsSSzLz81A4VhmpiSmFpYlFJalFxYtYhXwTUzKL8_N0FMIzi5Pz84oz83awMgIARRb-T1sAAAA&sa=X&ved=2ahUKEwjn_rmN0MPvAhUTE4gKHePWBNQQmxMoATAlegQIGhAD)). The mRNA levels were analyzed by RT-PCR using Taq polymerase and quantified by qPCR using TOPrealTM qPCR 2X preMIX (Enzynomics, Daejeon, Republic of Korea). The primer sequences for analysis of gene expression profile and alternative transcripts of ARs are listed in Supplementary Table S1.

Western Blot Analysis

Western blot assays were performed as previous described [1]. Proteins were separated by SDS-PAGE, and then transferred onto a nitrocellulose blotting membrane (AmershamTM PortranTM Preminum 0.2 µM; GE healthcare, Little Chalfont, UK). The membrane was blocked, incubated with primary antibodies at 4oC overnight, and then incubated with secondary antibodies for 1 h. Band signals were visualized on X-ray films with ECLTM western blotting analysis system (GE Healthcare). The antibodies are listed in Supplementary Table S2.

CoImmunoprecipitation (Co-IP)

Co-IP assays were performed as previous described [1]. Cells were sonicated in 25 mM Tris-Cl (pH 8.0) buffer containing protease inhibitors. Supernatant was collected and then processed to Co-IP assays using protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology, Dallas, Texas, USA) and anti-hnRNP A2B1, anti-hnRNP E1/E2, anti-HUR, anti-AR or anti-TR3 antibodies. Proteins were then detected by western blot analysis.

Chromatin Immunoprecipitation (ChIP)

Chromatin immunoprecipitation assay was performed as previously described [1]. ChIP assays were performed using CWR22rv cells infected with AdTR3 or Ad-Ctrl. Recruitment of TR3 protein to putative TR3 binding sites within the promoter and intron regions of AR gene (P and A–C regions) was determined by ChIP assays using anti-TR3 antibody. Changes in TR3 enrichment at putative TR3 binding sites (P, A, B, and C) were examined using PCR. The loading control (Ctrl) was *β*-actin. The primers for ChIP assay are listed in Table S1.

Generation of TR3-overexpressing adenoviral (AdTR3) constructs

TR3-overexpressing adenovirus (AdTR3) was generated as previously described [2]. FLAG-TR3 fragment was excised from pcDNA3.FLAG-TR3 using *Kpn*I and *Xho*I and inserted into pAdTrack-CMV within *Kpn*I and *Xho*I sites to generate pAdTrack-CMV.FLAG-TR3. To generate the stable homologous recombinant adenovirus, purified *Pme*I-linearized pAdTrack-CMV.FLAG-TR3 was introduced into BJ5183 bacterial cells harboring the supercoiled backbone vector (AdEasy-1 cells). The recombinant adenoviral construct was screened using *Pac*I digestion and then transiently transfected into recombinant adenovirus E1-expressing HEK-293 (Ad293) cells (Agilent Technologies, Inc., Santa Clara, CA 95051, USA) to generate the first virus generation (AdTR3). Viruses were further amplified and purified for infection experiments.

Subcellular localization

HEK 293T cells were transiently transfected with GFP-AR-FL or GFP-AR-NTD, with or without TR3, for 12 h. The cells were fixed using 3.7% paraformaldehyde in PBS, blocked with BSA, incubated with primary mouse anti-TR3 antibodies overnight at 4 °C, and then incubated with secondary goat anti-mouse Alexa Fluor568. Nuclei were stained using TOPRO-3. Subcellular localizations of ARs (green GFP signal) and TR3 (red Alexa Fluor568 signal) were analyzed using a Nikon A1 laser-scanning Leica TCS SPE confocal microscope equipped with an ACS APO ×63/1.30 numerical aperture immersion objective. Images were analyzed using the ImageJ software (<http://imagej.nih.gov/ij>, ImageJ 1.46r, Wayne Rasband, National Institutes of Health, USA).

Protein purification

HEK 293T cells were infected with AdTR3 for 24 h and harvested for the purification of TR3 and splicing factors (hnRNP A2B1, hnRNP E1/E2, and HUB/HUR). Proteins were purified through two-step gradient anion-cation exchange chromatography. The purity of eluted fractions was examined using Coomassie Brilliant Blue R-250 staining following separation using SDS-PAGE. The fractions containing TR3 or each splicing factor (hnRNP A2B1, hnRNP E1/E2, and HUB/HUR) were confirmed through western blot analysis and then used in *in vitro* RNA-protein interaction assays.

Cell proliferation and mobility assays

For cell proliferation assays, cells were transfected with expression constructs or siRNA, or infected with adenovirus. After 12 h starvation, cells were treated with 1 nM DHT, 100 ng/ml IL6, or 50 *µ*M FSK for 24-72 h. The medium was replaced each day using fresh media containing inducers. The percent cell growth and cell numbers were obtained using MTS assays, which were measured using a microplate reader system at λ of 490 nm.

For cell viability assays, CWR22rv cells were infected with AdTR3 or AdCtrl, or transfected with siTR3 or siCtrl. Cells were maintained for 24 h, seeded on coverslips (104 cells per well) for 24–72 h, and then processed for cell viability assays. Three random fields of 0.5% crystal violet-stained cells were imaged using ZEISS microscopy at 20X magnification and average cell numbers per field were plotted using Graphpad Prism 5.

For cell migration assays, CWR22rv cells were infected with AdTR3 or AdCtrl, or transfected with siTR3 or siCtrl. Cells were maintained for 24 h and then seeded in 12-well plates (16 x 104 cells per well). The scratch wounds were generated when cell confluence reached 70%. The floating cells were removed through replacement with fresh medium. The scratch spaces were monitored and imaged daily.

For cell invasion, TR3-overexpressing or -silenced CWR22rv cells were seeded onto a 24-well Costar chamber comprising a 8-*µ*m polycarbonate membrane, which was precoated with 20% phenol red-free Matrix gel (8×104 cells per well), and then allowed to grow and invade for 48 h. Cells were then stained using 0.5% crystal violet. Cells on the inner side of the chamber were gently removed by scraping with a cotton swab and rinsed several times with PBS to remove excess dye. Three random fields of stained cells were imaged using ZEISS microscopy at 20X magnification, and the average cell numbers per field were plotted in Graphpad Prism 5.

**Supplementary references**

[1] T.T. Tran, C.H. Song, K.J. Kim, K. Lee, A new compound targets the AF-1 of androgen receptor and decreases its activity and protein levels in prostate cancer cells, American journal of cancer research, 10 (2020) 4607-4623.

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