

Regulation of Protein Kinase C-related Kinase (PRK) signalling by the TP α and TP β isoforms of the human Thromboxane A₂ receptor: Implications for Thromboxane- and Androgen-dependent Neoplastic and Epigenetic Responses in Prostate Cancer.

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ABBREVIATIONS:

ADT, androgen deprivation therapy; AR, androgen receptor; ARE, androgen response element; AA, arachidonic acid; BCR, biochemical recurrence; ChIP, chromatin immunoprecipitation; COX, cyclooxygenase/prostaglandin G₂/H₂ synthase; CRPC, castrate resistant prostate cancer; CS-NS, charcoal stripped-normal serum; C-tail, carboxyl-terminal tail; DHT, dihydrotestosterone; ERK, extracellular signal regulated protein kinase; FBS, foetal bovine serum; H3Thr11, Histone H3 threonine 11; H3K9, Histone H3 lysine 9; H4K16, Histone H4 lysine 16; KLK, kallikrein; LSD, lysine-specific demethylase; MOF, male absent on the first; NTD, amino-terminal domain; PCa, prostate cancer; PDK-1, 3-phosphoinositide-dependent protein kinase-1; PI3'K, phosphatidylinositol 3'kinase; PK, protein kinase; PRK, protein kinase C-related kinase; PKN, protein kinase novel; PSA, prostate-specific antigen; RBD, Rho binding domain; *siRNA*, *small interfering RNA*; T-loop, phosphorylation/activation loop; TAU, transcriptional activation unit; TMPRSS2, transmembrane protease serine 2; TP, T Prostanoid receptor; TXA₂, thromboxane; WDR5, WD repeat-containing protein 5

ABSTRACT

The prostanoid thromboxane (TX) A₂ and its T Prostanoid receptor (the TP) are increasingly implicated in prostate cancer (PCa). Mechanistically, we recently discovered that both TP α and TP β form functional signalling complexes with members of the protein kinase C-related kinase (PRK) family, AGC- kinases essential for the epigenetic regulation of androgen receptor (AR)-dependent transcription and promising therapeutic targets for treatment of castrate-resistant prostate cancer (CRPC). Critically, similar to androgens, activation of the PRKs through the TXA₂/TP signalling axis induces phosphorylation of histone H3 at Thr11 (H3Thr11), a marker of androgen-induced chromatin remodelling and transcriptional activation, raising the possibility that TXA₂-TP signalling can mimic and/or enhance AR-induced cellular changes even in the absence of circulating androgens such as in CRPC. Hence the aim of the current study was to investigate whether TXA₂/TP-induced PRK activation can mimic and/or enhance AR-mediated cellular responses in the model androgen-responsive prostate adenocarcinoma LNCaP cell line. We reveal that TXA₂/TP signalling can act as a neoplastic- and epigenetic-regulator, promoting and enhancing both AR-associated chromatin remodelling (H3Thr11 phosphorylation, WDR5 recruitment and acetylation of histone H4 at lysine 16) and AR-mediated transcriptional activation (e.g of the *KLK3*/prostate-specific antigen and *TMPRSS2* genes) through mechanisms involving TP α /TP β mediated-PRK1 and PRK2, but not PRK3, signalling complexes. Overall, these data demonstrate that TP α /TP β can act as neoplastic and epigenetic regulators by mimicking and/or enhancing the actions of androgens within the prostate and provides further mechanistic insights into the role of the TXA₂/TP signalling axis in PCa, including potentially in CRPC.

RESEARCH HIGHLIGHTS:

- TP α and TP β isoforms of the TP complex with PRK1 and PRK2, not PRK3, in LNCaP cells (84)
- TP-PRK signalling promotes, &/or enhances AR-mediated, proliferation and migration (83)
- TP-PRK signalling promotes, and enhances AR-mediated, epigenetic responses (75)
- TP-PRK signalling promotes WDR5 & AR chromatin binding at androgen-responsive genes (84)
- TP-PRK signalling promotes, and enhances, AR-mediated gene expression (70)
- TP α /TP β act as neoplastic and epigenetic regulators in PCa, mimicking the androgens (84)

1. INTRODUCTION

Following prostatectomy, androgen deprivation therapy (ADT) remains the cornerstone treatment for men with locally advanced or metastatic prostate cancer (PCa). Despite reduced circulating androgen levels however, prostate cancer can recur and progress to the terminal castrate-resistant prostate cancer (CRPC) stage for which no cure is yet available¹. While the cellular mechanisms that lead to CRPC have not been fully delineated, it is thought that aberrant signalling by a number of growth factor, cytokine and/or kinase signal transduction pathways can activate the androgen receptor (AR) in the absence of androgens (e.g. testosterone or its 5 α -reductase metabolite dihydrotestosterone/DHT) or show a synergistic effect with low/reduced androgens to facilitate progression to castrate-resistance². The protein kinase C-related kinases (PRKs), also known as protein kinase novel/PKN, represent a subfamily of AGC kinases that play an essential role in the epigenetic regulation of AR-dependent gene expression/transcriptional activation in the prostate and have been strongly implicated in the mechanisms underpinning CRPC³⁻⁵. As downstream effectors of both Rho GTPase and phosphatidylinositol 3' kinase (PI3'K) oncogenic signalling cascades⁶⁻⁸, dysregulated expression and signalling by each or all of the 3 PRK isozymes, namely PRK1/PKN α , PRK2/PKN γ and PRK3/PKN β , are strongly yet differentially implicated in various neoplasms, particularly in androgen-associated PCa^{4, 8-11}.

Mechanistically, upon androgen stimulation, AR and PRK1 bind as functional complexes to androgen response elements (AREs) within AR-target genes resulting in PRK1-catalysed phosphorylation of histone H3 at Thr11 (H3Thr11) which, in turn, serves as a highly specific androgen-induced chromatin mark resulting in the subsequent recruitment of a series of chromatin-modifying enzymes to facilitate AR-dependent gene expression^{4, 5}. For instance, phosphorylation of H3Thr11 by PRK1 enhances the removal of repressive methylation marks from histone H3 at lysine 9 (H3K9) by lysine-specific demethylase (LSD)-1 and JMJD2C^{4, 12}. Furthermore, phosphorylated H3Thr11 serves as a binding platform for WD repeat-containing protein 5 (WDR5), an epigenomic scaffold which represents a core component of several histone remodelling complexes that catalyse the deposition of activating histone modifications at AR-target genes, such as the acetylation of histone H4 at lysine 16^{5, 13}. Therefore, due to their ability to complex with and regulate the AR, the PRK isozymes play a central role in the epigenetic priming of prostate tumour cells and aberrant stimulation of PRK signalling represents a key mechanism that can regulate AR-transcriptional activity, even in the absence of- or in reduced- circulating androgen levels, leading to castrate-resistance in PCa^{5, 14, 15}.

Through detailed mechanistic studies, we recently uncovered a novel pathway in prostate tumour cells whereby members of the PRK family were also shown to be activated in response to stimulation of the thromboxane (TX) A₂/T Prostanoid receptor (TP) signalling axis, thereby greatly adding to the complexity of PRK signalling in PCa^{16, 17}. The prostanoid TXA₂, synthesised via the sequential actions of cyclooxygenase (COX)-1/COX-2 and TXA₂ synthase (TXS), plays a central role in haemostasis regulating platelet activation status and vascular smooth muscle tone¹⁸. In addition and more recently, TXA₂ and its T Prostanoid receptor (the TP) has been increasingly implicated in promoting neoplastic progression including in prostate, lung, breast and bladder cancers, among others^{19, 20}. For example, within the prostate, expression of the TP directly correlates with Gleason score/pathologic stage and localises to areas of perineural invasion, a mechanism by which PCa cells penetrate the prostatic capsule and spread to other tissues²¹. Moreover, the benefits of daily Aspirin use in reducing both the incidence and mortality associated with several prevalent cancers, including PCa, further highlights the importance of the COX-derived prostanoid pathway, including the TXA₂/TP axis, in tumorigenesis and progression²²⁻²⁴.

In humans and other primates, TXA₂ actually signals through two isoforms of the TP, referred to as TP α and TP β , which differ exclusively in their intracellular carboxyl (C)-tail domains²⁵. Both

TP α and TP β are encoded by the same *TBXA2R* gene and arise by differential splicing of a 1^o RNA transcript²⁵. Moreover, TP α and TP β are differentially expressed in various cell/tissue types due to their transcriptional regulation by two distinct promoters, designated as promoter (Prm) 1 and Prm3 respectively, within the *TBXA2R*²⁶⁻²⁹. Functionally, TP α and TP β both couple to G α_q -mediated phospholipase C β and to G α_{12} -mediated RhoA activation^{25, 30, 31}, and to activation of the extracellular signal regulated protein kinase (ERK) and PI3'K signalling cascades³²⁻³⁴, but are subject to isoform-specific mechanisms of agonist-induced homologous^{35, 36} and heterologous desensitisation^{29, 37, 38}.

As stated, we recently discovered that the TP α /TP β isoforms can directly complex with and regulate signalling by PRK1 in the androgen-resistant PC-3 and androgen-responsive LNCaP prostate adenocarcinoma cell lines¹⁶. Mechanistically, the associations of TP α /TP β with PRK1 were found to be important not only in mediating TXA₂-induced cell proliferation and migration, but also enables the TXA₂/TP axis to induce phosphorylation of histone H3 at Thr11¹⁶. In a subsequent study carried out exclusively in PC-3 cells, it was established that the TP α and TP β isoforms can not only form signalling complexes with PRK1 but also with the related PRK2, but not with PRK3¹⁷. Collectively and critically, these findings were the first demonstration that an agent other than androgens, namely TXA₂, can induce this essential phosphoH3Thr11 chromatin mark and thereby suggested a potential role for the TXA₂-TP α /TP β axis in epigenetic regulation and/or chromatin remodelling in PCa^{16, 17}. Indeed, the findings also raised the exciting possibility that through its ability to induce PRK1/2-catalysed phosphorylation of H3Thr11, TXA₂ may function as a potential driver of aberrant AR-reactivation and transcriptional regulation of AR-target genes which can occur even in the absence of- or in reduced- testicular androgens, such as occurs in CRPC. However, whether the TXA₂/TP-PRK axis can mediate the subsequent chromatin modifications necessary for AR-dependent transcription downstream of H3Thr11 phosphorylation (e.g. WDR5 recruitment and H4K16 acetylation) remains to be elucidated. Moreover, whether TXA₂-induced activation of PRK signalling can directly stimulate the transcriptional activity of the AR, thus enabling the TXA₂/TP axis to promote androgen-independent AR-target gene expression in PCa remains unanswered.

Hence, using the model androgen-responsive LNCaP prostate adenocarcinoma cell line, the aim of the current study was to investigate if activation of the TXA₂/TP signalling axis might regulate signalling by PRK1, PRK2 and/or the related PRK3 to mimic or even enhance the effects of androgens (e.g. dihydrotestosterone/DHT) in PCa, including establishing whether TXA₂-TP/PRK signalling promotes the key steps of androgen-induced chromatin remodelling and, in turn, leading to transcriptional activation of AR-target genes within the prostate. We reveal that TXA₂/TP can act both as a neoplastic- and epigenetic-regulator, mimicking and enhancing the actions of androgens to promote chromatin remodelling (H3Thr11 phosphorylation, recruitment of the epigenomic scaffold WDR5 and consequent acetylation of histone H4 at lysine 16) and upregulation of AR-target gene expression (e.g PSA/*KLK3* and *TMPRSS2* genes) in LNCaP cells through mechanisms dependent on the ability of TP α /TP β to form functional, activation complexes with PRK1 and PRK2, but not with PRK3. These data show that TP α /TP β can act as neoplastic and epigenetic regulators mimicking and/or enhancing the actions of androgens within the prostate providing further mechanistic insights into the role of the TXA₂/TP signalling axis in PCa, including as a potential driver of CRPC.

2. MATERIALS AND METHODS

2.1 Materials

U46619 (16450) and SQ29548 (19025) were obtained from Cayman Chemical Company. *Anti-PRK1* (sc-1842), *anti-PRK2* (sc-6979), *anti-PRK2* (sc-271971), *anti-histone H3* (sc-8654), *anti-histone H4* (N-18; sc-8657) and *anti-AR* (sc-816x) antibodies, normal rabbit immunoglobulin G (IgG; sc-2027), goat *anti-mouse* HRP (sc-2005), goat *anti-rabbit* HRP (sc-2004) and mouse anti-goat HRP (sc-2354) secondary antibodies were obtained from Santa Cruz Biotechnology. The Brilliant III Sybr Green QPCR kit (600882) was from Agilent. LY294002, GSK2334470, PKC412 (Midostaurin), colcemid, thiazolyl blue tetrazolium bromide (MTT), Protein A-Sepharose, sodium butyrate and dihydrotestosterone (DHT) were obtained from Sigma. RPMI 1640 media, L-glutamine, fetal bovine serum (FBS), Superscript reverse transcriptase and TRIzol were from Invitrogen Life Sciences. Enzalutamide (ENZ) was from Selleck Biochem. *Anti-HDJ2* (DNAJ) (225PIABX) was from Neomarker. Dharmafect 3 transfection reagent was from ThermoFisher Scientific. RQ1 RNase-Free DNase was from Promega. *Anti-phospho-H3Thr11* (39151) and *anti-histone H4K16Ac* (39167) antibodies were obtained from Active Motif. *Anti-PRK3* (NBP1-30102) antibody was obtained from Novus Biologicals. *Anti-WDR5* (A302-429A) antibody was obtained from Bethyl Laboratories. *Anti-phospho-PRK1^{Thr774}/PRK2^{Thr816}* (#2611) antibody was obtained from Cell Signalling Technology. *Anti-TP α* and *anti-TP β* isoform specific antibodies have been previously described^{31,34}. Boyden chamber cell culture inserts (#353097) were obtained from BD Biosciences. H-89 dihydrochloride, Gö6983, salmon sperm DNA/protein A agarose beads and fibronectin (FC010) were obtained from Merck Millipore. All oligonucleotides were synthesized by Genosys Biotechnologies and all *small interfering (si)*RNAs were from Eurofins Scientific.

2.2 Cell Culture

The prostate adenocarcinoma LNCaP cell line was obtained from the American Type Culture Collection (ATCC) and was cultured in RPMI 1640, 0.2% (v/v) L-glutamine and 10% FBS (complete culture medium). For serum-free media conditions, cells were cultured in RPMI 1640 growth medium in the complete absence of FBS (0%) for the times specified for the given study. For studies involving dihydrotestosterone (DHT), cells were cultured in media supplemented with 10% charcoal stripped-normal serum (CS-NS) for at least 24 hr prior to performing the study. All mammalian cells were grown at 37 °C in a humid environment with 5% CO₂ and were confirmed mycoplasma free.

2.3 Disruption of PRK1/2/3 Expression by small interfering (si) RNA

For *siRNA* experiments, LNCaP cells were routinely plated at a density of 1 x 10⁶ cells/10 cm diameter dish in complete medium (10 ml) and transiently transfected with 30 nM *siRNA* specifically targeting either PRK1 (*siRNA*_{PRK1}, 5'-CCUCGAAGAUUUCAAGUUC-3'), PRK2 (*siRNA*_{PRK2}, 5'-GCACCAUUUUUCCGGCUA-3') or PRK3 (*siRNA*_{PRK3}, 5'-GGAAAUACUACGCCAUCAA-3'), or with a nonsense scrambled control *siRNA* oligonucleotide (*siRNA*_{Control}, 5'-AATTCTCCGAACGTGTCACGT-3'), using Dharmafect 3 transfection reagent (8 μ l/10 cm dish). All experiments involving *siRNA* were validated using an additional independent series of *siRNA* sequences directed against each of the PRK1, PRK2 or PRK3 isozymes: *siRNA*_{PRK1-2}, 5'-GCACUGUGCUUAAGCUGGA-3'; *siRNA*_{PRK2-2}, 5'-GCACCAUUUUUCCGGCUA-3' and *siRNA*_{PRK3-2}, (5'-AGACCUUUGUCAUCCACU-3'). To confirm *siRNA*-mediated knockdown of PRK1, PRK2, or PRK3 expression, cells were routinely harvested 72-96 hr post-transfection and whole cell lysates (20 μ g) were immunoblotted versus *anti-PRK1*, *anti-PRK2* or *anti-PRK3* isozyme-specific antibodies, respectively. Thereafter, membranes were screened with *anti-HDJ* to confirm equal protein loading. For analysis of cell proliferation, LNCaP cells were harvested 48 hr post-transfection and re-seeded onto 96 well plates in complete growth medium (4000 cells/well; 100 μ l)

before performing proliferation assays, as described below. For cell migration assays, some 72 hr post-transfection, cells were routinely placed in Boyden chambers (75,000 cells/well; 500 μ l) in serum-free media and cultured for a further 24 hr before analysis of cell migration, as outlined below.

2.4 Immunoprecipitations

Immunoprecipitations of endogenous TP-PRK complexes in LNCaP cells were performed as previously described for PC-3 cells ¹⁷.

2.5 Determination of PRK Activation Levels

To monitor changes in PRK1-3 activation status in response to TXA₂/U46619 in LNCaP cells, experiments were performed as outlined previously for PC-3 cells ¹⁷.

2.6 Proliferation Assays

To examine changes in proliferation, LNCaP cells (4000 cells/well) were seeded in complete culture medium (RPMI, containing 10% CS-NS; 100 μ l) in 96-well plates and grown for 24 hr at 37 °C. Thereafter, the media was gently aspirated and replaced with reduced-serum media (0.5% CS-NS; 100 μ l) containing U46619 (10 nM or, in concentration response studies, 5-250 nM), DHT (10 nM), SQ29548 (1 μ M), enzalutamide (10 μ M) or, as controls, vehicle (0.0001% EtOH), 10% serum, or docetaxel (20 nM) and the cells were cultured for an additional 72 hr. Thereafter, MTT reagent (5 mg/ml; 50 μ l) was added to each well and the cells were further incubated at 37 °C for 3 hr. The MTT/media mix was then removed and replaced with DMSO (200 μ l/well) to elute the purple formazan crystals. Levels of formazan were then measured by reading the absorbance at 540 nm from triplicate wells. Data is presented as mean fold-change in cell proliferation relative to vehicle-treated cells, assigned a value of 1 ($n \geq 3$, \pm SEM), where 'n' refers to the number of independent experiments performed and not to replicates within the same experiment.

2.7 Transwell Migration Assays

For analysis of cell migration, LNCaP cells were seeded on 10 cm dishes in complete culture medium (RPMI, containing 10% CS-NS; 8 ml) for 72 hr at 37 °C to achieve 80% confluency. Thereafter, the culture media was removed and cells were washed with serum-free media and cultured for a further 16 hr in serum-free media (0% FBS; 5 ml) before performing transwell assays, using Boyden Chambers (Falcon #353097, 8.0 μ M pore size, 24-well plate format). Prior to beginning experiments, Boyden chambers were coated for 1 hr with fibronectin (5 μ g/ml) and allowed to air-dry. For migration assays, 7.5×10^4 cells, in serum-free culture media (500 μ l), were placed in the top chamber/compartment. After the cells settled (approx. 30 min), culture medium (RPMI, containing 10% CS-NS; 500 μ l) was added to the bottom compartment and cells were incubated with U46619 (10 nM or, in concentration response studies, 5-250 nM), DHT (10 nM), SQ29548 (1 μ M), enzalutamide (10 μ M) or vehicle (0.0001% EtOH) using duplicate wells. Following incubation for 24 hr, the cells remaining in the upper compartment were removed by cotton swabs. The migrated cells attached to the underside of the Boyden Chamber membranes were fixed in PFA (3.7 %), stained with DAPI (1 μ g/ml in 0.1% TBS-tween), washed in H₂O and allowed to air-dry. The membranes were then detached using a scalpel blade and mounted in Mowiol mounting medium. The cells were viewed and imaged using a Zeiss Axioplan 2 microscope (10 \times magnification). For analysis, the numbers of migrated cells from at least five independent fields of view were counted, using duplicate wells. Data is presented as mean fold-change in cell migration relative to vehicle-treated cells, assigned a value of 1 ($n \geq 3$, \pm SEM), where 'n' refers to the number of independent experiments performed and not to replicates within the same experiment.

2.8 Investigation of Histone H3 Thr11 (H3Thr11) Phosphorylation and Histone H4 Lysine 16 (H4K16) Acetylation Levels

To measure changes in levels of phosphorylated Histone H3Thr11 and acetylated Histone H4K16 in response to TXA₂/U46619- and androgen-stimulation, LNCaP cells were seeded on 10 cm dishes in complete culture medium (8 ml; 10% CS-NS) and grown for 48 hr to achieve 85% confluency. Cells were stimulated with U46619 (50 nM, or in concentration-response studies, 0-1 μM; 0-240 min), DHT (10 nM, or in concentration-response studies, 1-100 nM; 0-24 hr) or, as controls, with colcemid (50 ng/ml; 24 hr), sodium butyrate (5 mM; 24 hr) or drug vehicle (0.0005 - 0.01% EtOH). The cells were then harvested by sequential extraction to remove soluble cytoplasmic and nucleoplasmic proteins and obtain histone-containing nuclear extracts as described previously^{16, 39}. For immunoblot analysis, nuclear extracts (0.8 μg) were resolved on 15% SDS-page gels and transferred onto PVDF membranes. The membranes were immunoblotted using *anti-phospho-H3Thr11* or *anti-H4K16* acetylation histone-modification specific antibodies and re-screened with *anti-Histone H3* to confirm uniform loading. In the case of Histone H4, parallel membrane blots were screened with *anti-Histone H4* antibodies to confirm uniform loading. Data presented is representative of three independent experiments (n = 3), where histone-containing nuclear extracts were isolated on each separate occasion, prior to analysis by SDS-PAGE.

2.9 Real time quantitative-reverse transcriptase (qRT)-PCR analysis

LNCaP cells were seeded at 1 x 10⁶ cells/10 cm dish in complete culture medium (8 ml) and grown for 48 hr to achieve 85% confluency. Thereafter, the media was replaced with reduced-serum media (5% CS-NS; 5 ml) and the cells were cultured for a further 24 hr, prior to stimulation with U46619 (50 nM), DHT (10 nM), SQ29548 (1 μM), enzalutamide (10 μM) or vehicle control (0.0005% EtOH) for 6, 16 or 24 hr. Total RNA was extracted using Trizol reagent and converted to first-strand (1^o) cDNA using Superscript reverse transcriptase from Invitrogen (1 μg RNA/20 μl reaction). All RNA samples were DNaseI treated (Promega #M6101; 10 μg RNA/30 μl reaction) prior to cDNA synthesis. Quantitative real-time reverse transcriptase PCR (qRT-PCR) analysis was carried out using the Brilliant III SYBR Green QPCR system (Agilent #600882), as previously described²⁸. Gene-specific primers were used to amplify a 136 bp region of PSA/*KLK3* mRNA, a 105 bp region of *KLK2* mRNA, a 115 bp region of *TMPRSS2* mRNA, a 102 bp region of *c-FOS* mRNA, or as a reference control, a 149 bp region of the human *18s rRNA* gene. The identities of the primers used for the qRT-PCR reactions, as well as their sequences are listed in *Supplemental Table S1*. The levels of PSA/*KLK3*, *KLK2*, *TMPRSS2* and *c-FOS* mRNA were normalised using corresponding *18s rRNA* expression levels to obtain ΔCt values and relative mRNA expression levels were calculated using the 2^{-ΔΔCt} method. Data is presented as mean changes in mRNA expression relative to the levels in control, vehicle-treated cells, assigned a value of 1. For all experiments, the QT-PCR data presented is representative of at least three independent experiments (n ≥ 3), including where RNA was isolated on each separate occasion, and analysed at least twice for each isolation by PCR.

2.10 Chromatin immunoprecipitation (ChIP) assays

Chromatin immunoprecipitation (ChIP) assays were performed in LNCaP cells essentially as previously described²⁸. Briefly, LNCaP cells were seeded on 10 cm dishes (~ 1 x 10⁶ cells/dish; 5 x dishes per treatment) in complete culture medium and grown for 48 hr to achieve 85% confluency. Thereafter, the cells were stimulated with either U46619 (50 nM), DHT (10 nM), or both, or vehicle control (0.0005 % EtOH) for 4 hr prior to harvesting by cell scraping. Harvested cells were collected by centrifugation (2000 g; 3 min) at 4 °C, washed twice in ice-cold PBS and re-suspended in 50 ml serum-free media. Chromatin was cross-linked by adding 1% formaldehyde dropwise to the cells with gentle rotation for 10 min, followed by addition of glycine (3 M) for 5 min. The cells were then collected by centrifugation (2000 g; 8 min), lysed to release the chromatin and sheared by sonication

to generate 350–1000 bp fragments. The sheared chromatin was then re-suspended in a final volume of 6 ml lysis buffer and pre-cleared by incubating with normal rabbit IgG, followed by incubation with 250 μ l salmon sperm DNA/protein A agarose beads, overnight with rotation. For ChIP assays, the pre-cleared chromatin was divided into aliquots (672 μ l/aliquot) and incubated overnight with *anti*-AR (10 μ g) or *anti*-WDR5 (2.5 μ g) antibodies, or as controls, with equivalent amounts of the normal rabbit IgG or in the absence of primary antibody (-AB). Input chromatin (270 μ l) of the pre-cleared lysates was taken prior to immunoprecipitation and stored at -20°C for later use. Samples were then incubated with salmon sperm DNA/protein A agarose beads for 1 hr and complexes were eluted from the beads, prior to incubation with RNase A at 65°C overnight, followed by incubation with Proteinase K at 45°C for 7 h, to reverse cross-linking. Thereafter, samples were precipitated and resuspended in H_2O (50 μ l). For quantitation of the relative abundance of the PCR products derived from the individual test (*anti*-WDR5 or *anti*-AR) or control immunoprecipitates relative to that of the corresponding input chromatins, real-time quantitative (QT)-PCR analysis was carried out for the same number of cycles (typically 40 cycles) using the Agilent MX3005P QPCR system to obtain cycle threshold (Ct) values. The relative PCR product intensities generated due to AR or WDR5 binding, or the corresponding IgG and -AB controls, were then calculated using the Relative Quantification method using the formula $2^{-\Delta\Delta\text{Ct}}$ ²⁸. Data is presented as mean product intensities of the individual test or control immunoprecipitates, expressed as a percentage of the products derived from the corresponding input chromatins. The identities and sequences of the primers used for the amplification of the androgen response element (ARE)-containing test regions of the *KLK3* and *TMPRSS2* genes, or the control region of the *GAPDH* gene are given in *Supplemental Table S2*. For all experiments, the QT-PCR data presented was obtained from at least 3 independent ChIP assays ($n = 3$) using chromatin extracted on 3 different occasions, where each ChIP assay was analysed on each occasion at least twice by PCR.

2.11 Statistical Analysis

Statistical analyses of differences were carried out using either the paired or unpaired Student's t tests or one-way ANOVA, followed by post-hoc Bonferroni's or Dunnetts multiple comparison t-tests, employing GraphPad Prism (V6) throughout. All values are expressed as mean \pm standard error of the mean (SEM). P values ≤ 0.05 were considered to indicate statistically significant differences; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

3. RESULTS

3.1 *TP α /TP β complex with PRK1 and PRK2 in androgen-responsive prostate cancer LNCaP cells, leading to T-loop phosphorylation/activation of PRK*

Despite reduced circulating androgen levels following ADT (androgen deprivation therapy), the AR can continue to signal suggesting that there are other yet undefined signalling pathways which can influence the transcriptional activity of the AR to facilitate progression to castrate-resistance (CRPC) in PCa⁴⁰. We recently uncovered a novel pathway in prostate tumour cells whereby both the TP α and TP β isoforms of the human TP can directly complex with and regulate signalling by PRK1, an AGC-type kinase that plays an essential role in the epigenetic regulation of AR-dependent transcription including in mechanisms underpinning CRPC^{4, 5, 17}. Critically, similar to androgens, activation of PRK1 through the TXA₂/TP signalling axis induces phosphorylation of H3Thr11, a marker of androgen-induced chromatin remodelling and transcriptional activation, and represented the first demonstration that an agent other than androgens can induce this essential chromatin mark¹⁶. Such findings raise the possibility that the TXA₂-TP axis can mimic and/or enhance AR-induced cellular responses, to function as a potential driver of aberrant AR-reactivation and transcriptional activation which occurs even in the absence of/in reduced circulating androgens including in CRPC^{16, 17}. Hence, using the model androgen-responsive prostate adenocarcinoma LNCaP cell line, the aim of this study was to investigate whether TXA₂/TP can regulate signalling by the other members of the PRK family and to establish whether such TP/PRK signalling might mimic and/or enhance the effects of androgens (e.g. DHT), to promote chromatin remodelling and AR-mediated transcriptional activation in PCa.

To address this, complex formation between TP α and TP β and the individual PRK isozymes, namely PRK1/PKN α , PRK2/PKN γ and PRK3/PKN β , was first investigated in LNCaP cells both in the absence and presence of the selective TXA₂ mimetic U46619. Consistent with previous findings in PC-3 cells¹⁷, in the absence of agonist, PRK1 was readily detected in immune complexes with both TP α and TP β and stimulation with U46619 did not alter the levels of PRK1 associated with either TP isoform (*Figure 1A & 1B*). In contrast and also in keeping with previous data in PC-3 cells¹⁷, the associations of the individual TP α and TP β with PRK2 were highly regulated in an agonist-dependent manner (*Figure 1A & 1B*). In the absence of agonist, PRK2 was detected in a specific complex with TP β , but not with TP α . However, at 10 min post-U46619 stimulation, PRK2 complexed with TP α while following prolonged agonist stimulation (at 60 min), the association of PRK2 with TP α had diminished to basal levels, observed in the absence of agonist (*Figure 1A & 1B*). For TP β , the TP β :PRK2 complex observed under basal conditions in the absence of agonist was lost at 10 min following stimulation with U46619 while at 60 min, levels of PRK2 associated with TP β were restored albeit to levels less than those observed in the absence of agonist (*Figure 1A & 1B*). Notably for PRK3, it was not found to complex with either TP α or TP β , either basally or following agonist-activation despite its expression in LNCaP cells, albeit at lower levels than either PRK1 or PRK2 (*Figure 1A*). Moreover, none of the PRK1, PRK2 or PRK3 isozymes were detected in the control pre-immune IgG immunoprecipitates (*Figure 1A*), confirming the specificity of the *anti*-TP α and *anti*-TP β immune complexes.

Structurally, the PRKs contain three highly conserved functional domains including an N-terminal Rho binding domain (RBD), an arachidonic acid-sensitive C2-like domain and a C-terminal catalytic kinase domain^{3, 41}. In response to upstream PI3'K/3-phosphoinositide-dependent kinase (PDK)-1 signalling, the PRK isozymes undergo phosphorylation at a conserved Thr residue located in the activation loop (T-loop) of the kinase domain, a modification that leads to catalytic activation of the individual PRKs^{6, 7, 42}. To determine whether PRK1/PRK2 undergo T-loop phosphorylation and activation in response to agonist (U46619)-induced activation of TP α /TP β in the androgen-responsive PCa cell line, LNCaP cells were stimulated with U46619 and cellular extracts analysed using an *anti*-

phospho-PRK1^{Thr774}/PRK2^{Thr816}/PRK3^{Thr718} antibody which specifically recognises the phosphorylated and activated T-loop site within the kinase domain of the PRKs. Initially, the *anti*-phospho-PRK antibody was confirmed to detect endogenous levels of both phosphorylated PRK1 and PRK2 present in LNCaP cells which were identified as a closely migrating doublet band following electrophoresis (*Figure 2A*). The identities of the individual PRK species within the doublet to be those of PRK1 and PRK2 were confirmed through the use of *small interfering* (*si*)RNAs, whereby specific silencing of PRK1 and PRK2, but not PRK3, expression inhibited detection of the phosphorylated forms of PRK1 (lower band) and PRK2 (upper band), respectively (*Figure 2A*). Upon stimulation with U46619, both PRK1 and PRK2 underwent rapid (10 min) and significant increases in their T-loop phosphorylation in LNCaP cells, an increase which was sustained at 60 min post agonist-stimulation (*Figure 2B & 2C*). The specificity of this effect was confirmed by inclusion of the pan-PRK inhibitor PKC412 which blocked the U46619-induced T-loop phosphorylation of both PRK1 and PRK2. Furthermore, the role of the upstream kinases PI3'K and PDK-1 in the U46619-induced PRK1/PRK2 phosphoactivation was confirmed whereby selective inhibition of either PI3'K (LY94002) or PDK-1 (GSK2334470) impaired the sustained T-loop phosphorylation and activation of PRK1/PRK2 in response to TP stimulation (*Figure 2B & 2C*). In contrast, inhibition of the second-messenger regulated protein kinase (PK) A or PKC with H-89 or Gö9688, respectively, had no effect on the U46619-induced T-loop phosphorylation of PRK1/PRK2, thereby confirming that TP-mediated T-loop phosphorylation of the PRKs occurs in a PI3'K/PDK-1-specific mechanism (*Figure 2B & 2C*).

In summary, these data confirm that both PRK1 and PRK2 complex with TP α /TP β in androgen-responsive prostate cancer LNCaP cells and, following agonist activation of TP α /TP β , undergo increased T-loop phosphorylation/activation which occurs in a PI3'K/PDK-1-dependent mechanism.

3.2 PRK1 and PRK2 are essential for TP- and AR-induced proliferation and migration by androgen-responsive LNCaP cells

The PRK isozymes each play a role in neoplastic transformation such as promoting cancer cell proliferation and metastasis^{8, 11, 13}, including in response to signalling by both the TPs (TP α /TP β) and the AR^{5, 16, 17}. Hence, to investigate whether agonist-stimulation of the TPs can indeed mimic and/or enhance androgen-induced cellular responses through TP α /TP β -mediated PRK signalling complexes in PCa, the effect of U46619-, androgen/DHT- or co-stimulation with both agents together on cell proliferation and migration in the androgen-responsive LNCaP cells was investigated.

Stimulation with the TXA₂ mimetic U46619 resulted in significant concentration-dependent increases in both LNCaP cell proliferation and migration, with maximal responses occurring at 10-50 nM U46619 in both cases (*Figure 3A & 3C; Supplemental Figure 1A & 1B*). DHT also significantly increased proliferation and migration by androgen-responsive LNCaP cells (*Figure 3A & 3C*). Moreover, co-stimulation with U46619 significantly enhanced DHT-induced LNCaP cell migration (*Figure 3C & Supplemental Figure 1C*), while co-stimulation with U46619 and DHT combined had no significant effect on proliferation of LNCaP cells compared to cells stimulated with DHT alone (*Figure 3A*). In all cases, the specificity of the observed effects was confirmed whereby the selective TP antagonist SQ29548 or the AR antagonist enzalutamide, blocked the U46619- or DHT-induced cell proliferative and migratory responses, respectively (*Figure 3A & 3C*). In addition, stimulation of LNCaP cells with either SQ29548 or enzalutamide alone did not significantly affect levels of proliferation or migration by LNCaP cells (*Figure 3A & 3C*).

To gain insight into the role of each of the individual PRKs in both the TP α /TP β - and AR-induced cell proliferative and migratory responses, LNCaP cells were transfected with a scrambled control *siRNA* (*siRNA*_{Control}) or with *siRNA* sequences specifically directed to either PRK1, PRK2 or PRK3. Initially, effective *siRNA*-mediated disruption of the PRKs in LNCaP cells was confirmed

where each *siRNA* resulted in specific and almost complete silencing of PRK1, PRK2 or PRK3 expression up to 6 days post-transfection (*Figure 3E*). Consistent with previous findings (*Figure 3A & 3C*), stimulation with U46619 and with DHT significantly increased proliferation and migration in LNCaP cells transfected with the control *siRNA*, while co-stimulation with both U46619 and DHT further specifically enhanced cell migration but not proliferation by the LNCaP cells (*siRNA*_{Control}; *Figure 3B & 3D*). Specific *siRNA*-mediated knockdown of either PRK1 (*siRNA*_{PRK1}) or PRK2 (*siRNA*_{PRK2}) expression significantly inhibited the U46619/TP- and DHT/AR-induced increases in LNCaP cell proliferation (*Figure 3B*) and migration (*Figure 3D*), while depletion of PRK3 (*siRNA*_{PRK3}) expression had no effect on LNCaP cell migration or proliferation irrespective of which receptor agonist (U46619/TP or DHT/AR) was used (*Figure 3B & 3D*).

Collectively, these data demonstrate that agonist stimulation of the TPs can mimic androgen/DHT-induced neoplastic prostate cellular responses, in a mechanism dependent on PRK1 and PRK2, but not PRK3. Furthermore, co-stimulation of the TXA₂-TP-PRK1/2 signalling axis can enhance androgen-induced cellular migration, while it mimics but does not enhance DHT-induced proliferation in the androgen-responsive LNCaP cell line.

3.3 Role of PRK1 on the Association of PRK2 with TP α and TP β in LNCaP cells

Through interactions involving its N-terminal domain, PRK2 is known to form homodimers, raising the possibility of the existence of heterodimers between members of the structurally related PRK family⁴². Moreover, both TP- and AR-induced neoplastic responses, including cell proliferation and migration show an almost equivalent dependence on PRK1 and PRK2 (*Figure 3B & 3D*), despite differences in relative PRK expression levels in LNCaP cells and in their agonist-regulated associations with TP α and TP β (*Figure 1A & 1B*). Therefore, to gain further insight into the composition of the TP α /TP β -PRK1/2 signalling complexes, we sought to determine if there might be any hierarchical relationship between the TP α /TP β -PRK1 and TP α /TP β -PRK2 signalling complexes formed in LNCaP cells. Hence, the effect of *siRNA*-mediated disruption of PRK1 expression on TP α /TP β -PRK2 complex formation, and *vice versa*, was examined. Notably, following *siRNA*-disruption of PRK1 expression, PRK2 was not found in the *anti*-TP α or *anti*-TP β immunoprecipitates formed either in the presence or absence of TP agonist (*Figure 3F*). Conversely, upon knockdown of PRK2 expression, levels of PRK1 associated with TP α and TP β were not significantly affected and were comparable to reference control levels in LNCaP cells transfected with *siRNA*_{Control} (*Figure 3F and 3G*). These data suggest that PRK1 can complex with both TP α /TP β independently of the expression of- and/or potential association with- PRK2. However, expression and/or prior association of PRK1 with TP α and TP β may be a prerequisite for PRK2 complex formation with the TPs.

3.4 TP α /TP β - and AR-agonist stimulation leads to PRK1/2-mediated phosphorylation of Histone H3 at Thr11 in androgen-responsive LNCaP cells

In addition to androgens, activation of PRK1 signalling through the TXA₂/TP axis induces phosphorylation of H3Thr11, the essential upstream chromatin marker and gatekeeper of androgen-induced chromatin remodelling, suggesting that the TXA₂-TP/PRK axis may likewise play a role in epigenetic regulation and/or chromatin remodelling in PCa^{4, 16, 17}. To establish if the TXA₂/TP axis can indeed mimic or even enhance the actions of androgens in PCa cells and, in turn, potentially promote some of the key steps of AR-associated chromatin remodelling and AR-transcriptional activation, we first investigated the influence of the TP α /TP β -PRK1 and TP α /TP β -PRK2 signalling complexes on H3Thr11 phosphorylation in the androgen-responsive LNCaP cell line.

To this end, a specific *anti*-phospho H3Thr11 antibody was used to measure changes in levels of phosphorylated H3Thr11 in LNCaP cells in response to TP α /TP β - and AR-mediated signalling, where cells growth-arrested in metaphase with colcemid served as a reference positive control (*Figure*

4Ai - 4Aiii). Consistent with previous reports by us^{16, 17}, stimulation with the TXA₂ mimetic U46619 (5 nM – 1 μM) induced significant, concentration-dependent increases in the levels of phosphorylated H3Thr11 in LNCaP cells, with maximal increases observed with 50 nM U46619 at 30-120 min post-stimulation (*Figure 4Ai & 4Aii*). Similarly, DHT-stimulation resulted in significant concentration- and time-dependent phosphorylation of H3Thr11 in LNCaP cells, with maximal responses following stimulation with the higher concentrations of DHT (50-100 nM) or stimulation with 10 nM DHT for pro-longed periods of time (8 – 24 hr; *Figure 4Bi & 4Bii*). Hence, agonist stimulation of the TPs can mimic the DHT-induced effect to induce phosphorylation of histone H3 at Thr11.

Thereafter, whether TXA₂/U46619-stimulation might also enhance androgen/DHT-induced H3Thr11 phosphorylation was investigated, where the role of the individual PRK isozymes was examined through *siRNA*-mediated knockdown studies in LNCaP cells. Consistent with previous results in the non-transfected LNCaP cells (*Figure 4A & 4B*), agonist-stimulation of either the TPs- or the AR- significantly increased H3Thr11 phosphorylation in LNCaP cells transfected with a scrambled control *siRNA* (*siRNA*_{Control}; *Figure 4Ci & 4Cii*). Furthermore, co-stimulation of LNCaP cells with U46619 (50 nM; 30 min) significantly enhanced the DHT-induced increases in H3Thr11 phosphorylation relative to those levels in the presence of DHT alone (2.5-fold versus 1.9 fold; *Figure 4Cii*). Moreover, specific *siRNA*-mediated disruption of either PRK1 or PRK2 expression significantly impaired both the individual and combined U46619- and/or DHT-induced H3Thr11 phosphorylation in LNCaP cells, when compared to the *siRNA*_{Control}-transfected LNCaP cells (*Figure 4Ci & 4Cii*). In contrast, *siRNA*-disruption of PRK3 expression had no significant effect on the U46619- and DHT-stimulated phosphorylation of H3Thr11 (*Figure 4C*). In all cases, rescreening of *anti*-phosphoH3Thr11 membranes with an *anti*-histone H3 antibody confirmed that changes in levels of phosphorylated H3Thr11 were not due to differences in histone H3 expression or protein loading (*Figure 4A – 4C*). Thus, TXA₂/TP-mediated activation of PRK1 and PRK2 signalling can both mimic and enhance androgens/DHT to induce hyper-phosphorylation of H3Th11 in prostate LNCaP cells.

3.5 TPα/TPβ- and AR-agonist stimulation leads to recruitment of WDR5 scaffold protein to AR-target genes in prostate adenocarcinoma LNCaP cells

Mechanistically, PRK1-catalysed phosphorylation of H3Thr11 acts as a highly specific androgen-induced chromatin mark which signals the subsequent downstream recruitment of a series of chromatin-modifying enzymes at androgen response elements (AREs) within AR-target genes to facilitate AR-dependent gene expression^{4, 5, 13}. More specifically, phosphorylated H3Thr11 serves as a direct binding platform for WDR5 to AREs, a WD-repeat containing protein which acts as an epigenomic scaffold, facilitating the assembly of histone-modifying complexes to promote AR-target gene expression including in PCa^{5, 13}.

Hence, given the propensity of the TPs to both mimic and enhance androgen-induced H3Thr11 phosphorylation in LNCaP cells (*Figure 4A–4C*), we next asked whether the TXA₂/TP axis might also stimulate chromatin occupancy/recruitment of WDR5 at AREs within the enhancer or promoter regions of select AR-target genes, the best characterised of which are prostate-specific antigen (PSA)/*KLK3* and transmembrane protease serine 2 (*TMPRSS2*) genes. To test this possibility, chromatin immunoprecipitation (ChIP) analyses were performed using chromatin isolated from LNCaP cells pre-incubated either with the TXA₂ mimetic U46619, the endogenous AR ligand DHT or with the drug-vehicle control and *anti*-WDR5 specific antisera. Consistent with previous reports⁵, real-time quantitative (QT)-PCR analysis confirmed that levels of WDR5 associated with the ARE-containing regions of both the *KLK3* (nucleotides -4107 to -3979) and the *TMPRSS2* (nucleotides -13850 to -13506) genes were significantly increased in *anti*-WDR5-immunoprecipitates from DHT-treated cells compared to the control (vehicle-treated) cells (*Figures 5A & 5B*). Furthermore, stimulation with U46619 led to significant increases in WDR5 associated with the ARE-containing

regions within both *KLK3* (Figure 5A) and *TMPRSS2* (Figure 5B) genes, albeit at levels that were approximately 2-fold lower than those cells treated with DHT (Figures 5A & 5B). No PCR amplicons based on those ARE-containing regions of either the *KLK3* or *TMPRSS2* genes were generated using ChIP complexes using pre-immune IgG or -AB control immunoprecipitates from either the U46619- or DHT-treated cells (Figures 5A & 5B). Furthermore, as additional controls, no PCR amplicons were obtained in *anti-WDR5*, *anti-IgG* or -AB ChIP complexes from either the vehicle-, U46619- or DHT-treated cells using primers specific for *GAPDH* gene (Figure 5C), a promoter region known not to contain an ARE^{5, 13}.

In summary, these data establish that activation of the TXA₂-TP α /TP β axis not only induces phosphorylation of H3Thr11 but, in turn, also leads to the recruitment of WDR5 to AR-target genes to mimic the effects of androgens/DHT in LNCaP prostate tumour cells.

3.6 TP α /TP β - and AR-agonist stimulation induces acetylation of histone H4 at K16 in prostate adenocarcinoma LNCaP cells

Acetylation of histone H4 at Lys16 (H4K16) represents a prevalent post-translational chromatin modification in eukaryotes, altering chromatin from a repressive to a transcriptionally active state⁴³. It has been recently proposed that in response to PRK1-catalysed H3Thr11 phosphorylation, WDR5 binds to AREs within AR-target genes and thereby co-localises with MOF (male absent on the first, also known as KAT8 or MYST1), a member of the MYST family of histone acetyltransferases. In turn, MOF acetylates chromatin-associated histone H4 at lysine 16, a highly specific mark required for transcriptional activation^{5, 13, 44}. Herein, given our demonstration of the ability of the TXA₂-TP/PRK axis to mimic and enhance androgen-induced H3Thr11 phosphorylation (Figure 4), as well as to enable the direct recruitment of WDR5 to select AR-target genes (Figure 5A & 5B), we next explored whether TXA₂ signalling might also induce acetylation of H4K16, the essential downstream modification necessary for AR-mediated transcriptional activation in the prostate.

Hence, using an *anti-acetyl-H4K16* antisera, changes in levels of histone H4 acetylated at K16 (H4K16) were investigated following stimulation of LNCaP cells with U46619-, DHT-, or co-stimulated with both agonists, where hyper-acetylation of H4K16 induced by sodium butyrate served as a reference positive control⁴⁵. Treatment of LNCaP cells with sodium butyrate resulted in substantial increases in hyper-acetylation of H4K16 (~3-fold), thereby validating the specificity of the *anti-acetyl H4K16* antibody and of the assay conditions used (Figure 6Ai & 6Aii). Stimulation with U46619 resulted in significant concentration- and time-dependent increases in H4K16 acetylation in LNCaP cells, where maximal responses (2-fold) were observed using 50 nM U46619 at 30-60 min post-TP stimulation (Figure 6Ai & 6Aii). Likewise, stimulation with DHT induced robust concentration- and time-dependent increases in H4K16 acetylation in LNCaP cells, with maximal responses occurring with lower concentrations of DHT (10-20 nM) and following more prolonged incubation times (8-24hr; Figure 6Bi & 6Bii). Hence, agonist stimulation of the TPs can mimic the effects of DHT to induce acetylation of histone H4 at K16.

Thereafter, it was sought to investigate if TXA₂/U46619-stimulation might also enhance androgen/DHT-induced acetylation of H4K16, potentially through a mechanism involving TP α /TP β -mediated PRK1 and/or PRK2 signalling. Hence, the effect of *siRNA*-mediated knockdown of PRK1, PRK2 or PRK3 expression on TP- and AR-induced H4K16 hyper-acetylation in LNCaP cells was next investigated. Agonist-stimulation of either the TPs (with U46619)- or the AR (with DHT)-significantly increased H4K16 acetylation in *siRNA*_{Control}-transfected LNCaP cells (Figure 6Ci & 6Cii). Furthermore, co-stimulation of LNCaP cells with U46619- and DHT- combined significantly enhanced levels of acetylated H4K16 compared to in cells incubated with DHT alone (Figure 6Ci & 6Cii). Notably, in the presence of U46619 and DHT, levels of acetylated H4K16 were ~ 2.2-fold higher than in the control, vehicle-treated cells (Figure 6C). Furthermore, *siRNA*-mediated

knockdown of either PRK1 (*siRNA_{PRK1}*) or PRK2 (*siRNA_{PRK2}*) expression specifically impaired both the U46619- and DHT-induced increases in acetylated H4K16 in LNCaP tumour cells (*Figure 6C*). Conversely, the PRK3-specific *siRNA* sequence (*siRNA_{PRK3}*) did not significantly inhibit TP- and AR-induced H4K16 acetylation (*Figure 6C*). To confirm that the agonist-induced changes in H4K16 acetylation were not due to discrepancies in the levels of total histone H4 expression or protein loading *per se*, parallel membranes were routinely screened with an *anti*-histone H4 antibody itself (*Figure 6A-6C*). Hence, agonist-stimulation of the TPs induces histone H4K16 acetylation and enhances levels of acetylated H4K16 in response to DHT and occurs through a PRK1- and PRK2, but not PRK3-dependent mechanism. Collectively, these data reveal that the TXA₂-TP signalling axis can act as a novel agent which can not only mimic but also enhance androgen signalling to induce H4K16 acetylation, a chromatin modification essential for transcriptional activation.

3.7 TXA₂- and DHT-stimulation leads to association of the AR at AR-target genes in LNCaP cells

Studies presented herein have established that activation of the TXA₂-TP signalling axis not only enables TP α /TP β to mimic, but also to enhance, the key steps of androgen-induced chromatin remodelling (H3Thr11 phosphorylation, WDR5 recruitment and H4K16 acetylation) necessary for AR-dependent target gene expression/transcriptional activation and that these events occur in a PRK1- and PRK2-dependent mechanism. Therefore, we next sought to clarify whether agonist-activation of the TPs might stimulate direct chromatin occupancy/recruitment of the AR to AREs within AR-target genes. To address this, ChIP analyses were performed using chromatin isolated from U46619 and DHT-stimulated LNCaP cells and a validated *anti*-AR antibody. Consistent with published studies^{4,5}, QT-PCR analyses confirmed that DHT induced substantial binding of the AR to ARE-containing regions in both the *KLK3*/PSA (nucleotides -4107 to -3979) and *TMPRSS2* (nucleotides -13850 to -13506) genes (*Figure 7A & 7B*). In addition, U46619 also significantly increased AR binding at AREs within both the *KLK3* (*Figure 7A*) and the *TMPRSS2* (*Figure 7B*) genes, albeit at levels ~3-fold lower than in DHT-treated cells (*Figure 7A & 7B*). Co-stimulation of LNCaP cells with U46619 in the presence of DHT also induced robust AR recruitment to *KLK3* and *TMPRSS2* ARE-containing regions (*KLK3*, 8-fold; *TMPRSS2*, 5-fold) and at levels which were higher than in response to DHT-stimulation alone (*Figure 7A & 7B*). QT-PCR confirmed that no PCR amplicons were generated for the ARE-containing regions of either the *KLK3* or *TMPRSS2* genes in the control preimmune *anti*-IgG or -AB immunoprecipitates from LNCaP cells stimulated with U46619-, DHT- or both agents together (*Figure 7A & 7B*). Moreover, the specificity of AR-binding to AREs was further confirmed whereby no amplification of the *GAPDH* gene, acting as an additional negative control region, was found using either the U46619- or DHT-treated *anti*-AR, *anti*-IgG or -AB immunoprecipitates (*Figure 7C*). Thus, similar to androgens/DHT-AR signalling, activation of the TXA₂-TP axis can induce direct recruitment and binding of the AR to AREs within the regulatory promoter/enhancer regions of AR-target genes.

3.8 Influence of TP-agonist stimulation on AR-target gene expression in LNCaP prostate cancer cells

Using real-time quantitative reverse transcriptase (qRT)-PCR, we next investigated whether stimulation of the TXA₂-TP/PRK axis might either mimic and/or enhance the expression of key AR-target genes (e.g *KLK3*/PSA, *KLK2* and *TMPRSS2*) in LNCaP cells. Consistent with previous reports^{4,5}, the AR ligand DHT yielded significant time-dependent increases in PSA/*KLK3*, *KLK2* and *TMPRSS2* mRNA expression, with the highest increases (4.5-fold) occurring at 16 hr post-DHT stimulation, and where the effects were abolished by the AR antagonist enzalutamide (*Figure 8B*). Moreover, U46619 also significantly increased PSA/*KLK3*, *KLK2* and *TMPRSS2* mRNA expression in a time-dependent manner, with maximal responses (1.75-fold) observed at 16 hr post-agonist

stimulation in all cases (*Figure 8A*). The selective TP antagonist SQ29548 specifically impaired the U46619-induced increases in PSA/*KLK3*, *KLK2* and *TMPRSS2* mRNA expression, while stimulation with SQ29548 alone had no effect thereby confirming the specificity of the TP-mediated effects (*Figure 8A*). Moreover, co-stimulation of LNCaP cells with U46619 plus DHT further enhanced PSA/*KLK3*, *KLK2* and *TMPRSS2* mRNA expression (~5-fold) and, in all cases, at levels significantly higher than those induced by DHT alone (*Figure 8C*). As additional controls, agonist-stimulation of the TPs or the AR, either alone or in combination, did not significantly affect expression of *c-FOS* mRNA (*Figure 8C*), a transcript previously shown not to respond to androgen-induced gene expression^{5, 13}.

Thereafter, the role of the PRK isozymes in mediating both TXA₂- and androgen-induced transcriptional activation of the AR-target genes (*KLK3* and *TMPRSS2*) was investigated (*Figure 8D & 8E*). To this end, LNCaP cells were transfected with *siRNA* sequences specific to the individual PRK isozymes prior to stimulation with U46619 and DHT and changes in gene expression analysed by qRT-PCR. Notably, *siRNA*-mediated disruption of PRK1 and PRK2, but not PRK3, expression significantly impaired both the U46619-, DHT- and combined U46619/DHT- mediated induction of PSA/*KLK3* and *TMPRSS2* expression in LNCaP cells, relative to reference control cells transfected with a nonsense scrambled *siRNA* (*siRNA*_{Control}; *Figure 8D & 8E*). Hence, activation of the TXA₂-TP axis can mimic the effects of androgens/DHT in prostate tumour cells, leading to transcriptional activation of AR-target gene expression and can additionally enhance androgen-induced activation of those target genes, in a mechanism dependent on the ability of TP α /TP β to form functional, activation complexes with PRK1 and PRK2, but not with PRK3.

4. DISCUSSION

The prostanoid TXA₂ and its T Prostanoid receptor, the TP, are increasingly implicated in PCa¹⁹. Mechanistically, we recently discovered that similar to androgens, activation of PRK1 and PRK2 through the TXA₂-TP α /TP β signalling axis can induce phosphorylation of Histone H3 at Thr11 (H3Thr11), an epigenetic marker critical for androgen-induced chromatin remodelling and transcriptional activation¹⁷. Significantly, these findings represented the first demonstration that an agent other than androgens can induce this essential chromatin mark and raised the possibility that the TXA₂-TP axis might mimic and/or enhance AR-induced cellular changes, to function as a potential driver of aberrant AR-reactivation and AR-mediated transcription in PCa. Hence, using LNCaP cells as a model androgen-responsive prostate adenocarcinoma cell line, the current study sought to investigate if the TXA₂-TP/PRK signalling axis might promote and/or enhance the key steps of androgen-induced chromatin remodelling and, in turn, lead to transcriptional activation of AR-target genes in PCa.

In our study, we demonstrate that both PRK1 and PRK2, but not the related PRK3, complex with TP α and TP β in LNCaP cells and, in response to agonist (TXA₂/U46619)-activation of TP α /TP β , PRK1 and PRK2 undergo increased T-loop phosphorylation and activation which occurs in a PI3'K- and PDK1- dependent mechanism (*Figure 9*). Critically, as illustrated in *Figure 9*, we reveal that TXA₂/TP α /TP β -mediated activation of PRK1 and PRK2 signalling enables the TPs to both mimic and enhance androgen-induced cellular responses including: **(1)** H3Thr11 phosphorylation and, consequent **(2)** downstream acetylation of histone H4 at lysine 16, through a mechanism involving TXA₂-stimulated recruitment of **(3)** the androgen receptor (AR) and of **(4)** WDR5 to androgen-response elements (AREs), a WD-repeat containing protein which acts as an epigenomic scaffold, facilitating the assembly of histone-modifying complexes (e.g. SET/MLL1 and MOF complexes) to promote AR-target gene expression. In turn, TXA₂-stimulation **(5)** leads to transcriptional activation of AR-target genes, such as PSA/*KLK3*, *KLK2* and *TMPRSS2* and can enhance androgen-induced expression of AR-target genes, in a PRK1- and PRK2- but not PRK3-dependent mechanism. In summary, these data demonstrate that TP α /TP β can both mimic and enhance the actions of androgens (e.g. DHT) in prostate cells, revealing further mechanistic insight into how the TXA₂/TP signalling axis can exacerbate the pathologic state of PCa. Furthermore, our findings show that the TXA₂-TP/PRK1/2 signalling axis can stimulate aberrant transcriptional activation of AR-target genes, revealing insight into a novel pathway which can influence the transcriptional activity of the AR and potentially facilitate progression to castrate-resistance in PCa (CRPC), an end-stage form of PCa that can occur following androgen deprivation therapy (ADT)².

Mechanistically, transcriptional activation of the AR is primarily controlled via two transcriptional activation units (TAU)-1 and TAU-5 within its amino-terminal domain (NTD)⁴⁶. In fact, due to its essential role in the control of AR transcriptional activity, becoming activated by numerous alternative pathways in the absence of androgen signalling including by PKA, IL-6, epidermal growth factor (EGF) as well as in response to signalling by PRK1 and PRK2, targeting the NTD of the AR represents a key therapeutic approach in the development of novel therapies for CRPC^{2,47}. Moreover, one of the more common mutations that occurs in the AR in cases of CRPC, the so-called 'splice AR variant 7', actually maps to within the N-terminal TAU domain of the AR⁴⁸. As stated, PRK1 and PRK2 both directly interact with the TAU-5 region of the AR NTD to induce a transcriptionally active AR and this occurs both in the presence and absence of testicular androgens¹⁴. Therefore, our discovery that the TXA₂/TP-PRK axis can stimulate transcriptional activity of the AR is entirely consistent with the functional role for both PRK1 and PRK2 in the control of the transcriptional activation of the AR through its PRK-inducible TAU-5 domain. Moreover, the fact that PRK controls essential activating histone marks necessary for AR-target gene expression, including H3Thr11 phosphorylation and H4K16 acetylation and that this occurs not only in response

to androgens but also following agonist-stimulation of TPs further supports a role for TXA₂ signalling in the transcriptional activation of androgen-regulated genes in PCa.

Notably, our data demonstrate that both TP α /TP β - and AR-mediated neoplastic- and transcriptional- responses in prostate cells are not impaired by siRNA-disruption of PRK3/PKN β expression, while showing a near-on equivalent dependency on the expression of both PRK1 and PRK2. In addition, neither the TP α /TP β isoforms associate with PRK3 in prostate cancer LNCaP cells despite its expression therein, albeit at lower levels than PRK1 or PRK2. The isozymes of PRK differ in their tissue distribution and enzymatic functions, as well as showing preferential differences in binding partners^{10, 49-52}. For instance, PRK3 predominately interacts with RhoC over the related PRK1/PRK2, to control metastatic prostate tumour growth downstream of a hyperactive PI3'K pathway¹⁰. Hence, it appears that the more divergent PRK3 isozyme may function as an effector of another oncogenic signalling network in prostate cancer which is potentially independent of the TXA₂-TP α /TP β - and androgen/DHT-AR-signalling axes^{8, 10, 52}. In addition, while previous reports have indicated a role for both PRK1 and PRK2¹⁴, to our knowledge, this represents the first detailed mechanistic insight into the role of all three PRK isozymes in mediating androgen-induced responses in PCa. Although not found to occur in prostate cells, the TPs do have the propensity to associate with PRK3 e.g. in HEK293 cells¹⁷. The biological significance of TP α /TP β -PRK3 complex formation remains unknown and will be the subject of future investigations, particularly in other neoplastic settings where aberrant TP and/or PRK3 signalling are implicated.

While our data demonstrate that both TP α and TP β can contribute to prostate tumorigenesis e.g. through association/activation of PRK1/2-mediated signalling, this may not always be the case in all tumour settings. For example, in lung cancer, it has been shown that TP α , but not TP β , can stimulate tumour growth through up-regulation/activation of ERK²⁰. TP α and TP β exhibit both common, over-lapping but also several isoform-specific functional roles. For instance, while showing similar coupling to G α_q /phospholipase C β and G α_{12} /RhoA, TP α and TP β differentially regulate other effectors such as adenylyl cyclase and are subject to distinct mechanisms of both agonist-induced homologous and heterologous desensitisation²⁵. Moreover, TP α and TP β are differentially expressed in a range of cell/tissue types, being under the transcriptional regulation of distinct promoters within the *TBXA2R* gene²⁹. Hence, the individual TP isoforms display several critical differences in their modes of signalling and regulation, as well as unique patterns of expression which may clearly impact on their relative contribution to disease processes, including cancer progression in different cellular or clinical settings potentially accounting for documented differential contributions of the TP α and TP β to certain cancer types^{20, 53}. Therefore, although evidence is increasing, further studies are clearly warranted to determine the role of the individual TP α and TP β isoforms in different cancer settings.

Of particular importance to the current study is our finding that agonist-stimulation of TP α /TP β can mimic and enhance the effects of androgens/DHT to upregulate the expression of AR-responsive genes (e.g. *PSA/KLK3, KLK2, TMPRSS2*). Through immunohistochemical analysis of TP α and TP β expression in PCa tumour microarrays (TMAs), we recently demonstrated a clinical relationship and clinical significance between the TXA₂/TP signalling axis with the biochemical recurrence (BCR) of PCa in a large cohort of patients who underwent prostatectomy⁵³. Significantly, in that study we established that expression of both TP α and TP β was increased in prostate tumour relative to the benign prostate tissue where TP β and, to a lesser extent, TP α expression significantly correlated with pathologic staging and with increasing Gleason score/tumour severity. Moreover, it was found that elevated levels of TP α and/or TP β expression in PCa patients predicts a shorter time to biochemical recurrence of disease, signified by rising serum PSA levels, and hence also correlated with shorter disease-free survival times in patients' post-prostatectomy⁵³. Data presented herein also show that TXA₂/TP-stimulated up-regulation of AR-responsive gene expression, as well as increased PCa cell proliferation and migration are effectively blocked by the selective TP antagonist SQ29548 (Figure 9). It would therefore indeed be of interest to determine whether targeting TP α and/or TP β

through use of receptor antagonists, either alone or in combination with currently available ADTs, such as enzalutamide, would have added clinical benefit in the inhibition of prostate tumour growth/migration including in CRPC. While beyond the scope of the current study, such investigations will be the focus of future studies.

The tumour microenvironment or surrounding stromal tissue is increasingly recognized as a major driver of tumour development, providing the essential paracrine or autocrine mediators to promote tumour growth & metastasis, angiogenesis, immune-evasion and overall survival, particularly in the heterogenous PCa tissue environ⁵⁴. Interestingly, in our recent study, enhanced TP α /TP β expression was detected in prostate epithelial cells⁵³ but also in fibromuscular stromal smooth muscle cells as well as in regions of inflammatory cell infiltrate within the tumour tissue (*Mulvaney & Kinsella, unpublished data*). The tumour microenvironment is characterised by the recruitment of inflammatory cells such as tumour-associated macrophages (TAMs) and lymphocytes, which produce a variety of inflammatory mediators, including TNF α , IL-6 and the inducible COX-2, also leading to enhanced generation of prostanoids within the tumour tissue^{23, 55, 56}. Moreover, enhanced platelet activation leads to sustained release of pro-tumorigenic factors in the tumour environment, particularly TXA₂, the major product of platelet COX-1²³. Hence, our discovery that the TXA₂/TP-PRK axis can induce chromatin remodelling and transcriptional activation of AR-target genes, *albeit* at lower levels but in a faster, more dynamic manner than androgens/DHT itself, may be highly significant. Indeed, our discovery may provide a mechanism, at least in part, for the aberrant up-regulation of AR-target gene expression (e.g. elevated PSA) which can occur in CRPC through persistent autocrine signalling by TXA₂ generated within the tumour environment. Furthermore, our data provide a further mechanistic explanation for the prophylactic benefits of Aspirin in reducing certain cancer risk through continuous suppression of TXA₂ biosynthesis.

Overall, as summarised in *Figure 9*, our data demonstrate that the TXA₂-TP α /TP β axis can act both as a neoplastic and epigenetic regulator, mimicking and/or enhancing the actions of androgens in prostate cells, to exacerbate the pathological state of PCa. Moreover, our study highlights the potential for TP α and/or TP β as a therapeutic target in the treatment of prostate cancer, including potentially in CRPC.

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CONFLICT OF INTERESTS STATEMENT

The authors declare that they have no competing interests.

REFERENCES

1. Tuomela J, Härkönen P. Tumor models for prostate cancer exemplified by fibroblast growth factor 8-induced tumorigenesis and tumor progression. *Reproductive Biology*. 2014;14: 16-24.
2. Shafi AA, Yen AE, Weigel NL. Androgen receptors in hormone-dependent and castration-resistant prostate cancer. *Pharmacol Ther*. 2013;140: 223-238.
3. Mukai H. The structure and function of PKN, a protein kinase having a catalytic domain homologous to that of PKC. *J Biochem*. 2003;133: 17-27.
4. Metzger E, Yin N, Wissmann M, et al. Phosphorylation of histone H3 at threonine 11 establishes a novel chromatin mark for transcriptional regulation. *Nat Cell Biol*. 2008;10: 53-60.
5. Kim JY, Banerjee T, Vinckevicius A, et al. A role for WDR5 in integrating threonine 11 phosphorylation to lysine 4 methylation on histone H3 during androgen signaling and in prostate cancer. *Mol Cell*. 2014;54: 613-625.
6. Flynn P, Mellor H, Casamassima A, Parker PJ. Rho GTPase control of protein kinase C-related protein kinase activation by 3-phosphoinositide-dependent protein kinase. *J Biol Chem*. 2000;275: 11064-11070.
7. Dong LQ, Landa LR, Wick MJ, et al. Phosphorylation of protein kinase N by phosphoinositide-dependent protein kinase-1 mediates insulin signals to the actin cytoskeleton. *Proc Natl Acad Sci U S A*. 2000;97: 5089-5094.
8. Leenders F, Mopert K, Schmiedeknecht A, et al. PKN3 is required for malignant prostate cell growth downstream of activated PI 3-kinase. *EMBO J*. 2004;23: 3303-3313.
9. Galgano MT, Conaway M, Spencer AM, Paschal BM, Frierson HF, Jr. PRK1 distribution in normal tissues and carcinomas: overexpression and activation in ovarian serous carcinoma. *Hum Pathol*. 2009;40: 1434-1440.
10. Unsal-Kacmaz K, Rangunathan S, Rosfjord E, et al. The interaction of PKN3 with RhoC promotes malignant growth. *Mol Oncol*. 2012;6: 284-298.
11. Lachmann S, Jevons A, De Rycker M, et al. Regulatory domain selectivity in the cell-type specific PKN-dependence of cell migration. *PloS one*. 2011;6: e21732.
12. Metzger E, Wissmann M, Yin N, et al. LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. *Nature*. 2005;437: 436-439.
13. Kim J-Y, Yu J, Abdulkadir SA, Chakravarti D. KAT8 Regulates Androgen Signaling in Prostate Cancer Cells. *Molecular Endocrinology*. 2016;30: 925-936.
14. Metzger E, Muller JM, Ferrari S, Buettner R, Schule R. A novel inducible transactivation domain in the androgen receptor: implications for PRK in prostate cancer. *EMBO J*. 2003;22: 270-280.
15. Kohler J, Erlenkamp G, Eberlin A, et al. Lestaurtinib inhibits histone phosphorylation and androgen-dependent gene expression in prostate cancer cells. *PLoS One*. 2012;7: e34973.
16. Turner EC, Kavanagh DJ, Mulvaney EP, et al. Identification of an interaction between the TP α and TP β isoforms of the human thromboxane A2 receptor with protein kinase C-related kinase (PRK) 1: Implications for prostate cancer. *J Biol Chem*. 2011;286: 15440-15457.
17. O'Sullivan AG, Mulvaney EP, Hyland PB, Kinsella BT. Protein kinase C-related kinase 1 and 2 play an essential role in thromboxane-mediated neoplastic responses in prostate cancer. *Oncotarget*. 2015;6: 26437-26456.
18. Woodward DF, Jones RL, Narumiya S. International Union of Basic and Clinical Pharmacology. LXXXIII: Classification of Prostanoid Receptors, Updating 15 Years of Progress. *Pharmacological Reviews*. 2011;63: 471-538.
19. Ekambaram P, Lambiv W, Cazzolli R, Ashton AW, Honn KV. The thromboxane synthase and receptor signaling pathway in cancer: an emerging paradigm in cancer progression and metastasis. *Cancer Metastasis Rev*. 2011;30: 397-408.
20. Huang RY, Li MY, Ng CS, et al. Thromboxane A2 receptor alpha promotes tumor growth through an autoregulatory feedback pathway. *J Mol Cell Biol*. 2013;5: 380-390.
21. Dassel T, de Leval X, de Leval L, Pirote B, Castronovo V, Waltregny D. Activation of the thromboxane A2 pathway in human prostate cancer correlates with tumor Gleason score and pathologic stage. *Eur Urol*. 2006;50: 1021-1031.
22. Cuzick J, Thorat MA, Bosetti C, et al. Estimates of benefits and harms of prophylactic use of aspirin in the general population. *Annals of Oncology*. 2015;26: 47-57.

23. Dovizio M, Alberti S, Guillem-Llobat P, Patrignani P. Role of platelets in inflammation and cancer: novel therapeutic strategies. *Basic Clin Pharmacol Toxicol*. 2014;114: 118-127.
24. Rothwell PM, Fowkes FG, Belch JF, Ogawa H, Warlow CP, Meade TW. Effect of daily aspirin on long-term risk of death due to cancer: analysis of individual patient data from randomised trials. *Lancet*. 2011;377: 31-41.
25. Kinsella BT. Thromboxane A2 signalling in humans: a 'Tail' of two receptors. *Biochem Soc Trans*. 2001;29: 641-654.
26. Coyle AT, Miggin SM, Kinsella BT. Characterization of the 5' untranslated region of alpha and beta isoforms of the human thromboxane A2 receptor (TP). Differential promoter utilization by the TP isoforms. *Eur J Biochem*. 2002;269: 4058-4073.
27. Coyle AT, Kinsella BT. Characterization of promoter 3 of the human thromboxane A receptor gene. A functional AP-1 and octamer motif are required for basal promoter activity. *FEBS J*. 2005;272: 1036-1053.
28. Keating GL, Reid HM, Eivers SB, Mulvaney EP, Kinsella BT. Transcriptional regulation of the human thromboxane A2 receptor gene by Wilms' tumor (WT)1 and hypermethylated in cancer (HIC) 1 in prostate and breast cancers. *Biochim Biophys Acta*. 2014;1839: 476-492.
29. Kinsella BT. Transcriptional Regulation of the Human Thromboxane A2 Receptor Gene by Wilms' Tumour (WT)1. In: van den Heuvel-Eibrink MM, editor. *Wilms Tumor*. Brisbane AU, 2016:163-187.
30. Walsh M, Foley JF, Kinsella BT. Investigation of the role of the carboxyl-terminal tails of the alpha and beta isoforms of the human thromboxane A(2) receptor (TP) in mediating receptor:effector coupling. *Biochim Biophys Acta*. 2000;1496: 164-182.
31. Wikstrom K, Kavanagh DJ, Reid HM, Kinsella BT. Differential regulation of RhoA-mediated signaling by the TPalpha and TPbeta isoforms of the human thromboxane A2 receptor: independent modulation of TPalpha signaling by prostacyclin and nitric oxide. *Cell Signal*. 2008;20: 1497-1512.
32. Li Z, Zhang G, Le Breton GC, Gao X, Malik AB, Du X. Two waves of platelet secretion induced by thromboxane A2 receptor and a critical role for phosphoinositide 3-kinases. *J Biol Chem*. 2003;278: 30725-30731.
33. Miggin SM, Kinsella BT. Thromboxane A(2) receptor mediated activation of the mitogen activated protein kinase cascades in human uterine smooth muscle cells. *Biochim Biophys Acta*. 2001;1539: 147-162.
34. Miggin SM, Kinsella BT. Regulation of extracellular signal-regulated kinase cascades by alpha- and beta-isoforms of the human thromboxane A(2) receptor. *Mol Pharmacol*. 2002;61: 817-831.
35. Kelley-Hickie LP, Kinsella BT. Homologous desensitization of signalling by the beta (beta) isoform of the human thromboxane A2 receptor. *Biochim Biophys Acta*. 2006;1761: 1114-1131.
36. Kelley-Hickie LP, O'Keefe MB, Reid HM, Kinsella BT. Homologous desensitization of signalling by the alpha (alpha) isoform of the human thromboxane A2 receptor: a specific role for nitric oxide signalling. *Biochim Biophys Acta*. 2007;1773: 970-989.
37. Kelley-Hickie LP, Kinsella BT. EP1- and FP-mediated cross-desensitization of the alpha (alpha) and beta (beta) isoforms of the human thromboxane A2 receptor. *Br J Pharmacol*. 2004;142: 203-221.
38. Walsh M-T, Foley JF, Kinsella BT. The α , but Not the β , Isoform of the Human Thromboxane A2 Receptor Is a Target for Prostacyclin-mediated Desensitization. *Journal of Biological Chemistry*. 2000;275: 20412-20423.
39. Shechter D, Dormann HL, Allis CD, Hake SB. Extraction, purification and analysis of histones. *Nat Protoc*. 2007;2: 1445-1457.
40. Yuan X, Cai C, Chen S, Chen S, Yu Z, Balk SP. Androgen receptor functions in castration-resistant prostate cancer and mechanisms of resistance to new agents targeting the androgen axis. *Oncogene*. 2014;33: 2815-2825.
41. Mukai H, Kitagawa M, Shibata H, et al. Activation of PKN, a novel 120-kDa protein kinase with leucine zipper-like sequences, by unsaturated fatty acids and by limited proteolysis. *Biochem Biophys Res Commun*. 1994;204: 348-356.
42. Bauer AF, Sonzogni S, Meyer L, et al. Regulation of Protein Kinase C-related Protein Kinase 2 (PRK2) by an Intermolecular PRK2-PRK2 Interaction Mediated by Its N-terminal Domain. *Journal of Biological Chemistry*. 2012;287: 20590-20602.
43. Shia W-J, Pattenden SG, Workman JL. Histone H4 lysine 16 acetylation breaks the genome's silence. *Genome Biology*. 2006;7: 1-3.

44. Chen Q, Costa M, Sun H. Structure and function of histone acetyltransferase MOF. *AIMS Biophys.* 2015;2: 555-569.
45. Sealy L, Chalkley R. The effect of sodium butyrate on histone modification. *Cell.* 1978;14: 115-121.
46. Tan MHE, Li J, Xu HE, Melcher K, Yong E-I. Androgen receptor: structure, role in prostate cancer and drug discovery. *Acta Pharmacol Sin.* 2015;36: 3-23.
47. Andersen RJ, Mawji NR, Wang J, et al. Regression of castrate-recurrent prostate cancer by a small-molecule inhibitor of the amino-terminus domain of the androgen receptor. *Cancer Cell.* 2010;17: 535-546.
48. Bryce AH, Antonarakis ES. Androgen receptor splice variant 7 in castration-resistant prostate cancer: Clinical considerations. *Int J Urol.* 2016;23: 646-653.
49. Hutchinson CL, Lowe PN, McLaughlin SH, Mott HR, Owen D. Mutational analysis reveals a single binding interface between RhoA and its effector, PRK1. *Biochemistry.* 2011;50: 2860-2869.
50. Falk MD, Liu W, Bolanos B, et al. Enzyme Kinetics and Distinct Modulation of the Protein Kinase N Family of Kinases by Lipid Activators and Small Molecule Inhibitors. *Biosci Rep.* 2014;34: e00097.
51. Quilliam LA, Lambert QT, Mickelson-Young LA, et al. Isolation of a NCK-associated kinase, PRK2, an SH3-binding protein and potential effector of Rho protein signaling. *J Biol Chem.* 1996;271: 28772-28776.
52. Oishi K, Mukai H, Shibata H, Takahashi M, Ona Y. Identification and characterization of PKNbeta, a novel isoform of protein kinase PKN: expression and arachidonic acid dependency are different from those of PKNalpha. *Biochem Biophys Res Commun.* 1999;261: 808-814.
53. Mulvaney EP, Shilling C, Eivers SB, et al. Expression of the TPalpha and TPbeta isoforms of the thromboxane prostanoid receptor (TP) in prostate cancer: clinical significance and diagnostic potential. *Oncotarget.* 2016;7: 73171-73187.
54. Chen F, Zhuang X, Lin L, et al. New horizons in tumor microenvironment biology: challenges and opportunities. *BMC Med.* 2015;13: 45.
55. Mishra RK, Tewari AK. Inflammation and Its Role in Prostate Cancer. *Journal of Cancer Therapy.* 2014;Vol.05No.09: 9.
56. Huang Q-C, Huang R-Y. The cyclooxygenase-2/thromboxane A2 pathway: a bridge from rheumatoid arthritis to lung cancer? *Cancer Letters.* 2014;354: 28-32.

FIGURE LEGENDS

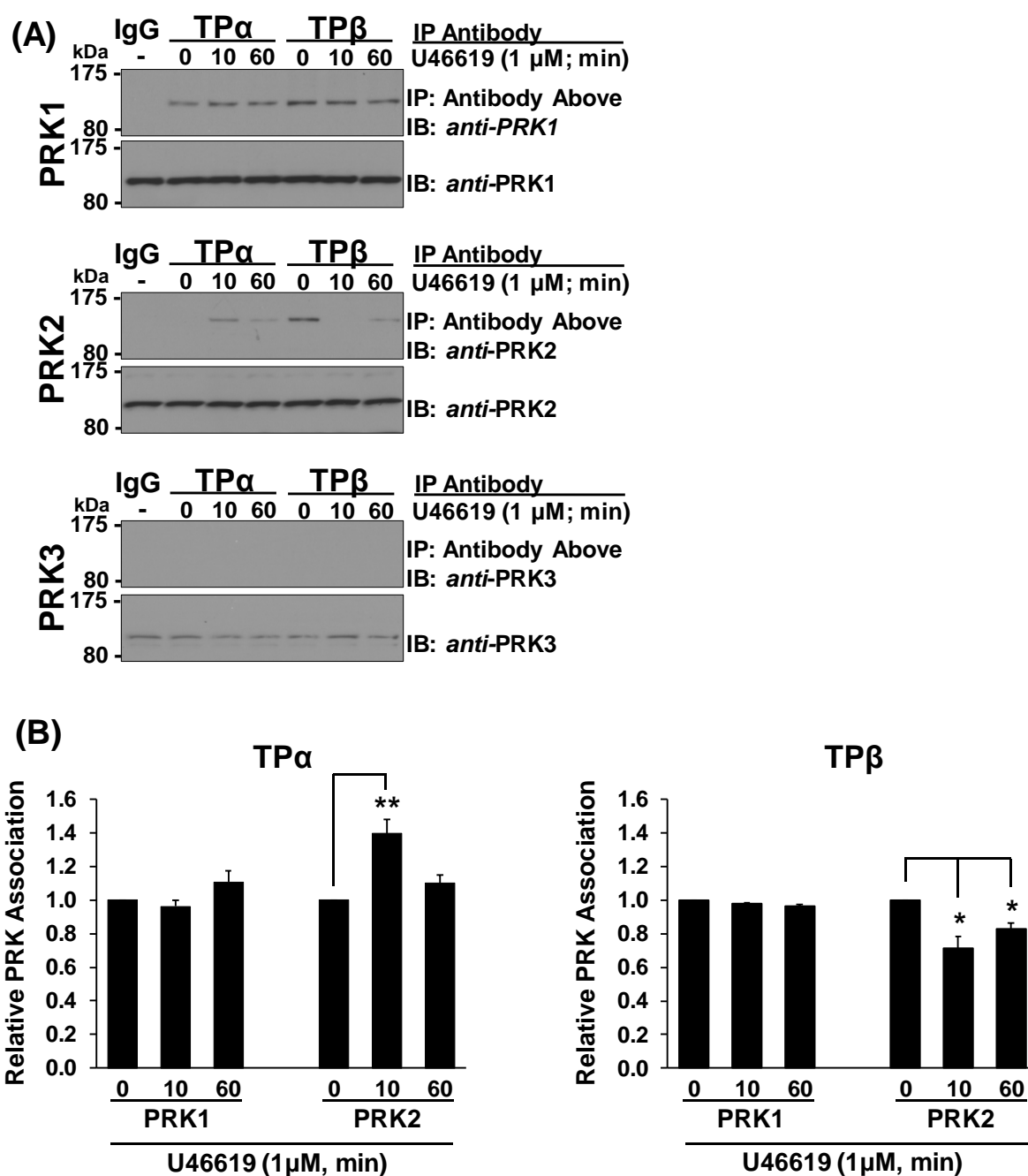


Figure 1: Association of TP α /TP β with PRK1 and PRK2 in prostate carcinoma LNCaP cells.

Panel A: LNCaP cells were stimulated with U46619 (1 μ M, 0 - 60 min) prior to immunoprecipitation with anti-TP α or anti-TP β isoform specific antisera or, as controls, with an equivalent concentration of the preimmune serum (IgG). Thereafter, immunoprecipitates (IP; upper panels) or equivalent aliquots of whole cell lysates (20 μ g/lane) were resolved by SDS-page and immunoblotted (IB; lower panels) with anti-PRK1, anti-PRK2 or anti-PRK3 antisera, as indicated. Data shown are representative of at least three independent experiments (n = 3). *Panel B:* Bar charts showing the mean relative levels of PRK1 and PRK2 associated with TP α or TP β immunoprecipitates, where levels in the absence of agonist (0 min) are assigned a value of 1 (n = 3, \pm SEM). The asterisks indicate where stimulation with U46619 led to significant changes in levels of TP α /TP β -associated PRK1 or PRK2, where * and ** represent $p < 0.05$ and $p < 0.01$, respectively.

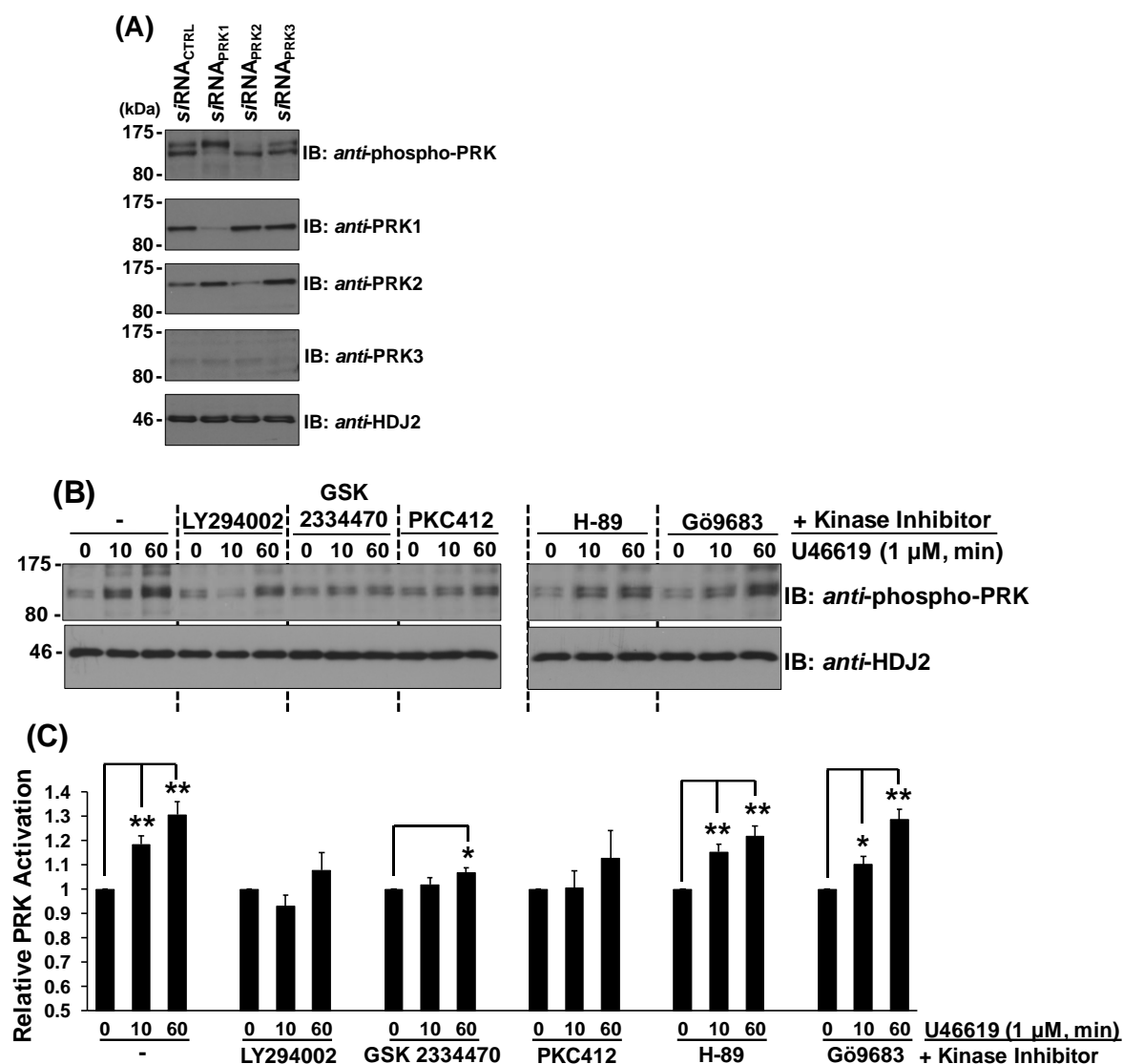
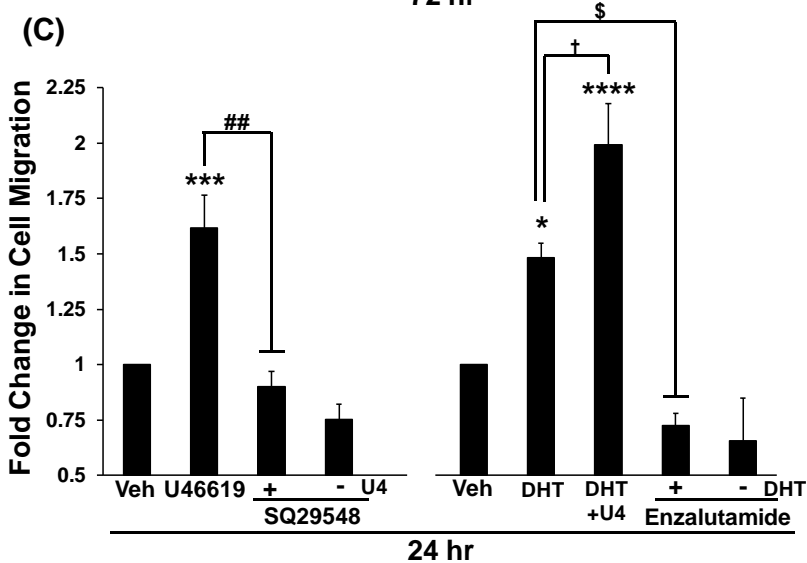
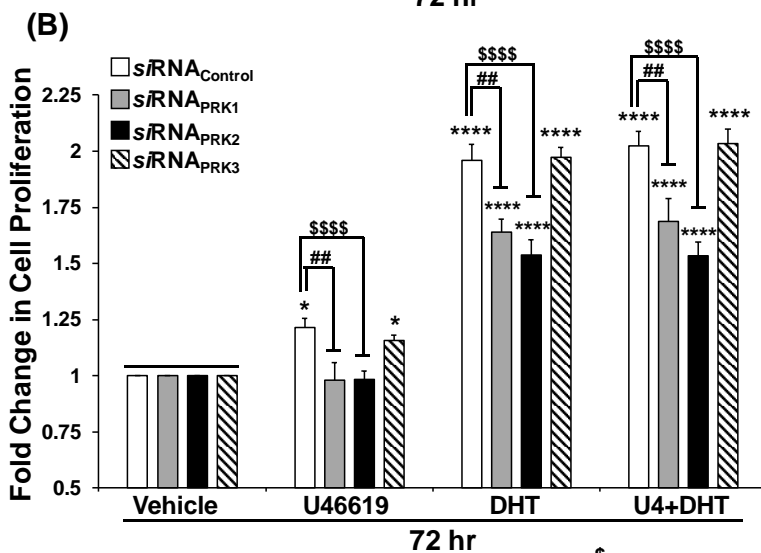
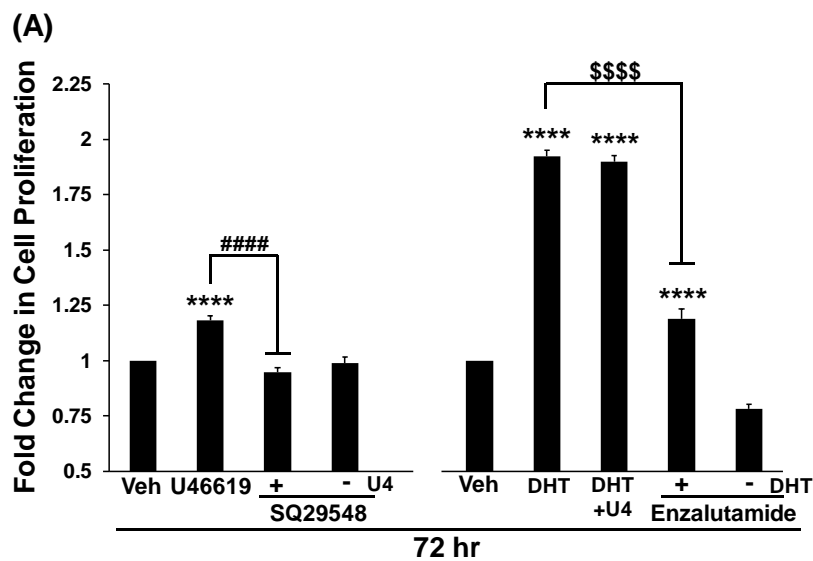


Figure 2: Agonist stimulation of TP α /TP β leads to T-loop phosphorylation/activation of PRK1 and PRK2 in prostate LNCaP cells.

Panels A-C: LNCaP cells were serum-starved (0% FBS) for 16 hr and pre-incubated for 30 min with the indicated kinase inhibitor, or as controls, with drug vehicle (0.01% DMSO), followed by stimulation with U46619 (1 μ M) for 0, 10, or 60 min (**Panels B & C**). To identify the species of PRK subject to U46619-induced T-loop phosphorylation, LNCaP cells were transfected with *siRNA*_{PRK1}, *siRNA*_{PRK2}, *siRNA*_{PRK3} OR with a scrambled control (*siRNA*_{Control}), before stimulation with U46619 (1 μ M; 60 min; **Panel A**). Thereafter, cells were harvested and aliquots (20 μ g/lane) were resolved by SDS-page and immunoblotted (IB) with *anti-phospho-PRK1*^{Thr774}/*PRK2*^{Thr816}/*PRK3*^{Thr718} to detect PRK activation/T-loop phosphorylation and with *anti-HDJ* antisera to confirm equal protein loading. Data shown are representative of at least three independent experiments ($n = 3$). The bar chart in **Panel C** shows the mean relative level of T-loop phosphorylation/PRK activation, as determined by quantitative densitometry, where levels in the absence of agonist (0 min) are assigned a value of 1 ($n = 3$, \pm SEM). The asterisks signify where stimulation with U46619 induced significant increases in levels of PRK1 and PRK2 activation, where * and ** represent $p < 0.05$ and $p < 0.01$, respectively ($n = 3$).



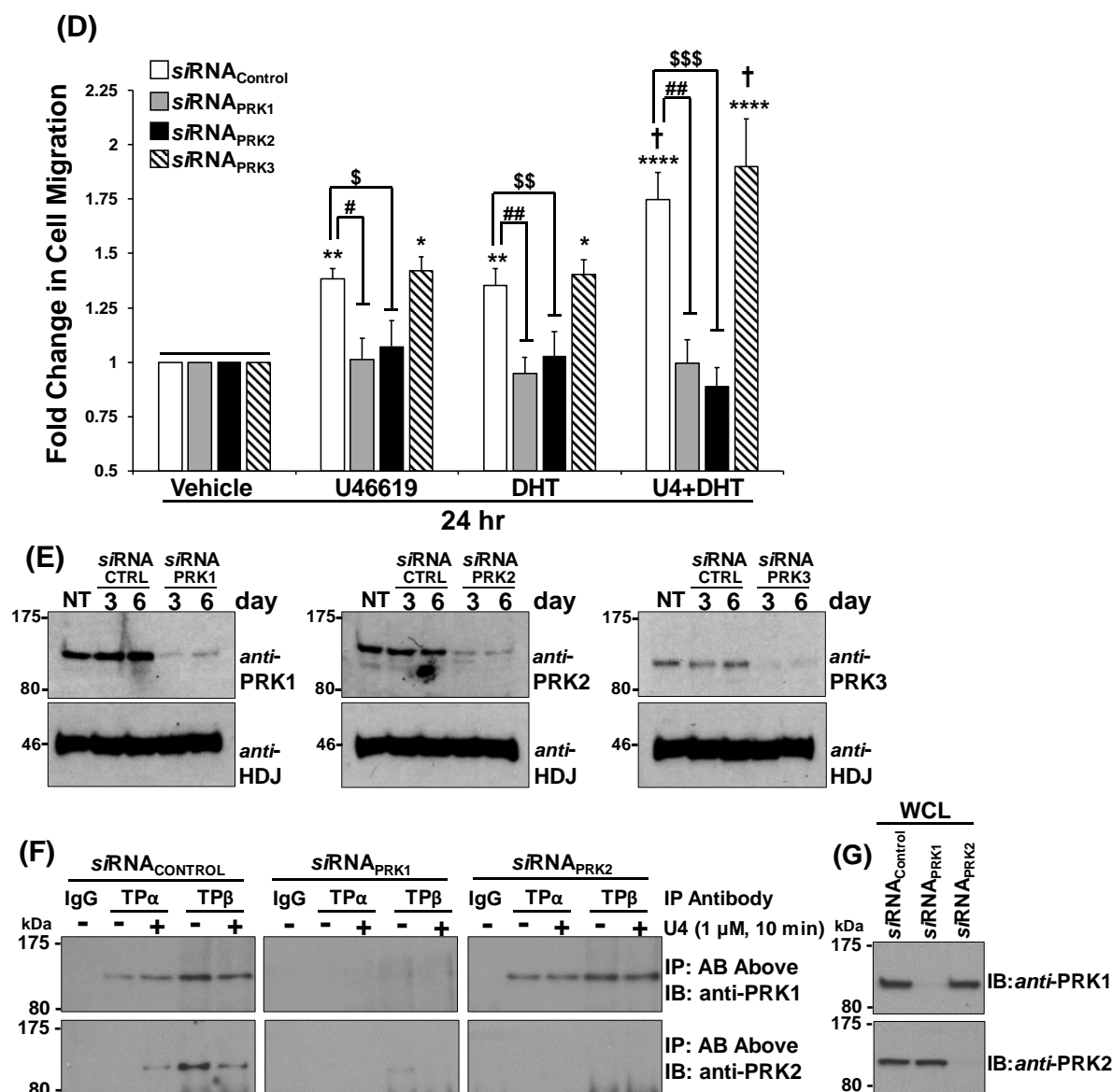


Figure 3: Effect of siRNA-disruption of PRK1, PRK2 and PRK3 expression on TP- and AR-agonist induced proliferation and migration of LNCaP cells.

Panels A-D: Proliferation (*Panels A & B*) and migration (*Panels C & D*) of LNCaP cells stimulated with either U46619 (U4; 10 nM), SQ29548 (1 μM), DHT (10 nM) or enzalutamide (10 μM), either alone or in combination, or with the drug vehicle (Veh; 0.0001% EtOH), for 24 or 72 hr as indicated. Results are expressed as mean fold-change in cell proliferation or migration relative to vehicle-treated cells, assigned a value of 1 ($n = 3$, \pm SEM). The *asterisks* indicate where stimulation with U46619 or DHT resulted in significant increases in LNCaP cell proliferation or migration. † indicates that combined U46619/DHT-stimulation resulted in significant increases in cell migration relative to DHT-stimulation alone. In *Panels A & C*, # and \$ signify that U46619- or DHT- stimulated cell proliferation/migration was significantly inhibited by SQ29548 or enzalutamide, respectively. In *Panels B & D*, LNCaP cells were transfected with 30 nM siRNA_{PRK1}, siRNA_{PRK2}, siRNA_{PRK3} or with a scrambled control siRNA (siRNA_{Control}) prior to stimulation with U46619 (50 nM), DHT (10 nM) or both, as indicated ($n = 5$). # and \$ indicate that levels of U46619- or DHT- stimulated cell proliferation or migration were significantly decreased in cells transfected with siRNA_{PRK1} or siRNA_{PRK2} respectively, relative to siRNA_{Control}. In all cases, single/double/triple/quadruple symbols indicate $p \leq 0.05/0.01/0.001/0.0001$ respectively, for one-way ANOVA, followed by Bonferroni or Dunnett's multiple comparison t-tests ($n \geq 3$). *Panel E:* Confirmation of siRNA-mediated disruption of PRK1,

PRK2 or PRK3 expression in whole cell lysates at 3 and 6 days post-transfection. *Panels F & G*: LNCaP cells transfected with *siRNA*_{PRK1}, *siRNA*_{PRK2}, or an *siRNA*_{Control} were stimulated with U46619 (1 μ M; 10 min) or vehicle (0.01% EtOH), prior to immunoprecipitation with *anti*-TP α , *anti*-TP β , or the preimmune serum (IgG) control. Thereafter, immunoprecipitates (IP; *Panel F*) or aliquots of the corresponding whole cell lysates (20 μ g/lane, whole cell lysate/WCL; *Panel G*) were analysed by immunoblotting (IB) with *anti*-PRK1 or *anti*-PRK2 antisera (Data n = 3).

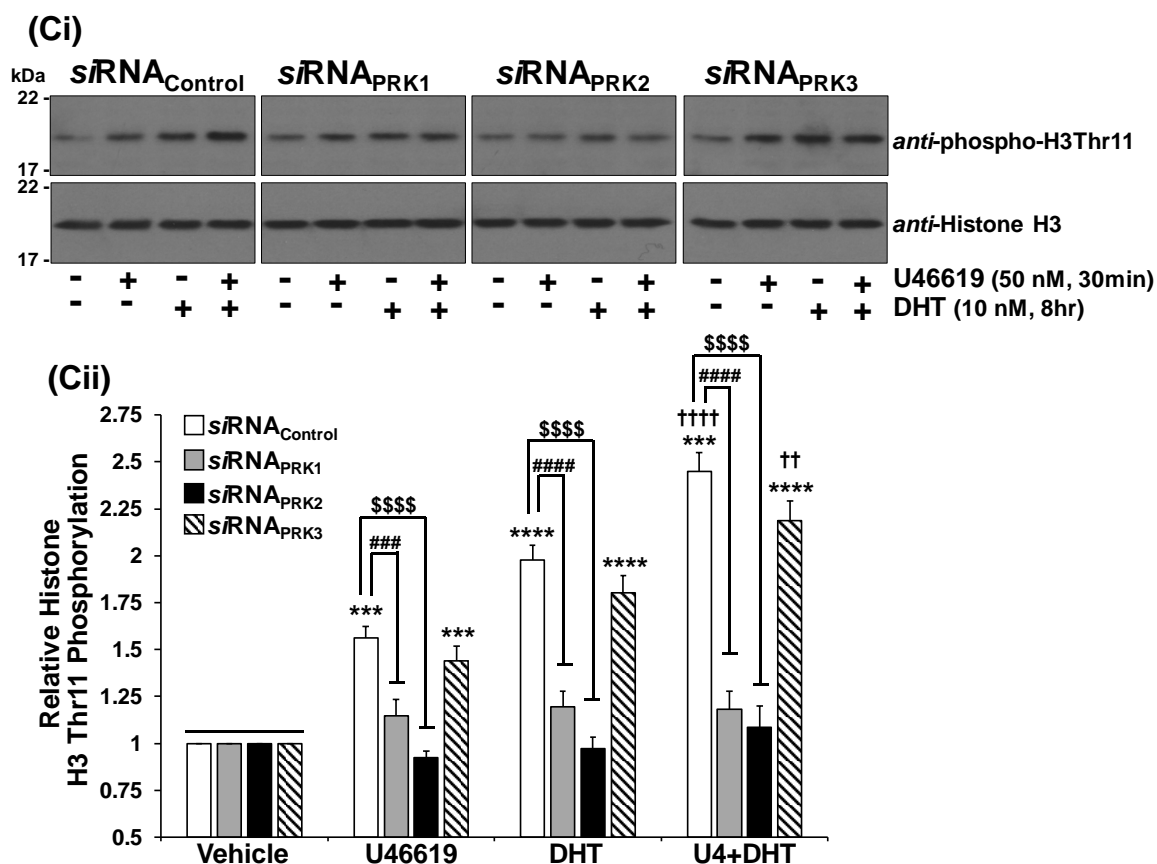


Figure 4: Effect of TP- and AR- agonist stimulation on Histone H3 at Thr 11 in prostate LNCaP cells.

Panels A-C: Immunoblot analysis of H3 Thr11 phosphorylation in LNCaP cells stimulated with either U46619 (5-1000 nM), DHT (1-100 nM), U46619 plus DHT (50 nM + 10 nM), or as controls, colcemid (50 ng/ μ l), or drug vehicle (0.0005 - 0.01% EtOH), for the indicated time. In all cases, isolated histones were resolved by SDS-PAGE and immunoblotted with *anti*-phospho-H3Thr11 and back-blotted with *anti*-Histone H3. The bar charts show the mean levels of H3 Thr11 phosphorylation in LNCaP cells relative to total histone H3 levels, as determined by quantitative densitometry, where levels in vehicle-treated cells were assigned a value of 1 (n = 3, \pm SEM). In *Panel C*, LNCaP cells were transfected with PRK isozyme-specific *siRNA* oligonucleotides, as indicated, or an *siRNA*_{Control}, prior to stimulation with U46619 (50 nM), DHT (10 nM) or both and immunoblot analysis of H3 Thr11 phosphorylation. In the bar charts associated with *Panels A-C*, the asterisks indicate that levels of H3Thr11 phosphorylation were significantly increased in response to U46619- or DHT-stimulation; † indicates that combined U46619/DHT-stimulation resulted in significant increases in levels of H3Thr11 phosphorylation relative to DHT-stimulation alone; # and \$ signify that levels of U46619- or DHT- stimulated H3Thr11 phosphorylation were significantly inhibited in cells transfected with *siRNA*_{PRK1} or *siRNA*_{PRK2}, respectively, relative to *siRNA*_{Control}. In all cases, single/double/triple/quadruple symbols indicate p \leq 0.05/0.01/0.001/0.0001 respectively, for one-way ANOVA, followed by Bonferroni or Dunnett's multiple comparison t-tests (n = 3).

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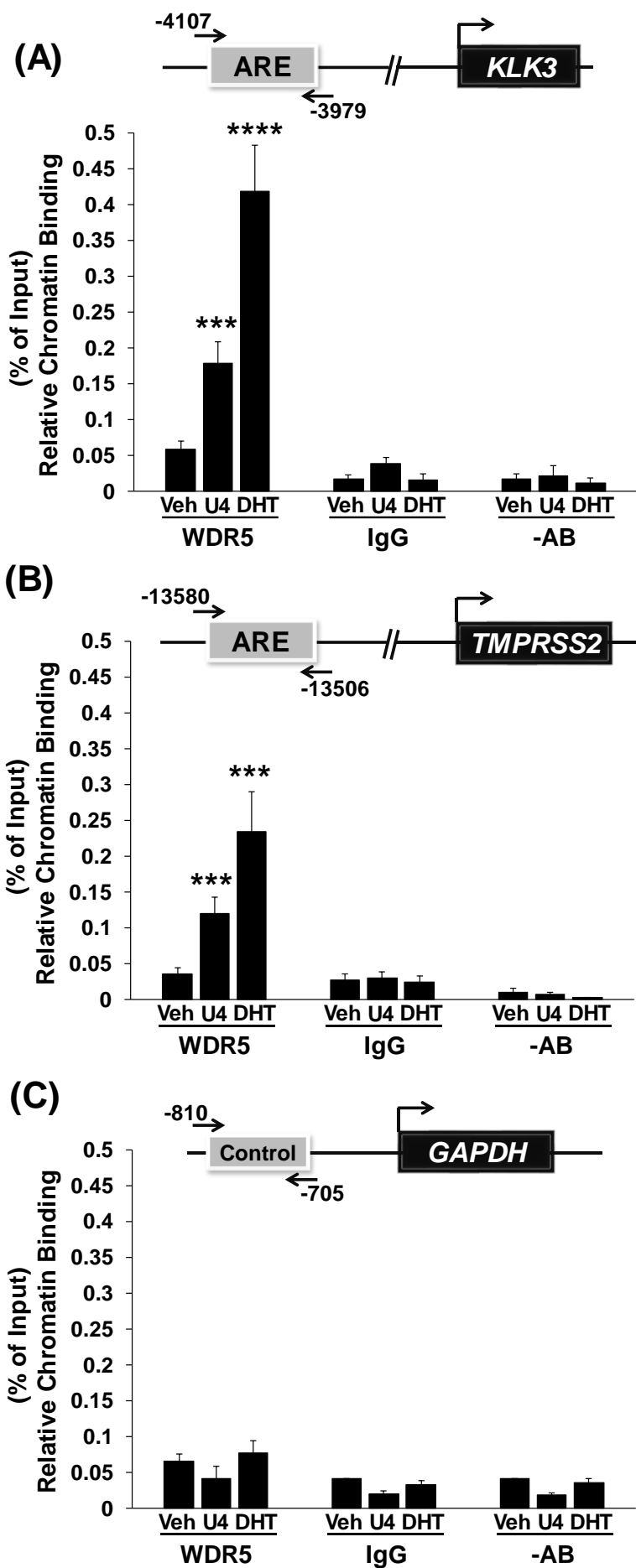
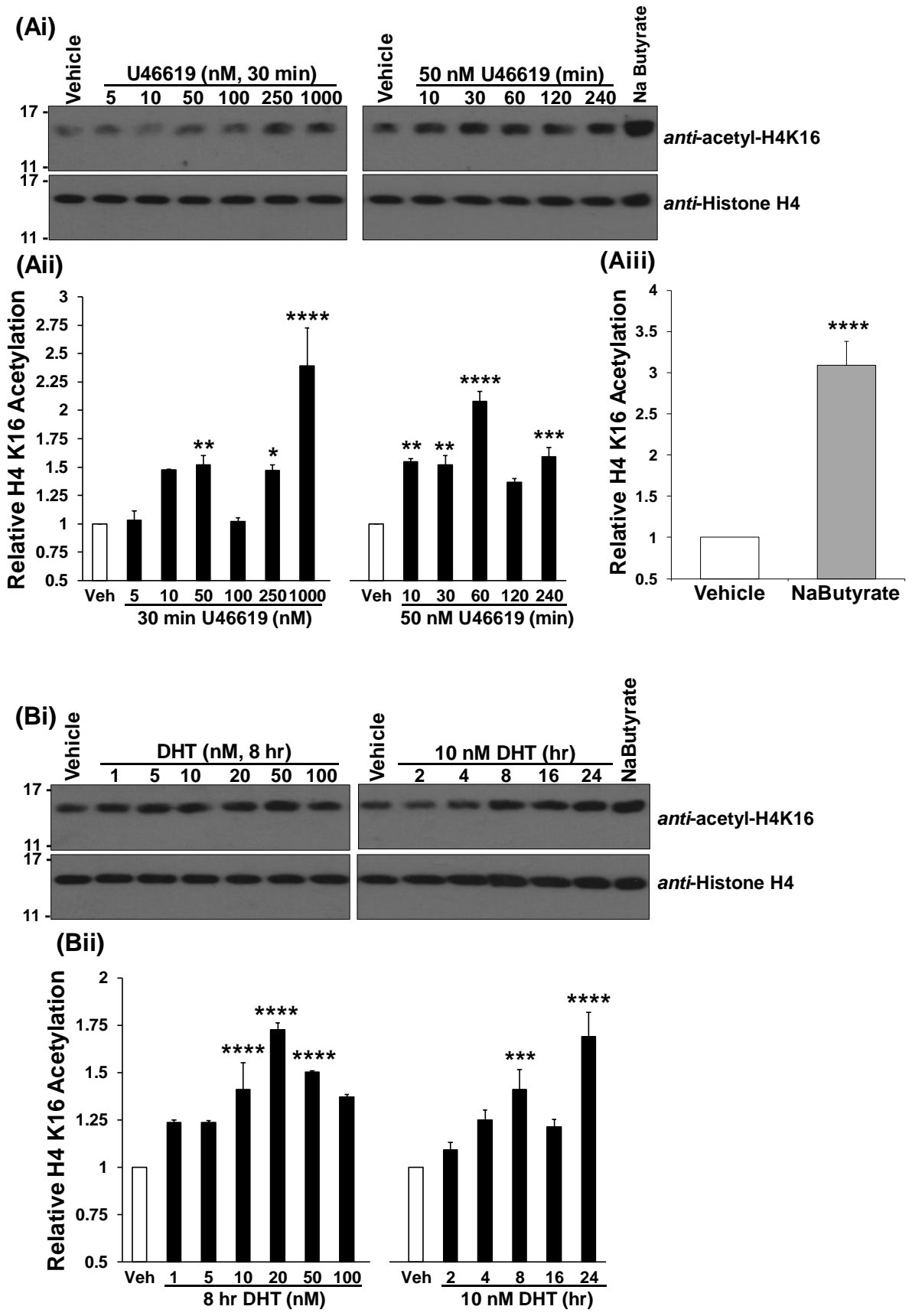


Figure 5: Influence of TP- and AR- agonist stimulation on the recruitment of WDR5 to AR-target genes.

LNCaP cells were stimulated with either 50 nM U46619, 10 nM DHT or the drug vehicle (Veh; 0.0005% EtOH), prior to cross-linking and chromatin extraction. For ChIP analysis of WDR5 binding, chromatin extracted from *anti*-WDR5, normal rabbit IgG or no 1° antibody (–AB) control immunoprecipitates was subjected to real time quantitative PCR (QT-PCR) analysis to amplify androgen response elements (ARE) within the *KLK3/PSA* (Panel A) or *TMPRSS2* (Panel B) AR-target genes or a non-androgen-responsive region of the *GAPDH* gene which served as negative control (Panel C). Results are presented as relative levels of PCR product generated from the *anti*-WDR5 or control immunoprecipitates, expressed as a percentage of those derived from the corresponding input chromatins (n = 3, ±SEM). The schematic above each bar chart shows the relative positions of the ARE within the *KLK3* (Panel A) or *TMPRSS2* (Panel B) genes, or the negative control region of the *GAPDH* gene (Panel C). The asterisks indicate where incubation with U46619 or DHT resulted in significant increases in WDR5 binding compared to vehicle control (***/**/** signified P ≤ 0.05/0.01/0.001/0.0001, respectively; n = 3).



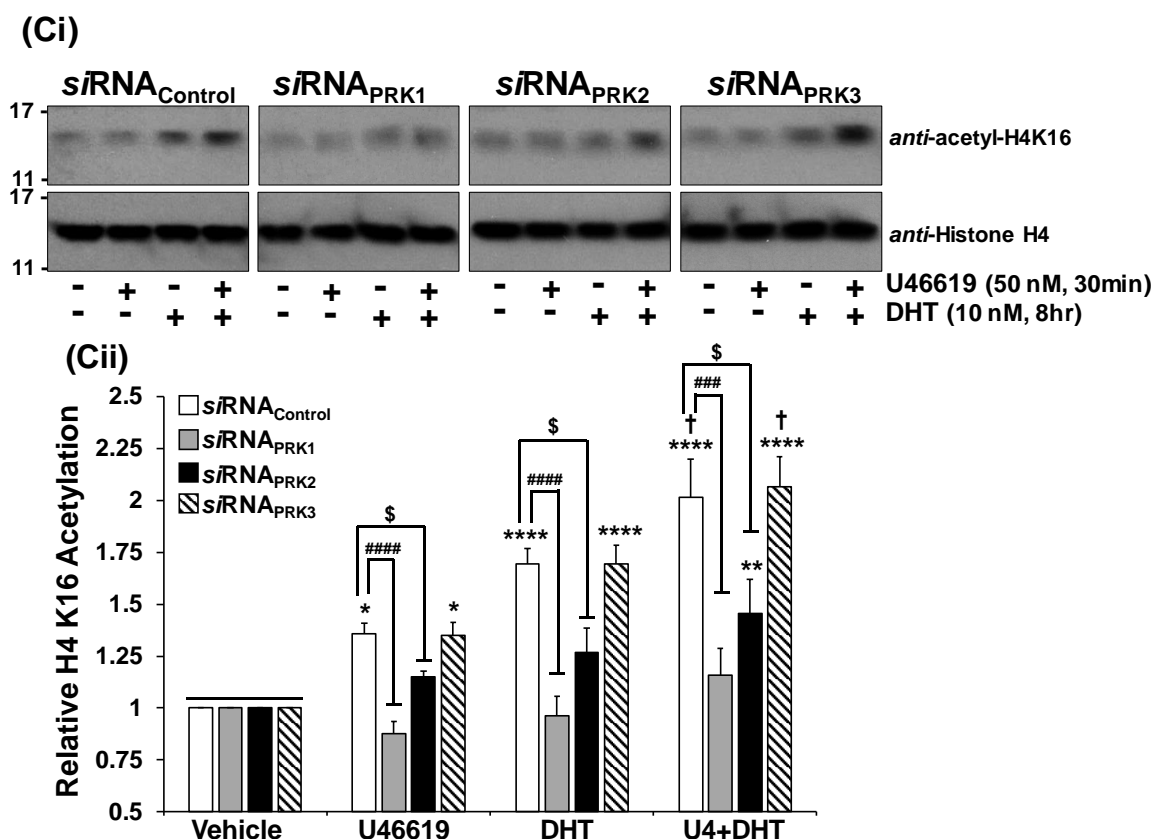


Figure 6: Effect of TP- and AR- agonist stimulation on Histone H4 K16 acetylation in prostate cancer LNCaP cells.

Panels A-C: Immunoblot analysis of Histone H4 K16 acetylation levels in LNCaP cells following stimulation with either U46619 (5-1000 nM), DHT (1-100 nM), U46619 plus DHT (50 nM + 10nM), or as controls, sodium butyrate (5 mM), or drug vehicle (0.0005 – 0.01% EtOH), for the indicated time. In all cases, isolated histones were resolved by SDS-PAGE and immunoblotted with an antibody which specifically detects acetylated H4 at K16 (*anti-acetyl-H4K16*). Parallel membrane blots were immunoblotted with an *anti-Histone H4* antibody to verify uniform histone H4 protein loading. The bar charts show the mean levels of acetylated H4 at K16 in LNCaP cells relative to total histone H4 levels, as determined by quantitative densitometry, where basal levels in vehicle-treated cells were assigned a value of 1 (\pm SEM, n = 3). In *Panel C*, LNCaP cells were transfected with *siRNA_{PRK1}*, *siRNA_{PRK2}*, *siRNA_{PRK3}* or an *siRNA_{Control}*, as indicated, prior to stimulation with U46619 (50 nM), DHT (10 nM) or both and immunoblot analysis of H4 K16 acetylation. In the bar charts associated with *Panels A-C*, the *asterisks* indicate that levels of H4 K16 acetylation were significantly increased in response to U46619- or DHT- stimulation; † indicates that combined U46619/DHT-stimulation resulted in significant increases in levels of H4K16 acetylation relative to DHT-stimulation alone; # and \$ signify that levels of U46619- or DHT- stimulated H4K16 acetylation were significantly inhibited in cells transfected with *siRNA_{PRK1}* or *siRNA_{PRK2}*, respectively, relative to *siRNA_{Control}*. In all cases, single/double/triple/quadruple symbols indicate $p \leq 0.05/0.01/0.001/0.0001$ respectively, for one-way ANOVA, followed by Bonferroni or Dunnett's multiple comparison t-tests (n = 3).

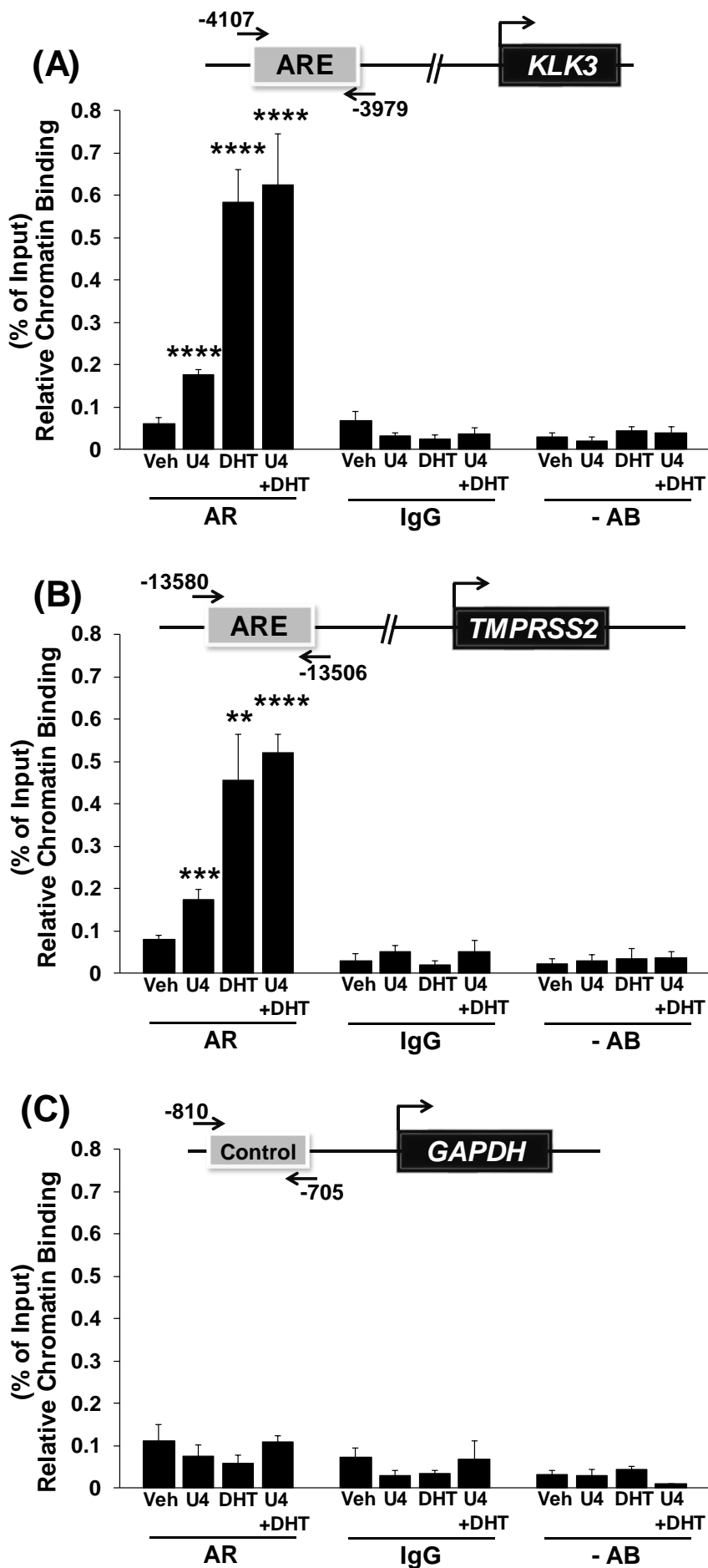
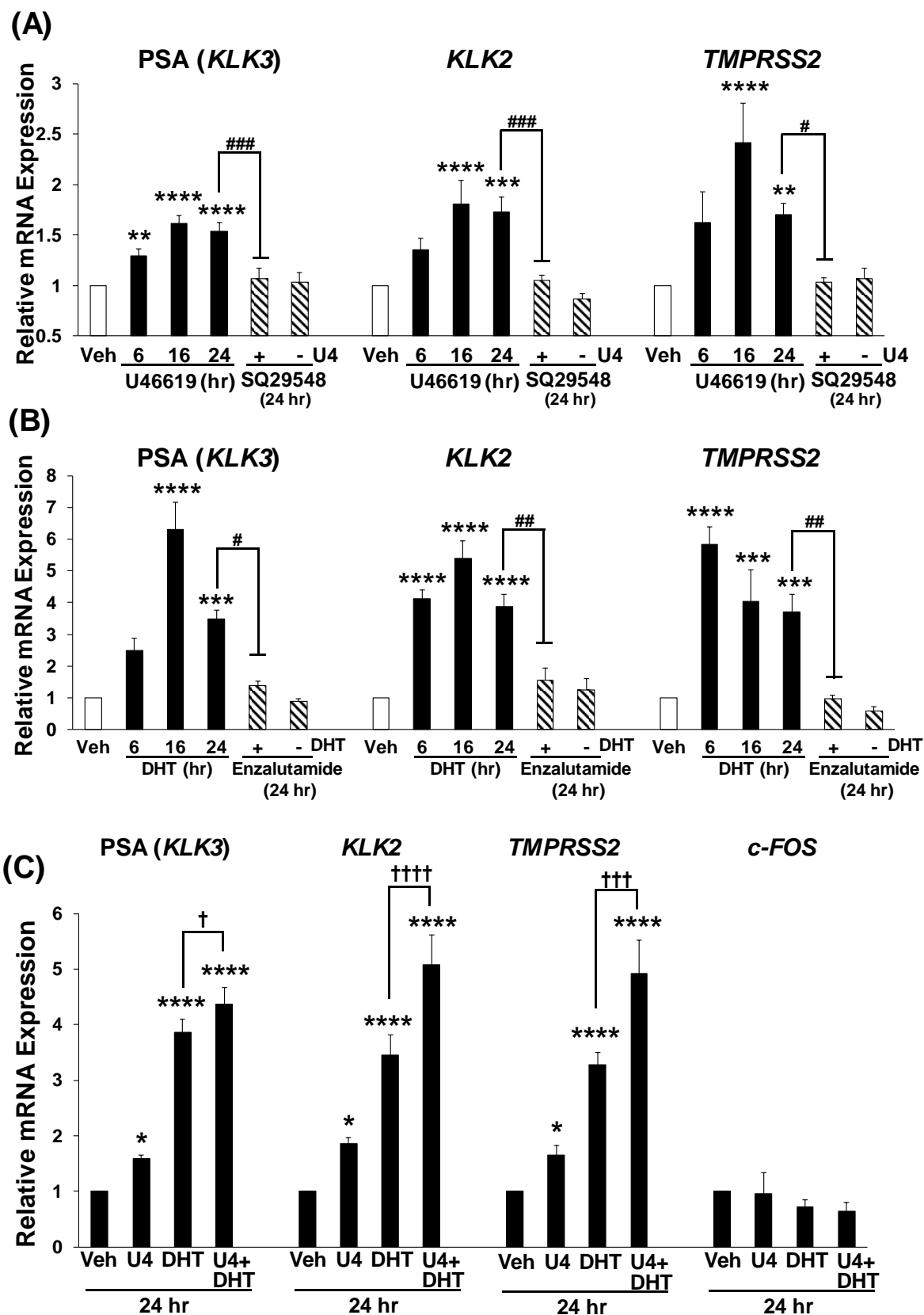


Figure 7: Influence of TP- and AR- agonist stimulation on the association of AR at AR-target genes.

LNCaP cells were stimulated with 50 nM U46619, 10 nM DHT, either alone or in combination, or with the drug vehicle (Veh; 0.0005% EtOH), prior to cross-linking and chromatin extraction. For ChIP analysis of AR binding, chromatin extracted from *anti*-AR, normal rabbit IgG or no 1^o antibody (-AB) control immunoprecipitates was subjected to real time quantitative PCR (QT-PCR) analysis to amplify androgen response elements (ARE) within the *KLK3* (Panel A) or *TMPRSS2* (Panel B) AR-target genes or a non-androgen-responsive region of the *GAPDH* gene which served as negative control (Panel C). Results are presented as relative levels of PCR product generated from the *anti*-AR or control immunoprecipitates, expressed as a percentage of those derived from the corresponding input chromatins (n = 3, \pm SEM). The schematic above each bar chart shows the relative positions of the ARE within the *KLK3* (Panel A) or *TMPRSS2* (Panel B) genes, or the negative control region of the *GAPDH* gene (Panel C). The asterisks indicate where stimulation with U46619, DHT, or both resulted in significant increases in relative AR binding compared to vehicle control, where *, **, *** and **** signifies $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$ and $P \leq 0.0001$, respectively (n = 3).



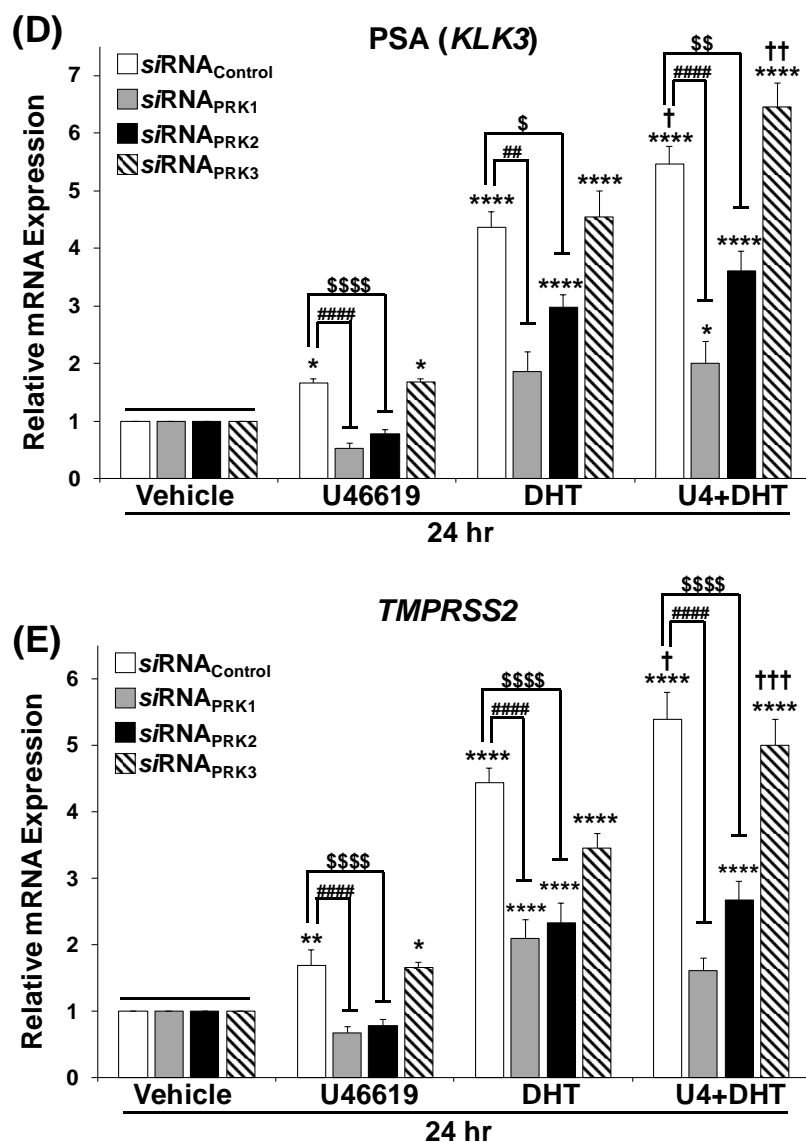


Figure 8: Influence of TP- and AR- agonist stimulation on AR-target gene expression in prostate cancer LNCaP cells.

Panels A-E: LNCaP cells were stimulated with either U46619 (U4; 50 nM), SQ29548 (1 μ M), DHT (10 nM) or enzalutamide (10 μ M), either alone or in combination, or with the drug vehicle (Veh; 0.0005% EtOH) for 6, 16, or 24 hr, as indicated. Thereafter, the relative mRNA expression levels of the AR-target genes, *KLK3* (PSA), *KLK2* and *TMPRSS2*, or as a control, the non-AR target gene *c-FOS* were determined by QT-PCR. Results are presented as mean *KLK3*, *KLK2*, *TMPRSS2*, or *c-FOS* mRNA expression, as indicated, relative to those levels in vehicle-treated cells set to a value of 1 (\pm SEM; $n \geq 3$). The asterisks signify that U46619- or DHT-stimulation significantly increased PSA, *KLK2* or *TMPRSS2* mRNA expression. † indicates that mRNA expression was significantly increased in response to combined U46619/DHT-stimulation, relative to DHT alone. *In Panels D & E*, LNCaP cells were transfected with 30 nM *siRNA*_{PRK1}, *siRNA*_{PRK2}, *siRNA*_{PRK3} or with a scrambled *siRNA* control (*siRNA*_{Control}), prior to stimulation with either U46619 (50 nM), DHT (10 nM) or both, as indicated. # and \$ indicate that U46619- or DHT- stimulated PSA/*KLK3* (*Panel D*) or *TMPRSS2* (*Panel E*) mRNA expression was significantly decreased in cells transfected with *siRNA*_{PRK1} or *siRNA*_{PRK2}, respectively, relative to *siRNA*_{Control}. In all cases, single/double/triple/quadruple symbols indicate $p \leq 0.05/0.01/0.001/0.0001$, respectively, for one-way ANOVA, followed by Bonferroni or Dunnett's multiple comparison t-tests ($n \geq 3$).

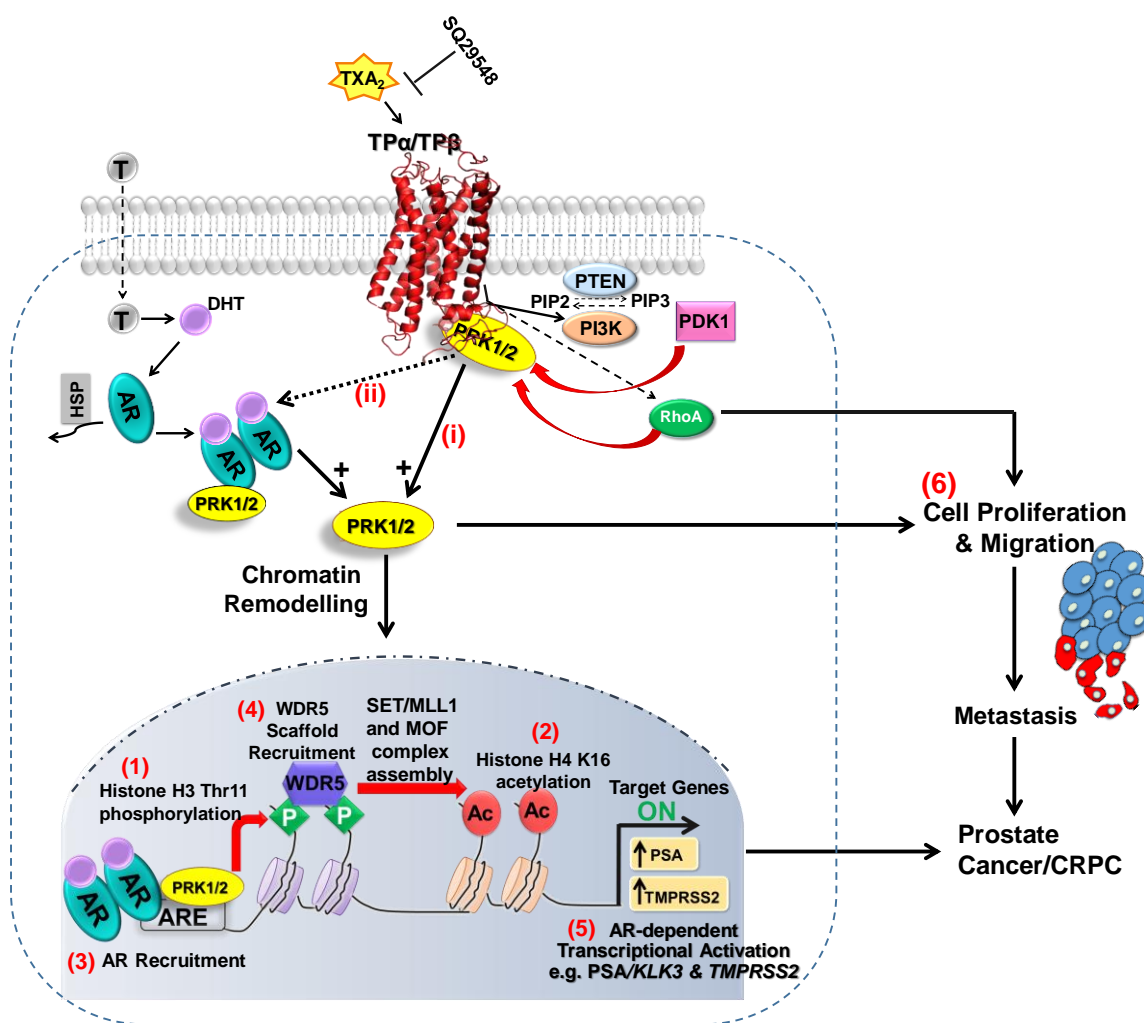
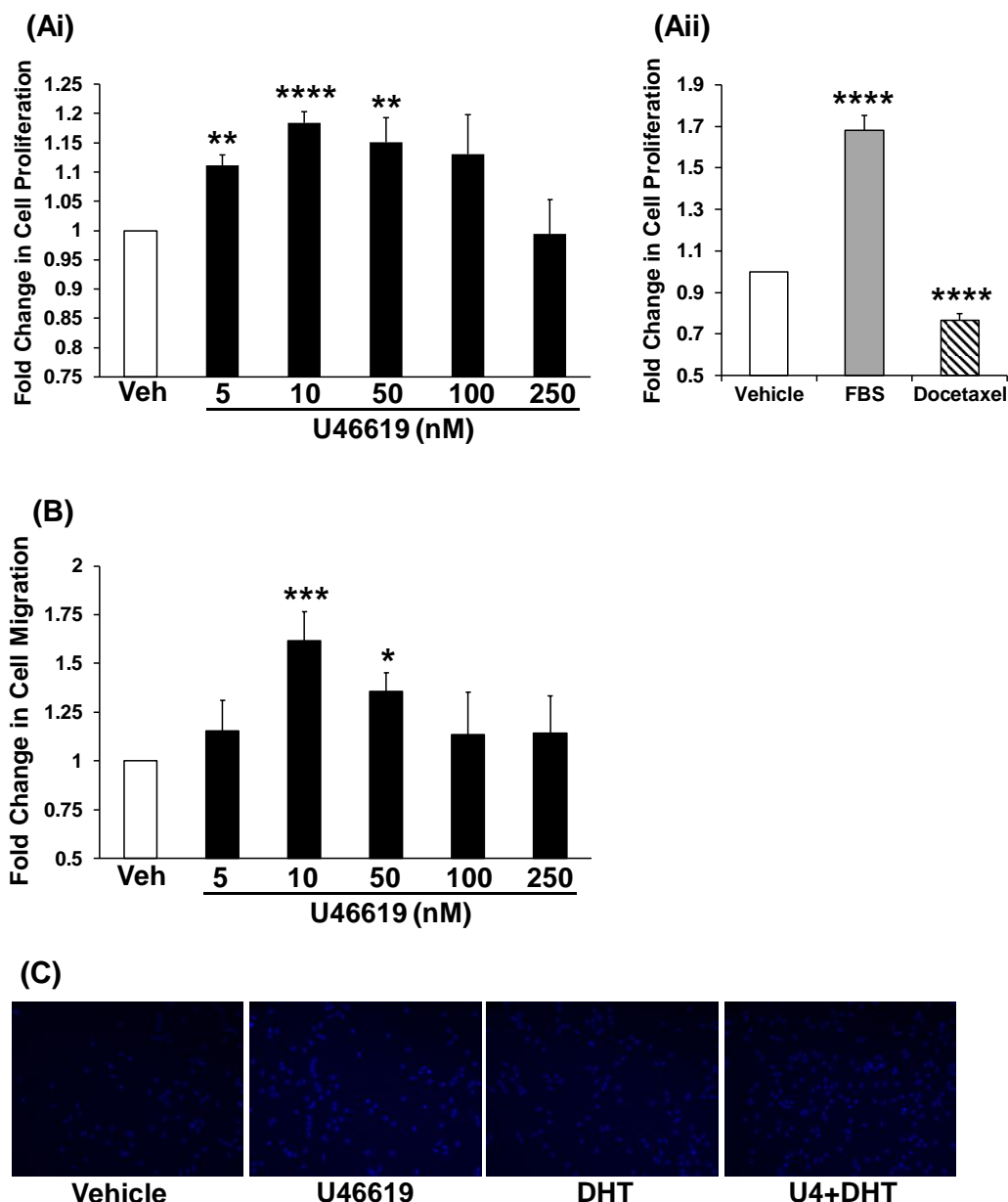


Figure 9: Model of signalling by the TXA₂/TP- and AR-axes through PRK1 and PRK2 in PCa.

In the prostate adenocarcinoma LNCaP cell line, the TPα/TPβ isoforms of the human T Prostanoid receptor (the TP) form functional signalling complexes with protein kinase C-related kinase (PRK)1 and PRK2. In response to TXA₂/U46619-stimulation, TPα/TPβ induces PDK-1-mediated T-loop phosphorylation and activation of PRK1/PRK2, downstream of phosphatidylinositol 3'kinase (PI3'K) and RhoA signalling. Critically, the TPα/TPβ-PRK1/2 activated signalling complexes enable the TPs to both (i) mimic and (ii) enhance androgen-induced cellular responses (e.g. by testosterone and/or its 5α-reductase metabolite dihydrotestosterone/DHT) in the androgen-responsive LNCaP cell line. Thus, TPα/TPβ-mediated activation of PRK1/PRK2 signalling can promote and enhance androgen-induced chromatin remodelling, including (1) phosphorylation of Histone H3 at Thr11 and consequent, (2) downstream acetylation of Histone H4 at K16, through a mechanism involving TXA₂-stimulated recruitment of the (3) androgen receptor (AR) and of the (4) epigenomic scaffold WDR5 to androgen response elements (AREs) within the promoter &/or enhancer regions of AR-target genes, leading to associated recruitment of histone remodelling complexes (e.g. SET/MLL1 and MOF complexes), to facilitate AR-dependent target gene expression. In turn, TXA₂-stimulation leads to transcriptional activation of AR-target genes, such as of the prostate specific antigen (PSA)/KLK3 and TMPRSS2 genes, and (5) enhances androgen-induced activation of those target genes. In addition, agonist-activation of TPα/TPβ promotes (6) prostate cancer cellular proliferation and migration through both PRK1/PRK2- and/or RhoA-dependent signalling mechanisms, and can enhance androgen-induced migration, but not proliferation by LNCaP cells. Hence, the TXA₂-TPα/TPβ signalling axis can act as neoplastic and epigenetic regulators by both mimicking and enhancing the actions of androgens/DHT in prostate cells, to exacerbate the pathologic state of prostate cancer, including potentially in castrate-resistant prostate cancer (CRPC).

SUPPLEMENTARY DATA



Supplemental Figure S1: Effect of TP- and AR- agonist stimulation on proliferation and migration of prostate cancer LNCaP cells

Panel Ai & Aii: For analysis of cell proliferation LNCaP cells were seeded at 4,000 cells/well (96 well format) in RPMI, 10% FBS and cultured for 24 hr. Thereafter, media was replaced with RPMI, 0.5% FBS and cells were stimulated with U46619 (5 - 250 nM) or, as controls, with the vehicle (Veh; 0.0001% EtOH), FBS (10%), or Docetaxel (Dox; 20 nM) for 72 hr, as indicated in the bar charts. *Panel B:* For analysis of cell migration, following serum starvation (0% FBS; 16 hr), LNCaP cells were stimulated with U46619 (5 - 250 nM) or, as a control, with the vehicle (Veh; 0.0001% EtOH) for 24 hr. In *Panels A & B*, the bar charts show the mean levels of LNCaP cell proliferation and migration, respectively, expressed as a fold change relative to those levels in vehicle-treated cells which was assigned a value of 1 (\pm SEM; $n \geq 3$). The *asterisks* indicate that levels of LNCaP cell proliferation (*Panel A*) or cell migration (*Panel B*) were significantly increased in response to U46619- or DHT-stimulation, compared with vehicle-treated cells. *Panel C:* Representative images of migrated LNCaP cells captured at 24 hr post-U46619 (10 nM), DHT (10 nM), U46619 plus DHT (10 nM each) or vehicle (0.0001% ethanol) stimulation using a Zeiss Axioplan 2 fluorescent microscope at 10X magnification are shown.

Supplemental Table S1: List of primers used for qRT-PCR analysis of AR-target gene expression

Target mRNA	Forward	Reverse
<i>18S rRNA</i>	5'-AACCCGTTGAACCCCATTCG-3'	5'-CGCTACTACCGATTGGATGG-3'
<i>PSA</i>	5'-CACCTGCTCGGGTGATTCTG-3'	5'-CCACTTCCGGTAATGCACCA-3'
<i>KLK2</i>	5'-GCTGCCCATTCGCCTAAAGAAG-3'	5'-TGGGAAGCTGTGGCTGACA-3'
<i>TMPRSS2</i>	5'-GGACAGTGTGCACCTCAAAGA-3'	5'-TTGCTGCCCATGAACTTCC-3'
<i>c-FOS</i>	5'-CTACCACTCACCCGCAGACT-3'	5'-AGTGCCAACCTTCATTCCCAC-3'

Supplemental Table S2: List of primers used for ChIP Analysis

Gene	Nucleotides	Forward	Reverse
<i>KLK3</i> (PSA)	Nu -4107 to -3979	5' TGATCTTGGATTGAAAACAGACC 3'	5' TCTCAGATCCAGGCTTGCTT 3'
<i>TMPRSS2</i>	Nu -13850 to -13506	5' TGGTCCTGGATGATAAAAAAAGTTT 3'	5' GACATACGCCCCACAACAGA 3'
<i>GAPDH</i>	Nu -810 to -705	5' CCGGGAGAAGCTGAGTCATG 3'	5' TTTGCGGTGGAAATGTCCTT 3'