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Identification of an interaction between the TPα and TPβ isoforms of the human thromboxane A2 receptor with protein kinase C-related kinase (PRK) 1. Implications for prostate cancer.

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Running title: Thromboxane receptor interaction with PRK1.

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In humans, thromboxane (TX)A2 signals through the TPα and TPβ isoforms of the TXA2 receptor, or TP. Herein, the RhoA effector protein kinase C-related kinase (PRK) 1 was identified as an interactant of both TPα and TPβ involving common and unique sequences within their respective carboxy-terminal (C)-tail domains and the kinase domain of PRK1 (PRK1460-942). While the interaction with PRK1 is constitutive, agonist-activation of TPα/TPβ did not regulate the complex per se but enhanced PRK1 activation leading to phosphorylation of its general substrate histone H1 in vitro. Altered PRK1 and TP expression and signalling are increasingly implicated in certain neoplasms, particularly in androgen-associated prostate carcinomas. Agonist-activation of TPα/TPβ led to phosphorylation of histone H3 at Thr11 (H3Thr11), a previously recognized specific marker of androgen induced-chromatin remodeling, in the prostate LNCaP and PC-3 cell lines but not in primary vascular smooth muscle or endothelial cells. Moreover, this effect was augmented by dihydrotestosterone in androgen-responsive LNCaP but not in non-responsive PC-3 cells. Furthermore, PRK1 was confirmed to constitutively interact with TPα/TPβ in both LNCaP and PC-3 cells and targeted disruption of PRK1 impaired TPα/TPβ-mediated H3Thr11 phosphorylation in, and cell migration of, both prostate cell types. Collectively, considering the role of TXA2 as a potent mediator of RhoA signalling, the identification of PRK1 as a bone fide interactant of TPα/TPβ, and leading to H3Thr11 phosphorylation to regulate cell migration, has broad functional significance such as within the vasculature and in neoplasms in which both PRK1 and the TPs are increasingly implicated.

The local control of haemostasis and vascular tone is a complex process involving platelets, the endothelium and vascular smooth muscle (VSM), various soluble coagulation factors, vasodilator/vasoconstrictor autocoids and other diverse mediators (1). Agents such as thromboxane (TX) A2 that signal through G protein coupled receptors (GPCRs) to promote platelet aggregation or VSM contraction can typically co-couple to Gq-mediated phospholipase (PL)Cβ and to Gq/G12-RhoA activation leading to both Ca2+-dependent and Ca2+-independent responses to promote myosin light chain phosphorylation and actin polymerization (2,3). Owing to the recognized role of the RhoA effector Rho kinase 1/2 as a therapeutic target in hypertension, many studies have focussed on TXA2 regulation of Rho kinase signalling but few have investigated its regulation of other RhoA effectors such as protein kinase C-related kinases (PRK) 1 and 2 (4). The PRKs, also referred to as protein kinase novel (PKN), constitute a sub-family of serine/threonine kinases comprising PRK1/PKNα, PRK2/PKNγ and PKNβ (5). They contain three highly conserved regions including an amino (N)-terminal regulatory domain spanning three repeats of charged amino acids (antiparallel coiled-coil fold or ACC finger domains) with leucine zipper-like sequences, a centrally located arachidonic acid-sensitive C2-like auto-inhibitory domain and a C-terminal catalytic domain (5). Binding of RhoA-GTP within the regulatory domain induces a conformational change in PRK1, for example, leading to release and full activation of its kinase domain by 3-phosphoinositide dependent protein kinase-1 (PDK1) phosphorylation (5). PRK1 activity has been implicated in numerous cellular
processes (5), as well as being widely implicated in androgen-associated prostate cancers and ovarian serous carcinomas (6-9).

As stated, the prostanoid TXA$_2$ plays an essential role within the vasculature acting as a potent regulator of platelet activation status, VSM contraction, proliferation and migration and is widely implicated in a number of cardiovascular disorders including thrombosis, systemic- and pregnancy-induced hypertension, vessel remodelling and atherosclerotic progression (10-11). In addition, recent evidence suggests aberrant TXA$_2$ signalling and TXA$_2$ receptor (TP) expression is associated with certain cancers, in particular prostate cancer with direct correlation of tumor Gleason score and pathologic state (12-14), through as yet unknown mechanisms but potentially through its capacity to regulate RhoA signalling and cell migratory responses. In humans and other primates, TXA$_2$ actually signals through two distinct TXA$_2$ receptor isoforms termed TP$_\alpha$ and TP$_\beta$ which differ exclusively in their intracellular carboxyl-terminal (C tail) domains (15). Whilst the physiologic requirement for two receptors for TXA$_2$ in humans is not fully understood, they greatly increase the complexity of TXA$_2$ signalling and there is increasing evidence that TP$_\alpha$ and TP$_\beta$ have distinct (patho)physiologic roles. For example, TP$_\alpha$ and TP$_\beta$ display distinct patterns of expression, being under the transcriptional control of different promoters (16-22). While they show similar coupling to Gq/PLC$_\beta$ and to Gq/G$_\beta_\gamma$-regulation of RhoA activation, their primary effectors, they differentially regulate other secondary effectors including adenyllyl cyclase (15,23). TP$_\alpha$ and TP$_\beta$ also undergo entirely distinct mechanisms of both agonist-induced (homologous) desensitization (24,25) and heterologous desensitization or cross-talk/regulation by other signalling systems such as prostacyclin and nitric oxide (23,26-29). Such differences in desensitization of TP$_\alpha$ and TP$_\beta$ signalling occur due to differences in their phosphorylation, occurring mainly within their unique C-tail domains, by the second-messenger regulated protein kinases including by protein kinase (PK) C, PKA, PKG, and/or by GPCR-regulated kinases 1/2 (23-27,29).

Hence, exploiting critical differences in their intracellular C-tail domains, the initial aim of the current study was to screen for possible novel interacting partners of either TP$_\alpha$ and/or TP$_\beta$ and thereafter to explore the functional relevance of any interaction(s) identified. We report the identification of a novel specific interaction between both TP$_\alpha$ and TP$_\beta$ with the RhoA effector PRK1. This interaction occurs between the kinase domain of PRK1 with common and unique regions within the intracellular C-tail domain of TP$_\alpha$ and TP$_\beta$. Bearing in mind the critical role of TXA$_2$ as a regulator of RhoA signalling coupled with the identification of its effector PRK1 as a direct interactant of TP$_\alpha$ and TP$_\beta$, the discovery of this novel interaction is likely to have substantial (patho)physiologic implications for processes in which both TXA$_2$ and RhoA/PRK1 are involved.

**EXPERIMENTAL PROCEDURES**

**Materials.** The MATCHMAKER™ human kidney cDNA library, HY4043AH, was obtained from Clontech; pGEX-5X-1 and glutathione sepharose 4B were from GE Healthcare; pCMVTag2a/b/c vectors and the QuickChange™ site-directed mutagenesis system were from Stratagene. The rabbit reticulocyte lysate *in vitro* transcription and translation system (TnT®) was from Promega. Anti-FLAG® M2 monoclonal antibody (F3165), mouse monoclonal anti-FLAG® horseradish peroxidise (HRP) -conjugate, histone H1, protein A-sepharose CL-4B, protein G-sepharose, dihydrotestosterone (DHT) were from SigmaAldrich; anti-GST (B-14; sc-138), anti-RhoA (26C4; sc-418), goat anti-PRK1 (C-19; sc-1842), rabbit anti-Histone H3 (FL-136; sc-10809) were from Santa Cruz; anti-phospho histone H3Thr11 (anti-phospho H3Thr11) antibody was from ActiveMotif; HRP-conjugated mouse anti-goat, HRP-conjugated goat anti-mouse and HRP-conjugated goat anti-rabbit antibodies were from Santa Cruz; rat monoclonal 3F10 anti-HA- HRP-conjugated antibody was from Roche; mouse monoclonal anti-hemagglutinin (HA)-(101)R antibody was from Eurogentec; U46619 and 17 phenyl trinor prostaglandin (PG) E$_2$ was from Cayman Chemical Company; [32P] ATP (6000 Ci/mmoll; 10 mCi/ml) was from PerkinElmer Life Sciences; *Escherichia (E) coli* Rossetta 2 (DE3) from Merck Biosciences. All oligonucleotides were synthesised by Genosys Biotechnologies and all small interfering RNA (siRNA) were from
Dharmacon. Cicaprost was kindly donated by Schering AG (Berlin, Germany).

Expression Plasmids.
The plasmids pGBKT7:TPα312-343 and pGBKT7:TPβ312-407 were generated by subcloning the cDNA subfragments encoding the C-terminal domains of TPα (amino acid 312 – 343) and TPβ (amino acid 312-407) into the EcoRI/BamHI sites of the yeast bait vector pGBKT7 (Clontech) such that inserts were c-Myc epitope tagged and in-frame with the DNA-binding domain (DBD) of the yeast GAL4 transcriptional regulator. Similarly pGBKT7:TPα312-328, pGBKT7:TPα329-343, pGBKT7:TPβ329-407, pGBKT7:TPβ312-366, pGBKT7:TPβ329-392, pGBKT7:TPβ329-366, pGBKT7:TPβ353-407, pGBKT7:TPβ353-392, pGBKT7:TPβ366-392 and pGBKT7:TPβ353-366 were generated by subcloning the respective fragments into pGBKT7. The plasmids pGBKT7:TPβ312-353 and pGBKT7:TPβ329-333 were generated by QuickChange™ site-directed mutagenesis (SDM) using pGBKT7:TPβ312-407 and pGBKT7:TPβ329-407 as templates, respectively. pGEX-5X-1:TPα312-343, pGEX-5X-1:TPα329-343, pGEX-5X-1:TPβ312-343, pGEX-5X-1:TPβ329-343, pGEX-5X-1:IC1210-129, pGEX-5X-1:IC1210-129 and pGEX-5X-1:IC3211-246 were generated by subcloning the respective PCR amplified cDNA subfragments into pGEX-5X-1, such that fragments were in-frame with glutathione S-transferase (GST). The plasmids pCMVTag2b:PRK1, pCMVTag2b:PRK11-137, pCMVTag2b:PRK11-594, pCMVTag2b:PRK1561-942 and and pCMVTag2b:PRK1966-IIE, encoding a dominant negative/kinase dead form of PRK1 (9), were generated by either subcloning of the respective PCR amplified subfragments into pCMVTag2b such that they were in-frame with the N-terminal FLAG™ epitope tag or by Quickchange™ SDM using the pCMVTag2b:PRK1 as template. Ala-scanning SDM of residues Leu316 – Leu323 within the alpha-helical 8 domain (α-H8) of TPα and TPβ, expressed in the yeast bait plasmids pGBKT7:TPα312-343 and pGBKT7:TPβ312-407, respectively, was carried out using Quickchange™ SDM. In all cases, sequences of the specific primers used are listed in Supplemental Table 1. All plasmids were validated by DNA sequence analysis.

Yeast two-hybrid screening and Yeast Matings.
The human kidney cDNA library (3.5 X 10^6 independent clones; HY4043AH), cloned in-frame with the activation domain of the yeast GAL4 transcriptional activator in the yeast prey plasmid pACT2 and pre-transformed into Saccharomyces cerevisiae (S.c) Y187, was obtained from Clontech. The MATa bait strains S.c AH109 (pGBKT7:TPα312-343/pGBKT7:TPβ312-407) were mated with the MATα S.c Y187 (pACT2; human kidney cDNA library) at a density of 2 x 10^6 cells per ml and a ratio of 30:1 bait:prey cells, where 1 x 10^9 individual diploids were screened. After 24 hr growth in SD/Trp, Leu (DDO, double dropout) at 30 °C, diploids were plated onto SD/Trp, Leu, Ade, His medium (quadraple drop-out (QDO) medium) and maintained at 30 °C for 15 days. Following selection on QDO media, recombinant pACT2 plasmid DNA was extracted from putative interactants and transformed into super-competent Escherichia (E) coli XL-1Blue cells. Following re-transformation of the pACT2-based plasmid from putative interactants into S.c Y187 and re-mating with an expanded range of bait strains, including S.c AH109 (pGBKT7:TPα312-343/pGBKT7:TPβ329-407/pGBKT7:TPβ312-392/pGBKT7/ pGBKT7.p53) or with the Ala-scanning variants of Leu316 – Leu323 in the plasmids pGBKT7:TPα312-343 and pGBKT7:TPβ312-407 (Supplemental Table 1B), diploids were selected on D0 media while positive interactants were selected on the basis of expression of HIS3, ADE2 and MEL1 reporter genes by growth on QDO medium and cleavage of 5-bromo-4-chloro-3-indolyl alpha-D-galactopyranoside (X-α-Gal). cDNA inserts from positive interactants were identified by DNA sequence analysis.

Cell culture and transfections.
Human embryonic kidney (HEK) 293 cells were obtained from the American Type Culture Collection (ATCC) and grown in minimal essential medium (MEM), 0.2 % (v/v) L-glutamine, 10 % (v/v) foetal bovine serum (FBS). Routinely, approximately 48 hr prior to transfection, cells were plated at a density of 2 x 10^6 cells/10 cm dish in 8 ml media. Thereafter, cells were transiently transfected with 400 ng of pADVA and 1.6 µg of pCMV-based vectors.
using Effectene (Qiagen) and routinely harvested 48 hr post-transfection. HEK.TPα, HEK.TPβ and HEK.β2AR cells, stably over-expressing HA-tagged forms of TPα, TPβ and the β2 adrenergic receptor (β2AR), respectively, have been previously described (27). HEK.β-galactosidase (HEK.β-Gal) cells stably over-expressing the HA-tagged β-Gal were established essentially as previously described (29). The prostate carcinoma PC-3 and LNCaP cell lines were obtained from the ATCC and were cultured in 90 % RPMI-1640, 10 % FBS. For transfection, PC-3 and LNCaP cells were plated at a density of 2 x 10⁶ cells/10 cm dish in 8 ml media 48 hr prior to transfection. Thereafter, cells were transiently transfected with 2 µg of pCMV-based vectors using either Effectene (Qiagen) or Lipofectamine LTX (Invitrogen), respectively, and harvested 48 hr post-transfection. For experiments using DHT treatments, both PC-3 and LNCaP cells were cultured in growth media containing charcoal-stripped FBS (90 % RPMI-1640, 10 % Charcoal-stripped FBS) for at least 24 hours prior to DHT treatment. EA.hy926 cells were obtained from the Tissue Culture Facility at UNC Lineberger Comprehensive Cancer Center, North Carolina and grown in Dulbecco's modified Eagle's medium (DMEM), 10 % FBS. Primary (1°) human coronary artery smooth muscle cells (1° h.CoASMCs) were from Cascade Biologies (C-007-5C) and routinely grown in Smooth Muscle Cell Growth Medium 2 supplemented with 0.5 ng/ml epidermal growth factor, 2 ng/ml basic fibroblast growth factor, 5 µg/ml insulin, 5 % (v/v) FBS (Promocell GMBH, C-22062). 1° human umbilical vein endothelial cells (HUVECs), from Lonza (IR9-048-0904D), were routinely cultured in M199 media (Sigma-Aldrich) supplemented with 0.4 % (v/v) Endothelial Cell Growth Supplement/Heparin (ECGS/H; Lonza), 20 % (v/v) FBS and 0.2 % (v/v) L-glutamine. 1° h.CoASMC and 1° HUVECs were routinely used between passages 2 - 8. All mammalian cells were grown at 37 °C in a humid environment with 5 % CO₂ and confirmed to be mycoplasma free.

**Glutathione-S-transferase Pull-down Assays.**

*E.coli* Rossetta 2 (DE3) transformants of pGEX-5X-1:TPα312-343, pGEX-5X-1:TPα329-343, pGEX-5X-1:TPβ312-407, pGEX-5X-1:TPβ329-407 were grown at 37 °C in LB selection medium, until OD₆₀₀ nm of ~0.8, and glutathione-S-transferase (GST) proteins were induced at 4 °C for 1 hr with 0.25 mM isopropyl β-D-1-thiogalactopyranoside. Cell pellets from 1 l cultures were lysed in Lysis Buffer (150 mM NaCl, 5 mM MgCl₂, 1 % (v/v) Triton-X-100, 50 mM Tris-Cl pH 7.5, 1 mM DTT, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1mM PMSF; 10 ml) and proteins purified on 1 ml (50% slurry) glutathione-sepharose-4B beads pre-equilibrated in Wash Buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 0.5 % (v/v) Triton-X-100, 1 mM DTT, 1 µg/ml aprotinin, 1 µg/ml leupeptin and 0.1 mM PMSF), according to standard procedures. HA.PRK1640-942 was translated from the PCR amplified product encoding the T7 promoter sequence, optimal translation initiation site (GAG CC ACC ATG), HA-epitope tag and amino acids 640-942 of PRK1 using the TNT® T7 coupled in vitro transcription/translation system (Promega, Protocol # PN058). Per GST pull-down assay, 10 µg of each purified GST.TPα312-343, GST.TPβ312-407, GST.TPα329-343, GST.TPβ329-407 and GST bound to glutathione sepharose 4B beads were initially pre-equilibrated at room temperature in 10 ml Pull-down Buffer A (20 mM HEPES pH 7.4, 100 mM NaCl, 2 mM MgCl₂, 0.1 % (v/v) Triton X-100) supplemented with 1 % (w/v) BSA for 30 – 60 min. Thereafter, bound proteins were washed in three successive changes of Pull-down Buffer A and beads containing 5 µg of each protein was combined with the in vitro translated product (HA.PRK1640-942; 3 µl) and incubated at room temperature for 2 hr with rotation. Beads were then washed by three changes of Buffer A and finally resuspended in 30 µl Immunoprecipitation (IP) Sample Buffer [10 % (v/v) β-mercaptoethanol, 2 % (w/v) sodium dodecyl sulphate (SDS), 30 % (v/v) glycerol, 0.025 % (w/v) bromophenol blue]. The input TNT® in vitro translated product HA.PRK1640-942 (1 µl) and protein-bound glutathione sepharose 4B beads were boiled for 10 min; resolved by SDS-PAGE, on 12.5 % acrylamide gels, and transferred to polyvinylidene fluoride (PVDF). Membranes were immunoblotted versus anti-HA (3F10) (1 : 1000 dilution in TBS/5 % (w/v) skimmed milk powder) or anti-GST antisera (1 : 1000 dilution in TBS/5 % (w/v) skimmed milk powder). For GST pull-down assays using mammalian cell extracts, 48 hr prior to pull-down experiments, HEK 293 cells were transfected with pCMVTag2b:PRK1.
Thereafter, cells were lysed in Buffer B (10 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.5 % Triton-X-100 (v/v), 10 µg/ml aprotinin, 10 µg/ml leupeptin, 0.5 mM PMSF). Cell debris was removed by centrifugation (14,000 rpm) at 4 °C for 20 min and cellular lysates (500 µg) were incubated at 4 °C for 2 hr with glutathione-sepharose beads pre-loaded with 10 µg GST.TPα12-343, GST.TPβ312-407 or, as a control, GST.IP299-386. Beads were washed in three changes of Buffer B followed by three changes of phosphate buffered saline (PBS). Thereafter, proteins were solubilised in IP Sample Buffer (50 µl), boiled for 10 min, resolved by SDS-PAGE (12.5 % acrylamide gels) and transferred to PVDF membranes. Blots were screened versus anti-Flag HRP (1 : 1000 dilution in TBS/5 % (w/v) skimmed milk powder) or anti-GST (1 : 1000 dilution in TBS/5 % (w/v) skimmed milk powder) antibodies followed by chemiluminescence detection.

**Immunoprecipitations.**
HEK.TPα, HEK.TPβ and HEK.β-Gal cells, transiently transfected with pCMVTag2b:PRK1, pCMVTag2b:PRK1561-942, pCMVTag2b:PRK1528-561-942, pCMVTag2b:PRK1528-561-942, pCMVTag2b:PRK1528-561-942 or pCMVTag2b, where specified, or 1° HUVECs, 1° h. CoASMCs, EA.hy926, LNCaP and/or PC-3 cells were plated onto 10-cm dishes to achieve ≈ 80 % confluency. In all cases, prior to immunoprecipitation, cells were washed in the appropriate serum-free media and either incubated with vehicle (serum-free media) or with 1 µM U46619 in serum-free media for 10 min at 37 °C, or as indicated in the figure legends. LNCaP and PC-3 cells were pre-incubated with either vehicle (0.01 % EtOH) or dihydrotestosterone (DHT; 1 nM) for 24 hr at 37 °C, as specified. Thereafter, cells were washed twice in ice-cold PBS and lysed in Radio-Immune Precipitation (RIP) buffer (20 mM Tris-Cl HCl, pH 8.0, 150 mM NaCl, 10 mM EDTA, 1 % (v/v) Nonidet P-40, 1 % (w/v) sodium deoxycholate, 0.1 % (w/v) SDS, 1 mM sodium orthovanadate, 1 mM PMSF, 4 µg/ml aprotinin, 2.5 µg/ml leupeptin; 800 µl/10 cm dish) for 20 hr at 37 °C with either 10 µg of the indicated purified GST protein or histone H1 substrate in 50 µl of reaction mixture [20 mM Tris-Cl pH 7.5, 4 mM MgCl₂, 40 µM ATP, 18.5 KBq/0.5 µCi of [γ³²P] ATP (3000 Ci/mmol, 10mCi/ml)]. Samples were resolved by SDS-PAGE (12.5 % acrylamide gels) and transferred to PVDF membrane. The phosphorylated substrate(s) were visualised by autoradiography and the PVDF membrane was subsequently stained with Ponceau S or screened by western blot analysis using anti-GST antibody. HEK.TPα, HEK.TPβ, HEK.β-Gal, LNCaP, PC-3, EA.hy926 cells, 1° h. CoASMCs or 1° HUVECs were grown in 10-cm dishes, as previous, to achieve ≈ 80 % confluency. Prior to immunoprecipitation, cells were stimulated at 37 °C either with vehicle or U46619 (1 µM) for 0 – 60 min. Thereafter, PRK1 was precipitated from cell lysates either directly using anti-PRK1 (1 : 1000 dilution) or due to interaction with HA.TPα or HA.TPβ using anti-HA (101R) (1 : 300 dilution). Precipitates were washed four times with RIP Buffer and four times with Kinase purified antibody directed to residues 329-343 and 392-407 of TPα and TPβ, respectively) antibodies or, as controls, using an equivalent amount of the pre-immune IgG (20 µg per 500 µl assay) antibodies to precipitate HA-tagged TPα, -TPβ or -β-Gal and PRK1, respectively, through overnight incubation at 4 °C with rotation. Thereafter, the precipitates were incubated for 1 hr with Protein-G-Sepharose (50 % slurry in RIP buffer; 10 µl) or Protein-A-Sepharose (50 % slurry in RIP buffer; 40 µl) for precipitates using anti-TPα /TPβ (#168), prior to washing with at least four changes of RIP buffer followed by four changes of PBS. Immunoprecipitates (IP) and whole cell lysates were resolved by SDS-PAGE, on 12.5 % acrylamide gels, and subjected to successive immunoblotting (IB) with either anti-Flag-POD, anti-PRK1, anti-RhoA, and anti-HA-POD (3F10) antibodies in Blocking Buffer (5 % (w/v) skimmed milk powder in TBS) or followed by chemiluminescence detection.

**Assay of PRK1 activity.**
GST.ICE152-70, GST.ICE1129-149, GST.ICE321-246, GST.TPα12-343 and GST.TPβ312-407 recombinant proteins were purified from E.coli Rosetta (DE3) as previously described. For phosphorylation assays, 200 ng of purified PRK1 (Cell Signalling) was incubated for 1 hr at 30 ºC with either 10 µg of the indicated purified GST protein or histone H1 substrate in 50 µl of reaction mixture [20 mM Tris-Cl pH 7.5, 4 mM MgCl₂, 40 µM ATP, 18.5 KBq/0.5 µCi of [γ³²P] ATP (3000 Ci/mmol, 10mCi/ml)]. Samples were resolved by SDS-PAGE (12.5 % acrylamide gels) and transferred to PVDF membrane. The phosphorylated substrate(s) were visualised by autoradiography and the PVDF membrane was subsequently stained with Ponceau S or screened by western blot analysis using anti-GST antibody. HEK.TPα, HEK.TPβ, HEK.β-Gal, LNCaP, PC-3, EA.hy926 cells, 1° h. CoASMCs or 1° HUVECs were grown in 10-cm dishes, as previous, to achieve ≈ 80 % confluency. Prior to immunoprecipitation, cells were stimulated at 37 °C either with vehicle or U46619 (1 µM) for 0 – 60 min. Thereafter, PRK1 was precipitated from cell lysates either directly using anti-PRK1 (1 : 1000 dilution) or due to interaction with HA.TPα or HA.TPβ using anti-HA (101R) (1 : 300 dilution). Precipitates were washed four times with RIP Buffer and four times with Kinase.
Buffer (20 mM Tris-Cl pH 7.5, 4 mM MgCl₂). The purified PRK1 was incubated at 30 °C for 1 hr with histone H1 (10 µg) in 20 mM Tris-Cl pH 7.5, 4 mM MgCl₂, 40 µM ATP, 18.5 KBq/ 0.5 µCi of \([\gamma^3P]ATP (3000 \text{ Ci/mmol, 10mCi/ml})\) in 50 µl final reaction volume. The reaction mixture (50 µl) was then subjected to SDS-PAGE (12.5 % acrylamide gels) and transferred to PVDF membrane. Phosphorylated histone H1 was detected by autoradiography and all membranes were subsequently immunoblotted (IB) with anti-PRK1 or anti-HA 101R to verify precipitations.

**Disruption of PRK1 expression by small interfering (si)RNA**

For siRNA experiments, PC-3 and LNCaP cells were plated at ~2.5 x 10⁵ cells/10-cm dish in 8 ml growth media where the cells were plated 48 hr previously at 2 X 10⁴ cells/10-cm dish in 8 ml growth media such that they were ≥ 90 % confluent 90 - 96 hr prior to transfection. Knockdown in expression of PRK1 was confirmed by immunoblotting using anti-PRK1 sera at 0 - 48 hr post-transfection. Membranes were also screened using anti-HDJ-2 antisera to confirm uniform Histone H3 loading. For migration assays, 24 hr post-transfection cells were placed in serum-free media for 24 hr before experiments were performed.

**Investigation of Histone H3 Thr11 (H3Thr11) Phosphorylation.**

To examine the effect of U46619 on histone H3 threonine 11 (H3Thr11) phosphorylation, 1⁰ HUVECs, 1⁰ hCoASMCs, PC-3 and LNCaP cells were routinely plated 48 hr previously at 2 X 10⁵ cells/10-cm dish in 8 ml growth media where the 10 % FBS in the respective growth media was replaced with 10 % charcoal-stripped FBS (PanBiotech). Cells were then either transfected with pCMVTag2b:PRK1 (2 µg), pCMVTag2b:PRK1K644E (2 µg), siRNA<sub>PRK1</sub> (30 nM) or siRNA<sub>control</sub> (30 nM) or incubated with vehicle [0.01 % EtOH] or DHT (1 nM) for 24 hr, followed by incubation with either vehicle [0.01 % DMSO] or the PRK1 inhibitor RO-31-8220 (10 µM) for 1 hr at 37 °C as indicated. The concentration of RO-31-8220 (10 µM) used is that routinely used to inhibit PRK1 in cells in culture (5). Cells were stimulated with U46619 (1 µM; 0 – 60 min), 17 phenyl-trinor PGE₂ (1 µM; 30 min), cicaprost (1 µM; 30 min) or appropriate vehicle at 37 °C, as indicated. As a control for H3Thr11 phosphorylation, mitotic cells were obtained by treatment with nocodazol (50 ng/ml) for 24 hr prior to harvesting. The cells were harvested by sequential extraction to remove soluble cytoplasmic and nucleoplasmic proteins to obtain nuclear extracts (containing histones) as described (9). Briefly, cells were washed with ice-cold PBS and lysed in hypotonic buffer (10 mM Tris-Cl pH 8.0, 1 mM KCl, 1.5 mM MgCl₂, 1 mM PMSF, 50 µM leupeptin and 1 mM DTT) for 30 min at 4 °C with rotation. The lysate and nuclei were separated by centrifugation (10,000g) and the nuclei re-suspended in 0.4 N H₂SO₄ and incubated overnight at 4 °C with rotation. Nuclear debris was removed by centrifugation (16,000g) and the histone-containing supernatant was trichloroacetic acid -precipitated. The resulting pellets were washed with ice-cold acetone 3 times to remove acid, air dried, re-suspended in ddH₂O and histone concentrations analysed by the Bradford assay. For western blot analysis, nuclear extracts were resolved on 15 % SDS-PAGE gels and electrophoretically transferred onto nitrocellulose membranes according to standard protocols. Membranes were screened using anti-phospho H3Thr11 sera (ActiveMotif) in 5 % non-fat dried milk in TBS (0.01 M Tris-HCl, 0.1 M NaCl, pH 7.4) for 1 hr at room temperature followed by washing and screening using goat anti-rabbit horseradish peroxidase followed by chemiluminescence detection. Membranes were also screened using anti-Histone H3 antisera to confirm uniform Histone H3 loading.

**Investigation of PC-3 and LNCaP Cell Migration.**

Boyden chamber assays were used to assess the effects of TP activation on prostate cancer cell migration. Briefly, prior to migration assays, PC-3 and LNCaP cells were plated in 10 cm dishes in growth media containing charcoal-stripped FBS either the presence or absence of DHT (1 nM) such that they were ≥ 90 % confluent 24 - 36 hr post-seeding. Alternatively, cells were seeded such that they were ≥ 50 % confluent after 24 - 36
hr and transfected with siRNA (siRNA<sub>PRK1</sub> or siRNA<sub>Control</sub>) followed by incubation for a further 48 hr. Cells were routinely serum-starved for 24 hr before harvesting and QCM<sup>TM</sup> 24-well colorimetric cell migration assay (Millipore; ECM508) was performed as per the manufacturer’s instructions. Briefly, the cells were initially rinsed in PBS and then 1.0 x 10<sup>6</sup> cells in 300 µl serum-free media either in the presence or absence of DHT (1 nM) and treated with either vehicle (0.01 % EtOH) or U46619 (1 µM) were placed in the top chamber. Aliquots of cells were retained for immunoblot analysis for endogenous PRK1 and HDJ-2 protein expression. After the cells settled (~1 hr), 500 µl complete growth media in the presence or absence of DHT (1 nM) and containing either vehicle (0.01 % DMSO) or U46619 (1 µM) was added to the bottom chamber. Cell migration was assessed after 4 hr at 37 °C. The cells remaining in the top chamber were removed by cotton swabs and then migrated cells were stained with a crystal violet dye (Millipore; #90144) and washed with H<sub>2</sub>O. The cells were then extracted using an extraction buffer (Millipore, #90145) and the OD<sub>560nm</sub> measured. Migration was expressed as percentage of basal cell migration. All experiments were performed at least in duplicate and each experiment was repeated at least two times.

Confocal Microscopy.
LNCap and PC-3 cells, were seeded at 2 X 10<sup>5</sup> cells in 2 ml RPMI 1640, 10 % FBS in 6-well plates pre-coated with poly-L-lysine (0.001 % for 1 h) and grown at 37 °C for 48 h. Thereafter, cells were transiently transfected with pEGFP-C1 PRK1 (31) encoding GFP-tagged PRK1 (GFP:PRK1; 2 µg using Lipofectamine LTX (Invitrogen) for LNCap cells; 1 µg using Effectene (Qiagen) for PC-3 cells). Some 48 hr post-transfection, cells were stimulated with 1 µM U41669 for 0-240 min prior to fixation in 3.7 % paraformaldehyde. Cells were permeabilized with 0.2 % Triton X-100 for 10 mins on ice, followed by staining with 4',6-diamidino-2-phenylindole (DAPI; 1 µg/ml in H<sub>2</sub>O). Images were captured at 63X magnification using Carl Zeiss Laser Scanning System LSM510 and Zeiss LSM Imaging software for acquiring multichannel images with filters appropriate for enhanced DAPI and GFP. Images presented in the figures are representative of 10 independent fields.

Data analyses.
Statistical analyses of differences were carried out using the unpaired Student’s t test, 2-way ANOVA or 1-way ANOVA followed by post-hoc Dunnett’s multiple comparison t tests, as indicated, throughout employing GraphPad Prism, version 4.00 package. P-values of less than or equal to 0.05 were considered to indicate a statistically significant difference. To investigate overall differences between time-dependent, U46619-induced H3Thr11 phosphorylation in the absence or presence of DHT, nonlinear regression (R<sup>2</sup>) and F-test analyses were carried out, where p-values of less than or equal to 0.05 were considered to indicate a statistically significant difference. As relevant, single, double, triple and quadruple symbols signify p ≥ 0.05, ≥ 0.01, ≥ 0.001 and ≥ 0.0001, respectively, for post-hoc Dunnett’s multiple comparison t-test analyses.

RESULTS
Identification of PRK1 as an interactant of TPαTPβ in yeast.
Herein a yeast-two-hybrid (Y2H) screen of a human kidney cDNA library was initially performed to identify proteins that may interact with the carboxyl-terminal (C-tail) domains of the TPα (TPα<sub>312-343</sub>) and TPβ (TPβ<sub>312-407</sub>) isoforms of the human TXA<sub>2</sub> receptor. Several positive clones from the TPβ screen encoded protein kinase C-related kinase (PRK) 1, or protein kinase (PK) N 1. The clones identified encode amino acids 640 – 942 of PRK1 (PRK1<sub>640-942</sub>) encompassing its catalytic kinase domain (5). To further investigate specificity, the interaction with PRK1<sub>640-942</sub> was examined by extended Y2H matings of strains encoding TPα<sub>312-343</sub>, TPβ<sub>312-407</sub>, TPα<sub>329-343</sub>, TPβ<sub>329-407</sub> and, as controls, the unrelated protein p53 or the empty vector (pGBKT7). While each of the bait and prey strains mated successfully to form diploids (Fig.1A; DDO), strains encoding either p53 or TPα<sub>329-343</sub>, corresponding to the unique C-tail domain of TPα alone, or harbouring pGBKT7 alone failed to show any interaction with PRK1<sub>640-942</sub> (Fig.1A; QDO). Conversely, the original baits, TPα<sub>312-343</sub> and TPβ<sub>312-407</sub>, or the unique region of TPβ (TPβ<sub>329-407</sub>) showed an interaction with PRK1<sub>640-942</sub> (Fig. 1A). Based on these data, the common region within the C-tail domains of TPα/TPβ
The role of individual residues within the common region of TPrx/TPβ in contributing to their interaction with PRK1 was also investigated (Supplemental Fig. 1). This region of particular interest as much of it (residues 316-323) is predicted to be organized into the alpha-helical 8 domain (α-H8), a structural feature of many GPCRs (32,33). Ala-scanning SDM and Y2H-based screening established that mutation of Leu316, Arg318, and Leu323 abolished the interaction of TPrx312-343 with PRK1 while mutation of all other residues only impaired it (Supplemental Fig. 1). In contrast, while mutation of each of the α-H8 residues impaired the interaction of TPβ312-407 with PRK1β640-942, none of those mutations per se completely disrupted the interaction (Supplemental Fig. 1).

Confirmation of the association between TPrx/TPβ and PRK1 using glutathione-S-transferase pull-down assays and co-immunoprecipitations in mammalian cells.

To further examine the interaction between PRK1 and TPrx/TPβ, in vitro glutathione S-transferase (GST) pull-down assays were performed. Consistent with the Y2H studies, PRK1β640-942 was found to bind GST.TPβ312-407, GST.TPβ329-407 and GST.TPrx312-343 but not to GST.TPrx329-343 or GST, despite near equivalent expression of all proteins (Fig. 2A). The ability of full-length PRK1 (residues 1-942, hereon in referred to as PRK1), expressed as a Flag-epitope tagged form in mammalian HEK 293 cells, to bind GST.TPrx312-343 and GST.TPβ312-407 was also investigated. PRK1 showed a specific interaction with GST.TPrx312-343 and GST.TPβ312-407 but not with GST.hIgG, a GST-protein expressing the C-tail domain of the human prostacyclin receptor (Fig. 2B).

The ability of PRK1 to associate with haemagglutinin (HA)-tagged forms of TPrx or TPβ stably expressed in the previously characterised HEK.TPrx and HEK.TPβ cell lines (27) was examined through co-immunoprecipitations. PRK1 was detected in the anti-HA immunoprecipitates from HEK.TPrx and HEK.TPβ cells, but not in corresponding immunoprecipitates from the HEK.β-Gal cells, encoding HA-tagged β-galactosidase (β-Gal), acting as an additional/alternative control (Fig. 2C). Thereafter, the effect of short term agonist-exposure (10 min) on the interaction between TPrx/TPβ and PRK1 was investigated in response to stimulation of cells with the selective TXA2 mimetic U46619. As previous, in the absence of agonist, PRK1 was specifically detected in the anti-HA immunoprecipitates from HEK.TPrx and HEK.TPβ cells, and not from HEK.β-Gal cells. Stimulation of cells with U46619 did not lead to an alteration in the amount of PRK1 associated with TPrx or TPβ (Fig. 2D). In all cases, failure to detect PRK1 in the anti-HA immunoprecipitates from the control HEK.β-Gal cells was not due to lack of PRK1 expression (Fig. 2C and 2D, lower panels) or failure of the immunoprecipitation per se in those cells (Fig. 2C and 2D, middle panels).

The C-terminal domain of PRK1 is important for association with TPrx/TPβ.

The N-terminal region of PRK1, spanning residues 1 to 511, contains a number of important regulatory domains which influence its C-terminal catalytic kinase domain (Fig. 3A). The three ACC, or HR1, domains are involved in RhoA-GTP binding (34,35) while the C2-like domain participates in arachidonic acid (AA)-induced kinase activation and lies adjacent to the kinase domain itself (36). Hence, herein, the interaction
between TPα and TPβ with PRK1 or with three of its subfragments comprising either the kinase domain (PRK1\textsubscript{561-942}), the ACC/HR1 domains alone (PRK1\textsubscript{1-357}) or ACC/HR1 domains along with the AA-binding site (PRK1\textsubscript{1-594}) were examined through co-immunoprecipitations, where possible interactions with the unrelated HA-tagged β\textsubscript{2}-adrenergic receptor (β\textsubscript{2}-AR) served as an additional independent control. As previous, PRK1 associated strongly and specifically with TPα and TPβ as evidenced by its detection in immunoprecipitates from HEK.TPα and HEK.TPβ, but not from control HEK.β\textsubscript{2}-AR, cells (Fig. 3B; upper panels). The kinase only PRK1\textsubscript{561-942} sub-fragment was also abundantly expressed in TPα and TPβ, but not β\textsubscript{2}-AR, immunoprecipitates and at levels comparable to that of PRK1. Conversely, lower levels of PRK1\textsubscript{1-594} were detected in the TPβ and, to an even lesser extent, in the TPα immunoprecipitates. Furthermore removal of the AA-binding site, as in the PRK\textsubscript{1-357} subfragment, completely abolished binding to TPα and substantially reduced binding to TPβ. The observed differences in immunoprecipitation of PRK1 or its subfragments were not due to variations in their expression levels in the cell lines used or in the efficiency of the immunoprecipitations per se (Fig. 3B; lower and middle panels, respectively). The identity of the faint, non-specific immunoreactive protein present in the anti-HA precipitates from HEK.β\textsubscript{2}-AR cells is unknown (Fig. 3B; upper panels). Additional evidence of the involvement of the kinase domain of PRK1 in interacting with the TPs was established whereby ectopic over-expression of PRK1\textsubscript{561-942}, but not PRK1\textsubscript{1-594} or PRK1\textsubscript{1-357}, competed and thereby partially impaired the association of PRK1 with TPβ and, to a lesser extent, with TPα (Fig. 3C). Collectively, these data confirm a constitutive physical interaction between TPα/TPβ and PRK1 in mammalian cells and point to a critical role for the C-terminal region of PRK1, comprising its AA-binding C2-like domain and its catalytic kinase domain, in that interaction.

**RhoA-association with the TP/PRK1 complex.**
PRK1 is a downstream effector of the GTPase RhoA (37). Moreover, both TPα and TPβ modulate RhoA signalling (23). Hence, it was sought to further examine the interaction of TPα and TPβ with PRK1 and to establish whether endogenous RhoA may associate with the complex. Initially, HEK.TPα, HEK.TPβ and the control HEK.β-Gal cell lines, co-transfected with pCMVTag2b:PRK1, were stimulated with U46619 for 0 - 120 min and the presence of PRK1 and endogenous RhoA in the anti-HA immunoprecipitates examined. In the absence of agonist, PRK1 was detected in the anti-HA TPα and TPβ, but not β-Gal, immunoprecipitates while neither short term or more prolonged U46619 stimulation led to measurable changes in the amount of PRK1 associated with the TPs (Supplemental Fig. 2A-D). Moreover, RhoA was detected in the anti-HA TPα and TPβ immunoprecipitates and at levels that were unaffected by U46619 stimulation (Supplemental Fig. 2A-D). Conversely, RhoA was not detected in HA-β-Gal immunoprecipitates despite its efficient immunoprecipitation and equivalent expression of endogenous RhoA in all cell lines (Supplemental Fig. 2A and 2B).

To exclude the possibility that the observed associations of PRK1 or RhoA with TPα or TPβ may be an artifact of over-expression of PRK1, the ability of endogenous PRK1 and endogenous RhoA to associate with the TP isoforms was examined. As with the over-expressed PRK1, similar levels of association between TPα/TPβ and PRK1 were observed in both non-stimulated and U46619-stimulated cells (Fig. 4A and 4B; Supplemental Fig. 2E and 2F). Moreover, consistent with previous findings, RhoA was associated with the TPα/TPβ-PRK1 immunoprecipitates in ternary complexes that were not affected by U46619 stimulation (Fig. 4A and 4B; Supplemental Fig. 2E and 2F). Collectively, these data confirm a physical interaction between TPα/TPβ and PRK1 in a constitutive ternary complex with the PRK1 effector RhoA in mammalian cells that is independent of TP-agonist activation.

**TPα and TPβ are not phosphorylation targets of PRK1.**
Several functional targets of PRK1 have been identified ranging from its ligand-dependent interaction and activation of the nuclear androgen receptor (8) to its interaction and phosphorylation of vimentin and glial fibrillary acidic protein, to inhibit filament formation (38). Moreover, both TPα and TPβ are recognized targets of PKC...
phosphorylation (24-26,28). Hence, in view of its many regulatory functions, it was sought to investigate whether PRK1 might target TPα and/or TPβ by direct phosphorylation. To this end, the ability of purified preparations of PRK1 to phosphorylate GST-fusion proteins encoding the intracellular loop domains (IC1 – IC3) and the C-tail domains of TPα or TPβ were examined in vitro. The known PRK1 substrate histone H1 served as a control in the in vitro kinase assays (39). While histone H1 was readily phosphorylated, none of the purified recombinant GST proteins encoding the respective intracellular sub-domains of TPα/TPβ were phosphorylated in vitro by PRK1 (Supplemental Fig. 3). Moreover, despite repeated attempts, whole cell phosphorylations in HEK.TPα or HEK.TPβ cells established that PRK1 did not lead to phosphorylation of TPα or TPβ either in the absence or presence of U46619 stimulation or following over-expression of PRK1 (data not shown).

**PRK1 activity is increased in response to U46619 stimulation.**

As stated, it has been previously established that agonist-activation of TPα and TPβ leads to robust activation of RhoA (23). Moreover, herein, it has been established that PRK1 constitutively interacts with both TPα and TPβ in a complex that also contains RhoA. Hence, it was sought to investigate whether endogenous PRK1 associated with the immune complexes is functionally active and whether its activity may respond to agonist-stimulation of TPα and/or TPβ either expressed in the respective clonal HEK.TPα, HEK.TPβ cell lines or endogenously in vascular endothelial EA.hy926 cells or primary (1°) HUVECs and in 1° h.CoASM cells. Initially, to confirm the specificity of PRK1 activation, HEK.TPα, HEK.TPβ and control HEK.β-Gal cells were stimulated with U46619 for 10 min and, following immunoprecipitation with anti-PRK1 antibody, precipitates were used as source of kinase for the in vitro phosphorylation assays using histone H1 as the specific PRK1 substrate. Results show substantial phosphorylation of histone H1 when PRK1 was immunoprecipitated from agonist-stimulated HEK.TPα and HEK.TPβ cells (Fig. 5A and 5B). Conversely, relative levels of histone H1 phosphorylation was significantly lower when PRK1 was precipitated from the control HEK.β-Gal cells despite near equivalent immunoprecipitation of PRK1 in all cell types (Fig. 5A and 5B). Moreover, time-course assays established that, in the absence of agonist, PRK1 resulted in histone H1 phosphorylation which was transiently increased in the presence of U46619, with maximal responses occurring at 10-30 min for both TPα and TPβ (Fig. 5C and 5D). The precise physiologic impact of the observed increase in PRK1 activity in response to TP agonist activation, as determined by analysis of histone H1 phosphorylation, is unclear.

PRK1 immunoprecipitated from EA.hy926, 1° h.CoASM cells or 1° HUVECs also led to increases in histone H1 phosphorylation in vitro in the response to agonist-simulation (Supplemental Fig. 4A and 4B; and data not shown) and were lower than those observed in HEK.TPα and HEK.TPβ cells. Such differences are most likely reflective of the levels of endogenous TPα and TPβ expressed in the former cell types (10-20 fmol/mg cell protein (40)) relative to those levels in the clonal HEK.TPα or HEK.TPβ cell lines (approximately 2 pmol/mg cell protein). Furthermore, PRK1 was not detected in immunoprecipitates from either 1° HUVECs or 1° hCoASMCs using an affinity purified antibody (#168) directed to both TPα and TPβ (Supplemental Fig.4C – 4E).

As a means of verifying that the increased PRK1-induced phosphorylation of histone H1 observed in the presence of U46619 was due to activation of TPα and/or TPβ, PRK1 present in the anti-HA TPα and anti-HA TPβ immune complexes from HEK.TPα and HEK.TPβ cells was also used as a source of kinase in the in vitro assays. Consistent with previous data, PRK1 was efficiently co-immunoprecipitated with both TPα and TPβ and not with the control β-Gal (Fig. 5E and 5F; lower panels). Endogenous PRK1 present in the anti-TPα and anti-TPβ precipitates resulted in efficient in vitro phosphorylation of histone H1 while, as expected, no phosphorylation was detected using the anti-HA immune complexes from HEK.β-Gal cells (Fig. 5E and 5F, upper left panels). Moreover, while PRK1 present in the anti-TPα and anti-TPβ precipitates phosphorylated histone H1 in vitro in the absence of agonist, phosphorylation was increased following U46619 stimulation in both cases (Fig. 5E and 5F, upper right panels). Hence, collectively, these data confirm that PRK1-
associated in immune complexes with TPα and TPβ is functionally active and that it undergoes enhanced activation in response to receptor stimulation.

**Histone H3 Thr 11 Phosphorylation in Prostate PC-3 and LNCaP Cells.**

PRK1 has been established to play a key role in the regulation of transcription by the nuclear androgen receptor (AR) through its specific phosphorylation of histone H3 at a critical Thr11 residue (H3Thr11), thus initiating chromatin remodeling and potentiating androgen-induced gene expression, such as within the prostate (8,9). Furthermore, this occurs through androgen-induced association of PRK1 with the AR (8,9). Hence, owing to the findings herein demonstrating a direct interaction between TPα/TPβ with PRK1, it was sought to explore whether U46619-mediated activation of PRK1 might actually induce phosphorylation of histone H3 at Thr11 (H3Thr11) in 1o HUVECs and 1o h.CoASMCs, somewhat similar to that now established for the androgens (8,9). To this end, H3Thr11 phosphorylation was analysed using a specific anti-phospho-H3Thr11, where cells growth-arrested in metaphase with colcemid served as a positive control for H3Thr11 phosphorylation and blots were co-screened with anti-histone H3 antibody to ensure uniform total histone H3 loading (9,41). While colcemid induced robust phosphorylation, the level of H3Thr11 phosphorylation in response to U46619 in either 1o HUVECs and 1o h.CoASMCs was unaltered over the course of the treatment (0 – 60 min; **Supplemental Fig. 4F and 4G**) suggesting that, in those cell types at least, TPα/TPβ - induced PRK1 activation is not associated with H3Thr11 phosphorylation.

As previously stated, in addition to PRK1, increased TXA2-induced signalling and TP isoform(s) expression have been associated with human prostate cancer (12-14). Hence, in view of the direct interaction of PRK1 with TPα and TPβ and of the, by now, established role of androgen-activated PRK1 in inducing H3Thr11 phosphorylation (8,9), it was sought to investigate whether U46619-mediated activation of TP/PRK1 signalling might induce H3Thr11 phosphorylation in the human prostate carcinoma PC-3 and LNCaP cell lines. Moreover, it was also sought to investigate whether TP-mediated PRK1 activation might affect androgen-induced responses by comparing the effect of U46619 on dihydrotestosterone (DHT) -induced H3Thr11 phosphorylation in the androgen-insensitive PC-3 relative to the androgen-sensitive human adenocarcinoma LNCaP cell lines, where colcemid-arrested cells served as a positive control for the assay in both cell lines (**Fig. 6A and 6B**). In contrast to 1o HUVECs and 1o h.CoASMCs, stimulation of both PC-3 and LNCaP cell types with U46619 led to significant increases in H3Thr11 phosphorylation with maximal responses occurring at 30 – 60 min in both cell lines (**Fig. 6A and 6B**). In all cases, rescreening of the anti-phospho H3Thr11 blots with anti-histone H3 itself confirmed that any differences in H3Thr11 phosphorylation in either PC-3 or LNCaP cells, e.g in response to U46619, were not due to discrepancies in histone H3 levels (**Fig. 6A and 6B**). While treatment of PC-3 cells with DHT did not induce a significant increase in H3Thr11 phosphorylation per se (**Fig. 6A**), co-stimulation with U46619 led to similar, time-dependent increases in H3Thr11 phosphorylation but to levels that were not significantly different than those in the absence of DHT (**Fig. 6A; F-test analysis, p = 0.9039**). Conversely, pre-treatment of LNCaP cells with DHT led to a significant increase in H3Thr11 phosphorylation (**Fig. 6B**). Moreover, co-stimulation of LNCaP cells with U46619 in the presence of DHT led to robust, time-dependent increases in H3Thr11 phosphorylation relative to those levels in the presence of U46619 alone (**Fig. 6B; F-test analysis, p < 0.0001**), with maximal responses occurring at 30 – 60 min post U46619-treatment. In point of fact, at 30 min post-agonist stimulation, maximal levels of H3Thr11 phosphorylation in LNCaP cells were up to 3-fold greater in the presence of DHT plus U46619 relative to basal levels in the absence of either agent (**Fig. 6B; 2-way ANOVA, P < 0.0001**). In addition, to investigate whether the increased H3Thr11 phosphorylation in the latter cell types is specific to the TPs or a more general phenomenon, both PC-3 and LNCaP cells were stimulated with 17-phenyl trinor prostaglandin (PG)E2 or cicaprost, selective agonists for the related Gq-coupled EP1 subtype of the PGE2 receptor or the Gs-coupled prostacyclin receptor, respectively, and their effect on H3Thr11 phosphorylation was compared with that of the TP
agonist U46619 (Supplemental Fig. 5A and 5B). While U46619 resulted in substantial increases in H3Thr11 phosphorylation in both PC-3 and LNCaP cells, it was established that neither 17-phenyl trinor PGE₂ nor cicaprost did so in either cell line (Supplemental Fig. 5A and 5B). The precise physiologic impact of the observed TP-mediated PRK1 activation, as determined herein by analysis of H3Thr11 phosphorylation, is not fully evident at this time. However, given the serious nature of enhanced H3Thr11 phosphorylation as a key marker of androgen-dependent gene expression, any change in H3Thr11 phosphorylation in response to TP activation, possibly leading to enhanced AR-dependent gene expression, is likely to be of substantial significance, such as in the context of the enhanced TP expression-associated with prostate cancer (12-14).

To investigate whether PRK1 actually interacts with the TPα/TPβ endogenously expressed in the PC-3 and LNCaP cells, immunoprecipitations were performed with an affinity purified antibody (#168) directed to both TP isoforms. In the absence of agonist, PRK1 was detected in the anti-TPα/TPβ immunoprecipitates from both PC-3 and LNCaP cells but not in corresponding immunoprecipitates using the pre-immune IgG (Fig. 7A and 7B). Consistent with previous data in HEK.TPα and HEK.TPβ cells (Fig. 4), stimulation of PC-3 or LNCaP cells with U46619, either in the presence or absence of pre-stimulation with DHT, did not alter levels of PRK1 associated with anti-TPα/TPβ immune-complexes in either cell type (Fig. 7A and 7B). Noteworthy, owing to the relatively low levels of TPα/TPβ endogenously expressed in either PC-3 or LNCaP cells (~ 120 fmol/10⁶ cells (14)), secondary screening of the immunoprecipitates precluded detection of either receptor isoform (data not shown). However, in parallel experiments, the specificity of the affinity purified antibody to immunoprecipitate both HA-tagged TPα and TPβ from HEK.TPα and HEK.TPβ cells was confirmed, while neither receptor isoform was present in immunoprecipitates using equivalent amounts of the pre-immune IgG (Supplemental Fig.4E). Furthermore, through preliminary image analysis using green fluorescent protein (GFP) tagged form of PRK1 (GFP:PRK1), it was confirmed that PRK1 underwent transient translocation from the cytosolic to the nuclear fraction following stimulation of LNCaP (Figure 7C) and PC-3 (Supplemental Fig.6) cells with U46619, where maximal nuclear localization was observed at 30 min post agonist-stimulation.

Further confirmation that the agonist-induced increases in H3Thr11 phosphorylation observed in both PC-3 and LNCaP cells is due to PRK1-induced mechanisms was established whereby the broad spectrum PRK1 inhibitor RO-31-8220 partially inhibited colcemid- and U46619- phosphorylation and the DHT- responses in LNCaP cells (Fig. 6C and 6D). As the PRK1 inhibitor RO-31-8220 can also inhibit other kinases including GSKβ, S6K, RSK, MSK and PKCα (42), additional approaches were used to examine PRK1 specificity. While overexpression of the wild type PRK1 led to modest increases in basal H3Thr11 phosphorylation, which was further increased on U46619 stimulation (Supplemental Fig.5C and 5D), over-expression of a kinase defective dominant negative PRK1K644E (9) impaired U46619-induced H3Thr11 phosphorylation in both PC-3 and LNCaP cells (Fig. 6E and 6F, respectively). Furthermore, small interfering (si)RNA directed to PRK1 (siRNAPRK1), but not to the scrambled control sequence (siRNACONTROL), substantially reduced PRK1 expression (Fig. 8A and 8B) and U46619-induced H3Thr11 phosphorylation, both in the absence or presence of DHT, in PC-3 and LNCaP cells (Fig. 8C and 8D). Moreover, the DHT-induced H3Thr11 phosphorylation, both in the absence and presence of U46619, observed in LNCaP cells was partially, but not completely, impaired by the siRNAPRK1 but not by the siRNACONTROL. The decreases in PRK1 expression in the presence of siRNAPRK1 were not due to unequal loading of the protein samples per se as evidenced by immunoblotting of membranes for the ubiquitously expressed molecular chaperone HDJ-2/DNA-J protein, which served as an internal loading control (Fig. 8A and 8B).

U46619-induced activation of TPα/TPβ endogenously expressed in PC-3 cells has been previously shown to increase cell motility and migration, possibly accounting for the increased correlation between TP expression and signalling in prostate cancers (14). Hence, herein, it was sought to explore TP-mediated cell migration in the androgen-responsive LNCaP and non-responsive PC-3 cell lines and to investigate
whether PRK1 expression may influence that migration (Fig. 8E and 8F). Stimulation with U46619 led to substantial increases in migration of both LNCaP and PC-3 cells (Fig. 8E and 8F; Non-transfected). Furthermore, DHT also increased migration of LNCaP, but not of PC-3, cells and this effect was augmented by co-stimulation of the former cells with U46619 (Fig. 8E and 8F; Non-transfected; 2-way ANOVA, P = 0.0004). Disruption of PRK1 expression with the siRNA_{PRK1}, but not the siRNA_{CONTROL}, specifically impaired U46619-induced cell migration in both PC-3 and LNCaP cells (Fig. 8E and 8F). In addition, siRNA_{PRK1} also partially impaired DHT-induced cell migration in LNCaP cells both in the absence and presence of U46619 (Fig. 8F).

Taken together, these data establish that agonist-induced activation of the TPs endogenously expressed in the prostate adenocarcinoma PC-3 and LNCaP cell lines lead to H3Thr11 phosphorylation, a previously recognized marker of chromatin remodeling exclusively associated with androgen-induced responses. Moreover, the TXA$_2$ mimetic U46619 can significantly augment the androgen-induced H3Thr11 phosphorylation in LNCaP but not in PC-3 cells. It was established that PRK1 directly interacts with TP$\alpha$/TP$\beta$ endogenously expressed in both PC-3 and LNCaP cells and disruption of PRK1, such as through targeted siRNA, substantially impairs TP-mediated H3Thr11 phosphorylation and cell migration in response to U46619. Collectively, these data provide a potential molecular basis for the role of TXA$_2$ and its receptor in prostate cancer and in other conditions in which aberrant TXA$_2$/RhoA/PRK1 signalling is implicated.

**Discussion**

In this study, we report the discovery of a novel interaction between the TP$\alpha$ and TP$\beta$ isoforms of the human TXA$_2$ receptor (TP) with PRK1, an effector of certain members of the Rho subfamily of monomeric GTPases. TP$\alpha$- and TP$\beta$-mediated intracellular signalling to Gq and G$_{12}$ is well characterized (15,23). However, less is known about their signalling to extracellular stimuli that do not involve direct coupling to heterotrimeric G-proteins. TP$\alpha$/TP$\beta$ can form homo/hetero dimers or oligomers (43), raising the possibility of multiple protein associations at a single TP complex. Moreover, the role of various GPCR interacting proteins, or GIPs, are increasingly recognized in regulating novel intracellular cascades through direct protein:protein interaction, most often involving the intracellular loops and/or C-tail domain(s) of the GPCR, and which do not necessarily involve classic G-protein signalling (44). A number of novel associations with either TP$\alpha$ and/or TP$\beta$ have been previously identified. The proteasome activator PA28$\gamma$ specifically interacts with TP$\beta$, enhancing its degradation by a proteasomal-dependent mechanism (45). Interaction of TP$\beta$ with the nucleoside diphosphate kinase Nm23-H2 leads to its Rac1-dependent endocytosis (46) while its interaction with Rab11 participates in its agonist-induced trafficking through the slow endosome pathway (47). More recently, interactions between TP$\alpha$/TP$\beta$ with angio-associated migratory cell protein (AAMP) (48) and with KIAA1005 were reported (49). Interestingly, both PRK1 and KIAA1005 contain C2 domains. However, the significance of these conserved functional domains in the two TP interactants was not examined in the current study and will be investigated in future studies.

The direct interaction between TP$\alpha$/TP$\beta$ and PRK1 identified in the current study was found to be constitutive in mammalian cells. While there was no agonist-dependent alteration in the association of TP$\alpha$/TP$\beta$ with either PRK1 or RhoA, TP agonist-stimulation enhanced PRK1 activation leading to phosphorylation of its general substrate histone H1. Although the precise nature of the interaction with PRK1 remains to be determined, Y2H studies identified two regions of importance within the intracellular C-tail domains of TP$\alpha$/TP$\beta$, namely the common region (residues 312 – 328), proximal to transmembrane (TM) 7, and a more distal region of TP$\beta$ (residues 366 – 392). In the absence of one or both of these regions, binding to PRK1$^{640-942}$ in yeast is severely reduced or completely abolished. Using GST-based in vitro approaches, the specific requirement of the common 312-328 region within the C-tail domains of TP$\alpha$ and TP$\beta$ as a critical binding determinant with both the kinase domain and PRK1 was confirmed. Examination of the sub-domains of PRK1 reaffirmed an essential role for the C-terminal
domain of PRK1, incorporating the AA binding site and the kinase domain, in the interaction with TPα/TPβ while the N-terminal ACC/HR1 domains, involved in Rho/Rac binding, was not required. Moreover, ectopic expression of PRK1\(^{1561-942}\), but not PRK1\(^{1-357}\) or PRK1\(^{1-594}\), specifically competed the interaction of PRK1 with TPβ and, to a lesser extent, with TPα. Additional experimentation is required to clarify why PRK1\(^{1-594}\) can bind to TPβ, and to a lesser extent to TPα, but does not compete with binding of the full length PRK1 to either receptor isoform.

More precise mapping of the regions within TPα/TPβ and PRK1 that contribute to the interaction is beyond the scope of this study and will be the subject of further investigations. Noteworthy, using the Y2H screening approach, we also investigated the role of the 3 intracellular (IC) loops in the interaction with PRK1 and its subdomains. However, owing to their limited sizes for Y2H-type interaction studies, results generated were inconclusive and, therefore, the possibility that any one of all of the IC domains may contribute to the interaction with PRK1 in mammalian cells cannot be excluded.

The role of the common 312-328 region, encoding the α-H8 domain (32,33) of TPα/TPβ in contributing to their interaction with PRK1 was also investigated herein. Located proximal to TM7, perpendicular to the heptahelical TM bundles, the α-H8 domain can play an essential role in mediating interactions between certain GPCRs and their GIPs in addition to influencing receptor expression and/or trafficking (50-52). Moreover, it may act as a conformational switch between the active and inactive states of certain GPCRs. Hence, our discovery of a role for the putative α-H8 in the interaction of TPα/TPβ with PRK1 is indeed consistent with its functional importance and in mediating protein:protein interactions. While mutation of certain residues (Leu\(^{318}\), Arg\(^{318}\) and Leu\(^{323}\)) within the α-H8 domain completely disrupted the interaction between TPα\(^{312-343}\) and PRK1, mutation of other residues only impaired it. In contrast, mutation of residues within α-H8 impaired the interaction of TPβ\(^{312-407}\) with PRK1 in each case while no single mutant completely disrupted the interaction per se. This is entirely consistent with the role of other residues, namely 366 -392 within the distal C-tail domain of TPβ in contributing to its interaction with PRK1. Greater insights into the molecular components contributing to the specificity of interaction of TPα and/or TPβ with PRK1 will require further investigations involving alternative approaches outside of the Y2H system including detailed biophysical approaches. However, in terms of specificity, of particular note was the finding that while residues within the common (312 -328) and more distal (366 -392) domains have also recently been implicated in the interaction of TPβ with angio-associated mitragary cell protein (AAMP (48)), Ala scanning SDM of the α-H8 domain within that region does not influence the interaction of TPβ with AAMP in yeast (Kinsella lab, unpublished data).

Moreover, the finding that the α-H8 domain contributes to the interaction of TPα/TPβ with PRK1 is indeed consistent with the growing body of evidence of its general importance in mediating, at least in part, protein: protein interaction between certain GPCRs and specific GIP(s) (50-52).

The cellular responses to TXA2 are subject to regulation by both agonist-dependent homologous desensitization (24,25) and by crosstalk with other signalling systems (23,27,29) which mainly occurs through direct phosphorylation of the TPα and/or TPβ isoforms themselves. For example, both TPα and TPβ undergo PKC phosphorylation within their IC (e.g Ser\(^{145}\), IC\(_{2}\)) and unique C-tail (e.g TPα @ Thr\(^{337}\), TPβ @ Thr\(^{399}\)) domains (24-26). As PRK1 is closely related to other members of the PKC family, particularly in its kinase domain, and is known to phosphorylate many, but not all, of its interactants, it was sought to establish whether PRK1 could phosphorylate any or all of the intracellular domains of TPα and/or TPβ in vitro or in whole cell phosphorylation assays. The finding herein the neither TPα nor TPβ are phosphorylated by PRK1 is, in fact, entirely consistent with previous reports by us which established the agonist-induced phosphorylation of TPα occurs through a combined PKC and PKG feedback mechanism while that of TPβ occurs through a combined PKC and GRK2/3 mechanism (24,25).

The precise mechanism by which agonist-stimulation of TPα or TPβ leads to PRK1 activation is unclear but is likely to involve RhoA, a known downstream effector of TP-mediated G\(_{12}/p115\)RhoGEF signalling (23). So one might
ask as to what is the functional requirement or basis for the direct interaction of TPα/TPβ with PRK1. One possible reason might be to facilitate very rapid activation of the RhoA effector PRK1 already recruited/present in the receptor complex in response to agonist-stimulation. Noteworthy, while endogenous RhoA was found associated with both over-expressed and endogenous PRK1 in constitutive ternary complexes with TPα and TPβ, agonist-activation of either TP isoform did not alter the level of RhoA associated with such complexes. Hence, other possibilities might include RhoA-independent effects. For example, as stated, PRK1 is essential for androgen-dependent transcription involving its phosphorylation of histone H3 at Thr 11 (H3Thr11), a hallmark of androgen-induced chromatin remodeling and transcriptional activation (8,9). In fact PRK1 is regarded as a gatekeeper of androgen receptor (AR)-dependent transcription making it an exciting therapeutic target in prostate and certain ovarian carcinomas (6,9). PRK1 directly interacts with the transcriptional activation unit 5 (TAU-5) within the N-terminal domain (NTD) of the AR leading to superactivation of AR-regulated genes through the recruitment of demethylases (8). Moreover, similar to that discovered herein for TPα/TPβ, (i) the AR interacts with PRK1 also within its kinase domain and (ii) PRK1 does not phosphorylate the AR (8). From a mechanistic point of view, phosphorylation of H3Thr11 by PRK1 accelerates histone H3 demethylation by the Jumonji C (JmjC)-domain-containing protein JMJD2C, thereby promoting AR-dependent transcription (9). Levels of PRK1 and phosphorylated H3Thr11 correlate with Gleason scores of prostate and of certain ovarian carcinomas and inhibition or knockdown of PRK1 blocks AR-induced tumor cell proliferation (6,9). Coupled with this, in the context of TXA2, aberrant expression of the TPs has also recently been associated with prostate cancer, where a significant correlation between activation of the TXA2 pathway with Gleason score and pathologic state has been identified (12). Furthermore, the migratory phenotype of PC-3 cells is regulated by the TXA2 mimetic U46619 through a RhoA-dependent mechanism (14). Collectively, these data suggest that while the TPs modulate PC-3 cell migration through RhoA, the discovery that TPα/TPβ also interact and regulate PRK1 also raises the possibility that, like the AR, TP-mediated activation of PRK1 might lead to H3Thr11 phosphorylation contributing to or exacerbating the pathologic state. Hence, herein, it was of significant interest to investigate whether TP-mediated PRK1 activation might possibly lead to H3Thr11 phosphorylation.

While there was no evidence that stimulation of the TPs led to H3Thr11 phosphorylation either in 1° HUVECs or 1° CoASMCs, under conditions examined at least, stimulation of the TPs endogenously expressed in the human prostate carcinoma PC-3 and LNCaP cell lines led to significant, time-dependent increases in H3Thr11 phosphorylation. Interestingly, screening of anti-TPα/TPβ immunoprecipitates from either 1° HUVECs or 1° CoASMCs did not permit detection of PRK1. While the reason for this is not fully clear, it is likely to be due to receptor expression levels. Moreover, it is somewhat consistent with the finding that U46619-stimulation of TPs endogenously expressed in either 1° HUVECs or 1° CoASMCs did not lead to histone H3Thr11 phosphorylation and resulted in only marginal increases in histone H1 phosphorylation by PRK1 immunoprecipitated from those cell lines. In contrast, as stated, evidence of U46619-induced PRK1 activation and histone H3Thr11 phosphorylation and the presence of PRK1 in anti-TPα/TPβ immune complexes were readily detected in the prostate PC-3 and LNCaP cell lines. Furthermore, DHT induced H3Thr11 phosphorylation in the androgen-responsive LNCaP cells but not in the androgen-insensitive PC-3 cell line, an effect that was substantially augmented by the TXA2 mimetic U46619. Moreover, a direct constitutive interaction between PRK1 and TPα/TPβ endogenously-expressed in both PC-3 and LNCaP cells was confirmed and disruption of PRK1 activity (RO31-8220 or over-expression of PRK1K644E) or PRK1 expression (siRNAPRK1) impaired TP-mediated H3Thr11 phosphorylation. Collectively, these data establish that TP-mediated PRK1 activation can independently lead to H3Thr11 phosphorylation in prostate carcinoma cell lines but that it can also co-operate to augment androgen-induced H3Thr11 phosphorylation. These findings are entirely significant in that they are the first to establish that agents other than androgens can induce H3Thr11 phosphorylation.
and that it occurs through a similar PRK1-dependent mechanism. Furthermore, in preliminary experiments, it was established that U46619-induced PRK1 translocation to the nucleus while disruption of PRK1 expression impaired TP-mediated cell migration in both cell types and blocked a U46619-induced augmentation of LNCaP cell migration in the presence of DHT. Hence, it is indeed tempting to speculate that the observed co-operativity between TP- and AR-mediated PRK1 activation leading to H3Thr11 phosphorylation may account for the documented association between increased TXA2 signalling, including TP expression, in androgen-driven prostate cancer (12,14). It will be of significant interest to establish whether activation of TP signalling, in particular through the novel pathway identified herein involving PRK1-mediated H3Thr11 phosphorylation, occurs in other carcinomas in which androgens are implicated such as in ovarian carcinomas showing elevated serum androgen levels (6).

The importance of PRK family members in transcriptional regulation is further endorsed by recent reports that key members of class 11a histone deacetylases (HDACs) are phosphorylated by PRK1, and/or by the related PRK2. More specifically, HDAC-5, -7 and -9, but not -4 are phosphorylated by PRK1/2 within their nuclear localization signal (NLS), thereby impairing HDAC nuclear import and promoting transcriptional activation (53). PRK1 has been also established to specifically interact with the tumour necrosis factor receptor (TNFR) - associated factor 2 (TRAF2), involving a direct interaction between residues 580 – 584 of PRK1 located in the linker region between its AA-sensitive C2-like and catalytic domains (54). TRAF2 is one of the major mediators of TNFR signalling, transducing TNFα signalling to its many functional targets including to activation of NF-κB- and cJun kinase (JNK)-mediated inflammatory responses and/or apoptotic cascades. Disruption of PRK1 expression impairs TRAF2-induced NF-κB activation linking PRK1 to the regulation of TNFα-mediated inflammatory responses (54). Interestingly, in a follow up study, PRK1 was found to specifically phosphorylate the related TRAF1, which lacks the JNK/IKK signalling effector domain, but not its interactant TRAF2 or other TRAF members, leading to the recruitment of TRAF1 to the TNFR, silencing the receptor complex by PRK1(55). Moreover, within the vasculature, PRK1 has been implicated in the mediation of VSM-specific gene expression promoting VSM differentiation, such as in response to transforming growth factor -β1 (56,57). Bearing in mind the central role of TXA2 within the vasculature, including in the mediation of inflammatory disease, coupled with the finding herein of a direct interaction with and activation of PRK1, it will be of significant interest to investigate the possible interplay between those critical pathways, be it at the cellular or (patho)physiologic levels.

In conclusion, as outlined in the model presented in Figure 9, this study provides evidence of a novel constitutive interaction between TPα/TPβ and PRK1, in complex with RhoA. Furthermore, results demonstrate an agonist-dependent increase in PRK1 activity and a significant TP-dependent increase in histone H3 Thr11 (H3Thr11) phosphorylation, and associated cell migration, in prostate carcinoma cell lines, a modification that was up until this study almost exclusively viewed as an androgen-induced marker of chromatin remodeling and transcriptional activation. While the involvement of RhoA in the TP-mediated H3Thr11 phosphorylation by PRK1 is currently unknown, requiring additional experimentation, the discovery herein of a direct interaction between TPα/TPβ with PRK1 is significant. For example, such a discovery is likely to impact on the understanding of a range of (patho)physiologic processes in which aberrant TXA2/TP, RhoA and PRK signalling is implicated, not least within certain neoplasms and in vascular and hypertensive disease. Further investigation will reveal a more complete understanding of the physiologic, and possible (patho)physiologic or clinical, significance of this interaction and whether TP-antagonism might offer a therapeutic advantage in such conditions.
REFERENCES:

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**ACKNOWLEDGEMENTS**

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**Abbreviations:**
AA, arachidonic acid; ACC, antiparallel coiled-coil fold; α-H8, alpha-helical 8 domain; AR, androgen receptor; C-tail, carboxyl-terminal tail; DHT, dihydrotestosterone; FBS, foetal bovine serum; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; GST, glutathione-S-transferase; HA, hemagglutinin; HEK, human embryonic kidney; H3Thr11, histone H3 threonine 11; PG, prostaglandin; PRK1, Protein kinase C-related kinase 1; siRNA, small interfering RNA; TP, thromboxane receptor; Y2H, yeast two hybrid.
Figure 1. Interaction of PRK1 with the intracellular domains of TPα and TPβ.
Panels A and B: the *S. c* Y187(pACT2:PRK1<sup>640-942</sup>) prey strain was mated with *S. c* AH109 bait strains transformed with recombinant pGBK7T7 encoding the listed TPα or TPβ subfragments and, as controls, with pGBK7T7 alone and pGBK7T7:p53. Diploids were selected on DDO media and interactants on QDO media due to the GAL4-dependent transcriptional activation of the *HIS3* and *ADE2* reporter gene owing to positive interaction between bait and prey proteins. Data are representative of at least three independent experiments (Data; n ≥ 3).
Figure 2. Interaction of PRK1 with TPα and TPβ in vitro and in mammalian cells.
Panel A: The in vitro translated (IVT) PRK1640-942 product (3 µl) was incubated at room temperature for 2 hr with Glutathione-Sepharose beads pre-loaded with purified GST alone (control) or the indicated GST-fusion proteins (5 µg). Panel B: Cellular lysates (500 µg) obtained from HEK 293 cells, transiently transfected with pCMVTag2b:PRK11-942, encoding Flag-tagged PRK1, some 48 hr previously, were incubated at 4 °C for 2 hr with Glutathione-Sepharose beads pre-loaded with 10 µg GST.TPα312-343, GST.TPβ312-407 or, as a control, GST.IP299-386. The input IVT (1 µl; panel A) and GST pull-down products (panels A and B) were resolved by SDS-PAGE and immunoblotted versus anti-HA (3F10), anti-Flag or anti-GST. To verify expression of the Flag.PRK1, an aliquot of whole cell lysate (25 µg) was also immunoblotted with anti-Flag antibody (Panel B; upper right). Panel C and D: HEK.TPα, HEK.TPβ or, as controls, HEK.β-Gal cells, each transiently transfected with either pCMVTag2b:PRK1 (PRK1) or pCMVTag2b, were subject to immunoprecipitation with anti-HA (101R) antibody (panel C), or were incubated with vehicle (-) or 1 µM U46619 (+) for 10-min before immunoprecipitation with anti-HA (101R) antibody (panel D). Immunoprecipitates in Panels C and D were resolved by SDS-PAGE and immunoblotted with anti-Flag or anti-HA (3F10). To verify uniform expression of the Flag.PRK1 protein throughout, aliquots of whole cell lysates (50 µg/lane) were also immunoblotted with anti-Flag antibody (Panels C and D; lower). The relative positions of the molecular size markers (kDa) are indicated. Data; n ≥ 3.
Figure 3. The C-terminal kinase domain of PRK1 is critical for its interaction with TPα and TPβ.
Panel A: Schematic of PRK1 and its subfragments generated for this study. Panels B and C: HEK.TPα, HEK.TPβ and HEK.β2AR cells, either transiently transfected with pCMVTag2b vector encoding Flag-tagged PRK1, PRK11-594, PRK11-357 and PRK1561-942 alone (Panel B) or transiently co-transfected with the latter plasmids either in the presence of pCMVTag2b vector (Ø) alone or encoding PRK1 (Panel C), were immunoprecipitated with anti-HA (101R). Immunoprecipitates (IP) were resolved by SDS-PAGE and were either immunoblotted (IB) versus anti-Flag (upper panels), or anti-HA (3F10) (middle panels) antisera. To verify uniform expression of the Flag.PRK1 protein throughout, aliquots of the whole cell lysates (50 µg/lane) were also immunoblotted with anti-Flag antibody (lower panels). The relative positions of the molecular size markers (kDa) are indicated. Data; n ≥ 3.
Figure 4. Agonist-dependent interaction of PRK1 and RhoA with TPα and TPβ.

HEK.TPα (Panel A) and HEK.TPβ (Panel B) or, as controls, HEK.β-Gal (Panels A & B) cells were incubated with vehicle (-) or 1 µM U46619 (+) for 10 - 120 min prior to immunoprecipitation with anti-HA (101R) sera. Immunoprecipitates (IP) were resolved by SDS-PAGE and immunoblotted (IB) versus anti-Flag, anti-HA (3F10), anti-RhoA or anti-PRK1 sera. Aliquots of whole cell lysates (50 µg/lane) were also immunoblotted with anti-Flag, anti-RhoA or anti-PRK1 sera. The relative positions of the molecular size markers (kDa) are indicated. Data; n ≥ 3.
Figure 5. Agonist-induced activation of PRK1 by TPα and TPβ.
HEK.TPα, HEK.TPβ and/or HEK.β-Gal cells were incubated with 1 µM U46619 (+) for 10 min (Panels A & B) or with 1 µM U46619 for 0 - 60 min (Panels C & D) prior to immunoprecipitation (IP) with anti-PRK1 antibody. Panels E & F: HEK.TPα, HEK.TPβ and/or HEK.β-Gal were incubated with 1 µM U46619 for 10 min (left panels) or with 1 µM U46619 for 0 or 10 min (right panels) prior to immunoprecipitation (IP) with anti-HA (101R) antibody. Resulting anti-PRK1 (Panels A-D) or anti-HA (Panels E & F) immunoprecipitates were used as source of PRK1 to examine U46619-induced in vitro phosphorylation of histone H1 (10 µg; 30 °C for 30 min). Phosphorylated histone H1 was visualised by autoradiography while all immunoprecipitates (IP) were immunoblotted (IB) with anti-PRK1 antibody (Panels A – F; upper and lower, respectively). The relative positions of the 30 kDa and 104 kDa molecular size markers are to the left of the panels. The bar charts represent mean percentage changes in phosphorylation relative to PRK1 expression in the anti-PRK1 immunoprecipitates, where basal levels in the absence of U46619 are assigned a value of 100%. The asterisks (*) indicate that levels of phosphorylation were significantly increased in response to U46619-stimulation, relative to vehicle-treated cells where ** signifies \( p \geq 0.01 \) for post-hoc Dunnett’s multiple comparison \( t \)-test analysis. Data; \( n \geq 3 \).
Figure 6. Effect TP- activation on H3Thr11 phosphorylation in PC-3 and LNCaP cells.
Panels A-D: Immunoblot analysis of H3Thr11 phosphorylation in PC-3 (Panel A and C) and LNCaP (Panels B and D) cells were stimulated with U46619 (1 µM; 0 – 60 min) for 30 min following pre-incubation with either vehicle (0.01 % EtOH; 24 hr), DHT (1 nM; 24 hr) and/or the PRK1 inhibitor RO-31-8221 (10 µM; 1 hr), as indicated. As a control for H3Thr11 phosphorylation, cells were growth-arrested by treatment with colcemid (50 ng/ml) for 24 hr. Panels E and F: Immunoblot analysis of H3Thr11 phosphorylation in PC-3 (Panel E) and LNCaP (Panel F) cells transiently co-transfected with either pCMVTag2b (Ø), or pCMVTag2b:PRK1<sup>K644E</sup> (PRK1<sup>K644E</sup>) and stimulated 48 hr post-transfection with U46619 (1 µM; 30 min). In all cases, isolated histones were resolved by SDS-PAGE and immunoblotted (IB) with anti-phospho H3Thr11 (upper) or anti-histone H3 (lower) antibody followed by chemiluminescence detection. The bar charts represent mean percentage changes in H3Thr11 phosphorylation relative to histone H3 levels and are expressed in arbitrary units (± S.E.M, n = 3) where basal levels in vehicle-treated cells and in the absence of U46619 are assigned a value of 100 %. The asterisks (*) indicate that levels of H3Thr11 phosphorylation were significantly increased in response to U46619-stimulation, relative to vehicle-treated cells. The hashes (#) indicate that levels of H3Thr11 phosphorylation were significantly increased in response to U46619-stimulation in the presence of DHT. The cross symbol (†) signifies that levels of H3Thr11 phosphorylation were significantly decreased in the presence of RO-31-8221. In these cases, single and double symbols signify $p \geq 0.05$ and $p \geq 0.01$, respectively, for post-hoc Dunnett’s multiple comparison t-test analysis. The dollar sign ($) signifies that levels of H3Thr11 phosphorylation were significantly increased in the presence of DHT where $$ indicates $P \geq 0.001$ for unpaired Student’s t test. The insets in Panels E and F confirm over-expression of Flag-tagged dominant negative PRK1<sup>K644E</sup> variant, where the Ø symbol signifies the empty vector pCMVTag2b.
Figure 7. Interaction of PRK1 with TPα and TPβ in PC-3 and LNCap cells.

PC-3 (Panel A) and LNCaP (Panel B) cells were pre-incubated with vehicle, U46619 (1 µM; 30 min), DHT (1 nM; 24 hr) or U46619 + DHT (1 nM DHT for 24 hr followed by 1 µM U46619 for 30 min), as indicated. Thereafter, lysates (~500 µg/assay) were subject to immunoprecipitation with affinity purified anti-TPα/TPβ (#168; 20 µg) or with an equivalent concentration of the pre-immune serum (IgG). Immunoprecipitates (IP) and/or aliquots of whole cell lysates (50 µg/lane) were resolved by SDS-PAGE and immunoblotted (IB) with anti-PRK1. The relative positions of the molecular size markers (kDa) are to the left of the panels. Data; n ≥ 3. Panel C: Confocal image analysis of GFP:PRK1 nuclear translocation in LNCap cells stimulated with U46619 (1 µM; 0 – 240 min). Images were captured at 63X magnification using Zeiss LSM imaging software, where the horizontal bars represent 10 µm. Data are representative of at least three independent experiments.
Figure 8. Effect PRK1 on H3Thr11 phosphorylation and PC-3 and LNCaP cell migration. Panels A & B: Immunoblot analysis of endogenous PRK1 expression in PC-3 (Panel A) and LNCaP (Panel B) cells before or 48 hr post transfection with siRNA PRK1 or the scrambled siRNA CONTROL. To validate uniform protein loading, immunoblots were also screened for the ubiquitous chaperone protein HDJ-2. Panels C & D: Immunoblot analysis of H3Thr11 phosphorylation (upper) or anti-histone H3 expression (lower) in nucleosomes extracted from PC-3 (Panel C) and LNCaP (Panel D) cells following transfection with either siRNA PRK1 or siRNA CONTROL. Some 48 hr post-transfection, cells, pre-incubated with either vehicle (0.01 % EtOH; 24 hr) or DHT (1 nM; 24 hr), were treated with U46619 (1 µM; 30 min). The bar charts represent mean percentage changes in H3Thr11 phosphorylation relative to histone H3 levels and are expressed in arbitrary units (± S.E.M, n = 3) where basal levels in siRNA CONTROL-transfected vehicle-treated cells and in the absence of U46619 are assigned a value of 100 %. Panels E & F: PC-3 (Panel E) and LNCaP (Panel F) cells were either transfected for 24 hr with siRNA PRK1 or siRNA CONTROL or, as a control, non-transfected, followed by pre-treatment for a further 24 hr with either vehicle (0.01 % EtOH) or 1 nM DHT prior to assessment of migration for 4 hr in either the vehicle (0.01 % EtOH), 1 µM U46619, 1 nM DHT or 1 µM U46619 plus 1 nM DHT. In all cases, mean cell migration in vehicle-treated cells was assigned a value of 100 % and agonist-stimulated migration expressed as a relative percentage. The asterisk (*), hash (#) and dollar ($) symbols signify that levels of H3Thr11 phosphorylation were significantly increased in response to U46619-, U46619 in the presence of DHT- or DHT-stimulation, relative to vehicle-treated cells. The cross symbol (†) signifies that levels of H3Thr11 phosphorylation were significantly decreased in cells transfected with siRNA PRK1 compared to siRNA CONTROL. In all cases, single and double symbols signify $p \geq 0.05$ and $p \geq 0.01$, respectively, for post-hoc Dunnett’s multiple comparison t-test analysis. The insets in Panels E & F represent immunoblot analysis of endogenous PRK1 expression in PC-3 and LNCaP cells, where blots were screened with anti-HDJ2 to confirm uniform protein loading.
Figure 9: Model of AR- and TP-dependent H3Thr11 Phosphorylation in response to PRK1 activation.

The cell permeable androgen testosterone (T) is converted to its active metabolite dihyrotestosterone (DHT) by the cellular 5-α-reductase. DHT, in turn, binds to the hormone binding domain of the androgen receptor (AR), leading to its release from an inactive complex with heat shock proteins (HSP), promoting AR dimerisation and activation. The ligand-bound AR can activate gene expression by (i) translocating to the nucleus leading to transcriptional activation of target gene(s) containing an androgen response element (ARE) within its promoter. In addition, (ii) the ligand-bound AR can activate PRK1 which, in turn, translocates to the nucleus where it specifically catalyses the phosphorylation of histone H3 at Thr 11 (H3Thr11) initiating chromatin remodelling, promoting AR-dependent transcriptional activation of target genes, such as within the prostate. Herein, it was established that PRK1 is recruited into a complex with TPα and TPβ. Agonist (U46619)-dependent activation of TPα and/or TPβ leads to activation of PRK1. It was also established that, similar to that of the AR, TPα/TPβ-dependent activation of PRK1 can also lead to increased H3Thr11 phosphorylation, enhancing transcriptional activation of AR-responsive target genes and promoting cell proliferation and/or migration, such as within the prostate. Additionally, in the androgen-responsive LNCaP cells, co-stimulation with the TP agonist and DHT augments PRK1-dependent H3Thr11 phosphorylation.
SUPPLEMENTAL DATA:

Supplemental Table 1A - List of PCR Amplification Primers

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<td>pGBKT7:TPβ&lt;sup&gt;329-392&lt;/sup&gt;</td>
<td>pHM6:TPβ</td>
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<td>CTCTGGAATCCCTACGTACGCCCGG</td>
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<td>pHM6:TPβ</td>
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*Sequences are shown 5’ → 3’ and the cloning sites are underlined in italics.
### Supplemental Table 1B - List of Mutator Primers used for QuickChange™ Site-Directed Mutagenesis

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<th>Template</th>
<th>Sense Mutator Primer**</th>
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<td>pCMVTag2b:PRK1</td>
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<td>pGBK7:TPα</td>
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<td>pGBK7:TPα</td>
<td>pGBK7:TPβ312-343, L316A</td>
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** Sequences are shown 5’ → 3’, where the mutated nucleotides are underlined in bold, and the sequence of the antisense primer is inferred.
Supplemental Figure 1. Role of residues within the common alpha-helical 8 (α-H8) domain of TPα and TPβ in mediating their interaction with PRK1.

The S.c Y187(pACT2:PRK1<sup>640-942</sup>) prey strain was mated with S.c AH109 bait strains transformed with recombinant pGBKT7 plasmids encoding the listed wild type or mutated subfragments of TPα or TPβ. Diploids were selected on DDO media and interactants on QDO media due to the GAL4-dependent transcriptional activation of the HIS3 and ADE2 reporter gene owing to positive interaction between bait and prey proteins. Data are representative of at least three independent experiments (Data; n ≥ 3).
Supplemental Figure 2. Agonist-dependent interaction of PRK1 and RhoA with TPα and TPβ.

Panels A and B: HEK.TPα, HEK.TPβ, and HEK.β-Gal cells, transiently co-transfected with pCMVTag2b:PRK1 encoding Flag-tagged PRK1, were incubated with vehicle (-) or 1 μM U46619 (+) for 10 - 120 min prior to immunoprecipitation with anti-HA (101R) sera. Immunoprecipitates (IP) were resolved by SDS-PAGE and immunoblotted (IB) versus anti-Flag, anti-HA (3F10), anti-RhoA or anti-PRK1 sera. Aliquots of whole cell lysates (50 μg/lane) were also immunoblotted with anti-Flag, anti-RhoA or anti-PRK1 sera. The relative positions of the molecular size markers (kDa) are indicated.

Panels C – F: Quantitative analyses of data presented in Panels A and B and also from Figure 4A and 4B.
respectively. In all cases, the bar charts represent mean percentage changes (± S.E.M, n ≥ 3) in PRK1 (upper graphs) or RhoA (lower graphs) expression relative to TPα/TPβ expression in the anti-HA immunoprecipitates, where basal levels in the absence of U46619 are assigned a value of 100%. Data; n ≥ 3.

Supplemental Figure 3. Phosphorylation of the intracellular domains of TPα and TPβ by PRK1 in vitro.

Purified GST fusion proteins (15 µg/assay) containing either the intracellular loop (IC; GST.IC1, GST.IC2, GST.IC3), or the C-tail domains of TPα (GST.TPα312-343) and TPβ (GST.TPβ312-407) or, as controls, GST and histone H1 (10 µg) were subject to in vitro phosphorylation with PRK1 (200 ng) at 30 °C for 30 min. Phosphorylated proteins were resolved by SDS-PAGE and visualised by autoradiography (upper panel). Thereafter, membranes were either screened by immunoblotting (IB) using anti-GST sera or stained with Ponceau S, as indicated in the lower panels. The relative positions of the molecular size markers (kDa) are indicated. Data; n ≥ 3.
Supplemental Figure 4. 

(A) EA.hy926 cells

B) 1°.CoASMCs

(C) 1° HUVECs

(D) 1°.h.CoASMCs

(E)
Supplemental Figure 4. Interaction of PRK1 with TPα and TPβ and the effect of TP-activation on histone H3 Thr11 phosphorylation in 1° HUVECs and 1° hCoASMCs.

Panels A & B: EA.hy926 cells and 1° h.CoASMCs were incubated with 1 µM U46619 for 0 - 60 min prior to immunoprecipitation (IP) with anti-PRK1 antibody. Resulting anti-PRK1 immunoprecipitates were used as source of PRK1 to examine U46619-induced in vitro phosphorylation of histone H1 (10 µg; 30 °C for 30 min). Phosphorylated histone H1 was visualised by autoradiography while all immunoprecipitates (IP) were immunoblotted (IB) with anti-PRK1 antibody. The relative positions of the 30 kDa and 104 kDa molecular size markers are to the left of the panels. Panels C – E: 1° HUVECs (Panel C) and 1° hCoASMCs (Panel D) cells, pre-incubated with vehicle or 1 µM U46619 for 30 min. Thereafter, lysates (~500 µg/assay) from the 1° HUVECs, 1° hCoASMCs and, as controls, HEK.TPα or HEK.TPβ cells (Panel E) were subject to immunoprecipitation with affinity purified anti-TPα/TPβ (#168; 20 µg) or with an equivalent concentration of the pre-immune serum (IgG; 20 µg). Immunoprecipitates (IP) and/or aliquots of whole cell lysates (50 µg/lane) were resolved by SDS-PAGE and immunoblotted (IB) with anti-PRK1 or anti-HA (3F10) sera, as indicated. Panels F and G: Immunoblot analysis of histone (H)3 Thr11 (H3Thr11) phosphorylation in 1° HUVECs (Panel F) and 1° hCoASMCs (Panel G) stimulated with U41669 (1 µM; 0 – 60 min). As a control for H3Thr11 phosphorylation, cells were growth-arrested by treatment with colcemid (50 ng/ml) for 24 hr. Isolated histones were resolved by SDS-PAGE and immunoblotted (IB) with anti-phospho H3Thr11 (upper) or anti-histone H3 (lower) antibody followed by chemiluminescence detection. The bar charts represent mean percentage changes in H3Thr11 phosphorylation relative to histone H3 levels and are expressed in arbitrary units (± S.E.M, n ≥ 3) where basal levels in the absence of U46619 are assigned a value of 100 %. Data; n ≥ 3.
Supplemental Figure 5. Effect TP- activation on H3Thr11 phosphorylation in PC-3 and LNCaP cells. Panels A and B: Immunoblot analysis of H3Thr11 phosphorylation in PC-3 (Panel A) and LNCaP (Panels B) cells stimulated with U46619 (1 μM; 0 – 60 min) for 30 min with vehicle (0.01 % EtOH), 1 μM 17 phenyl trinor PGE₂ or 1 μM cicaprost, as indicated. Panels C and D: Immunoblot analysis of H3Thr11 phosphorylation in PC-3 (Panel C) and LNCaP (Panel D) cells transiently co-transfected with either pCMVTag2b (Ø) or pCMVTag2b: PRK1
FL (PRK1
FL) and stimulated 48 hr post-transfection with U46619 (1 μM; 30 min). In all cases, isolated histones were resolved by SDS-PAGE and immunoblotted (IB) with anti-phospho H3Thr11 (upper) or anti-histone H3 (lower) antibody followed by chemiluminescence detection. The bar charts represent mean percentage changes in H3Thr11 phosphorylation relative to histone H3 levels and are expressed in arbitrary units (± S.E.M, n = 3) where basal levels in vehicle-treated cells and in the absence of U46619 are assigned a value of 100 %. The asterisks (*) indicate that levels of H3Thr11 phosphorylation were significantly increased in response to U46619-stimulation, relative to vehicle-treated cells, where *, **, ***, **** signifies p ≥ 0.05, 0.01, 0.001, 0.0001, respectively. The insets in Panels C and D confirm over-expression of Flag-tagged full length PRK1, where the Ø symbol signifies the empty vector pCMVTag2b.
Supplemental Figure 6. U46619-induced nuclear translocation of GFP:PRK1 in PC-3 cells.

Panel A: Confocal image analysis of GFP:PRK1 nuclear translocation in PC-3 cells stimulated with U46619 (1 µM; 0 – 120 min). Images were captured at 63X magnification using Zeiss LSM imaging software, where the horizontal bars represent 10 µm. Data are representative of at least three independent experiments.