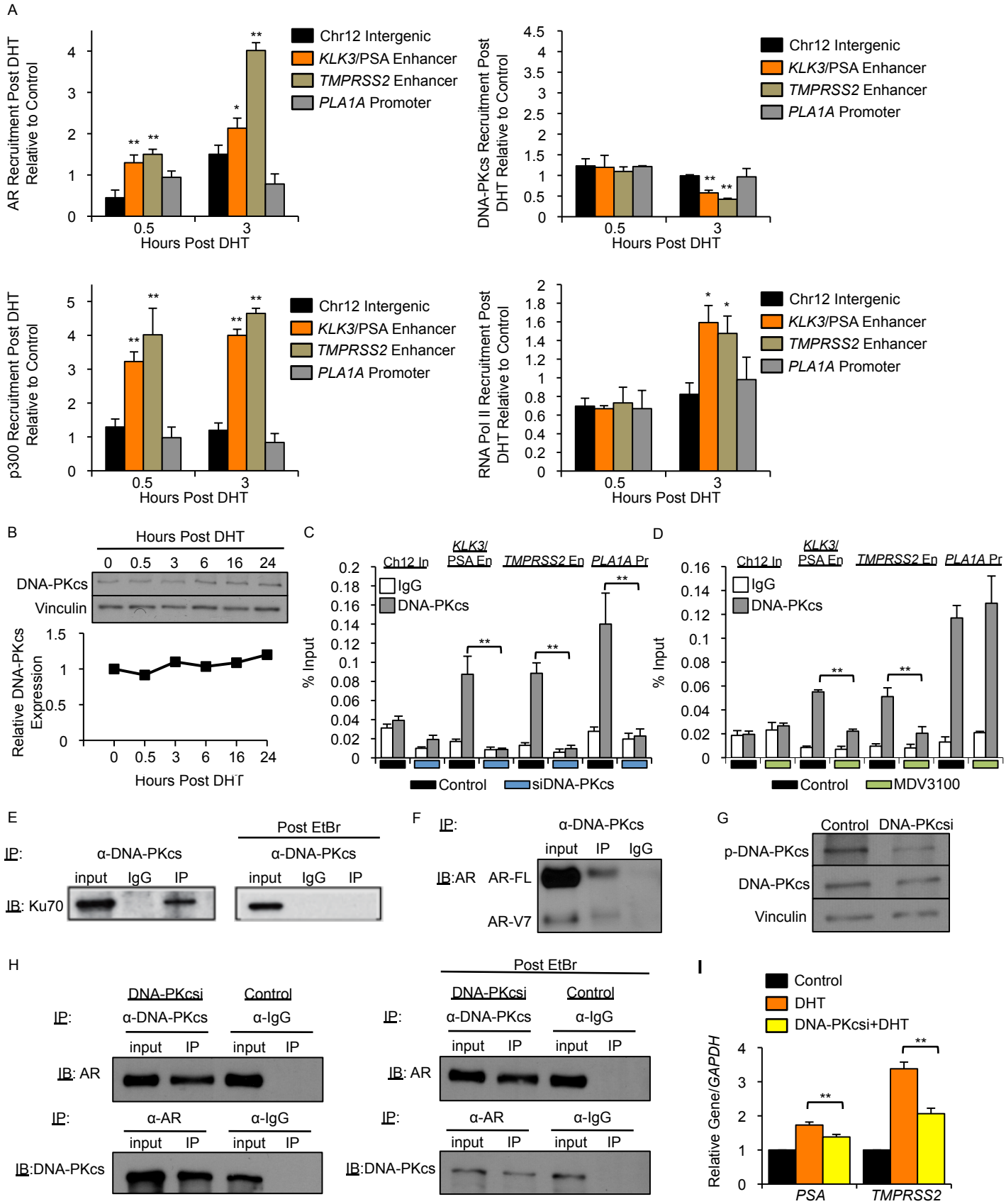

Figure S1, related to Figure 1: DNA-PKcs binds AR and is recruited to sites of AR action. (A) 0.5 and 3 hour timepoints from main text Figure 1C . **(B)** C4-2 cells treated with 10 nM DHT were harvested for immunoblot analysis of total DNA-PKcs at the indicated timepoints with quantification performed using ImageJ software. **(C,D)** C4-2 cells (C) depleted of DNA-PKcs or (D) treated with 10 μM MDV3100 for 24 hours were harvested for ChIP-qPCR analysis and percent (input) occupancy of DNA-PKcs reported. Black bar represents control, colored bar represents treatment. **(E)** C4-2 were harvested and co-immunoprecipitation performed in the presence or absence of 50ug/mL ethidium bromide **(F)** 22Rv1 cells were harvested for co-immunoprecipitation with immunoblot for full length AR (AR-FL) and an AR splice variant (AR-V7). **(G,H)** C4-2 cells were treated with 1μM NU7441 (DNA-Pkcsi) for 24 hours and harvested for (F) immunoblot analysis of phospho-S2056 and total DNA-PKcs or (G) co-immunoprecipitation performed in the absence or presence of 50ug/mL ethidium bromide. **(I)** C4-2 cells were pre-treated for 24 hours with 1 μM NU7441 then treated with 10nM DHT and relative expression of indicated transcripts analyzed and normalized to *GAPDH* mRNA. Data are reported as mean +/- SD. *p<0.05 **p<0.01 compared to control.

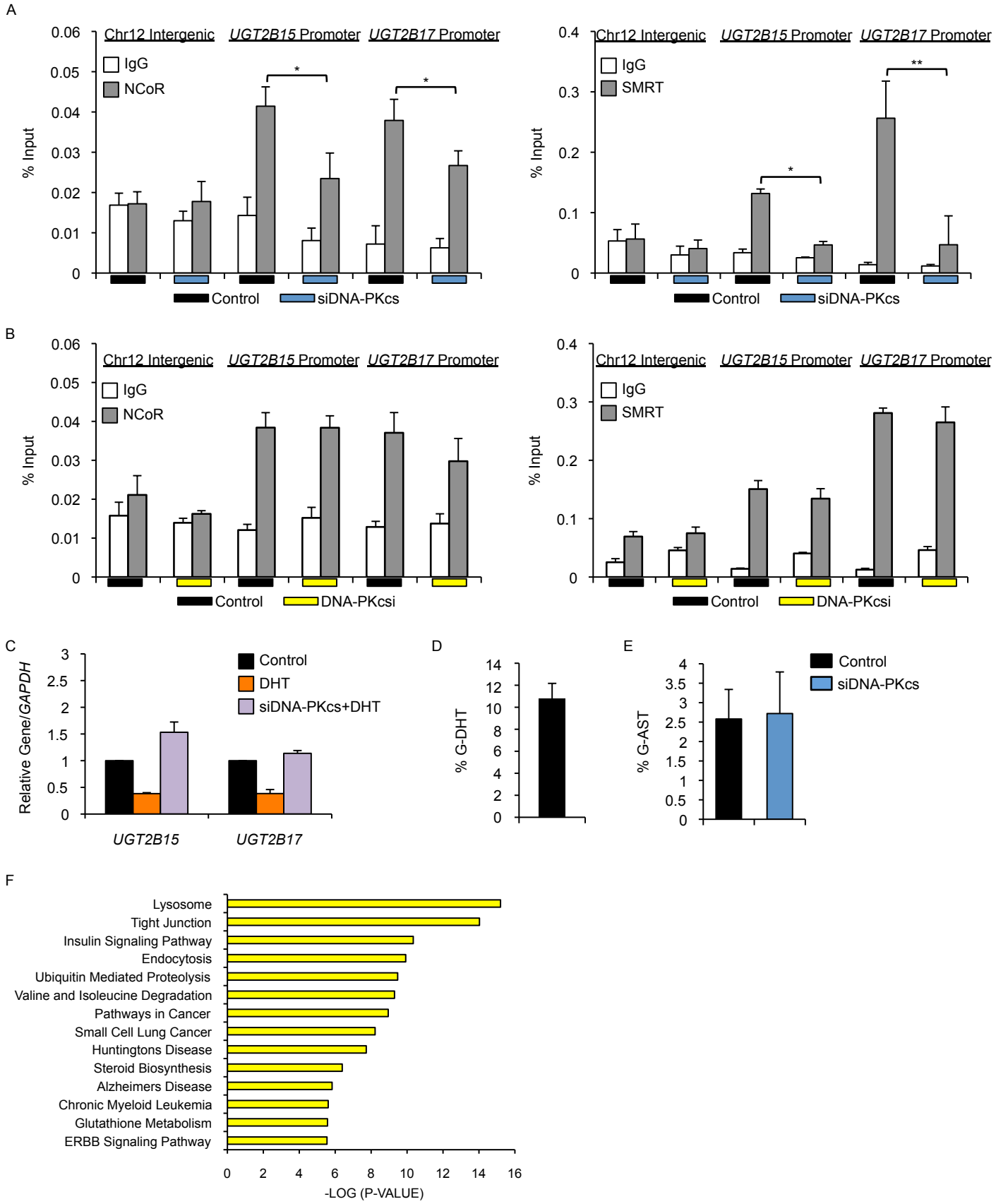

Figure S2, related to figure 3: DNA-PKcs inhibition negatively regulates gene expression networks. (A,B) C4-2 cells depleted of DNA-PKcs or treated for 24 hours with 1 μM NU7441 were harvested for ChIP-qPCR analysis and percent (input) occupancy of NCoR (left) or SMRT (right) reported. Black bar represents Control, blue bar represents siDNA-PKcs, yellow bar represents NU7441. **(C)** C4-2 cells depleted of DNA-PKcs and treated with 10 nM DHT were harvested for qPCR analysis with the indicated transcripts set relative to *GAPDH* mRNA. **(D)** G-DHT levels 24 hours after 100 nM DHT treatment in 22Rv1 cells were determined by HPLC. **(E)** G-androsterone (G-AST) levels in C4-2 cells depleted of DNA-PKcs were determined by HPLC. **(F)** GSEA KEGG pathway analysis of genes identified to be upregulated by ≥1.5 fold compared to control after DNA-PKcs inhibitor treatment.

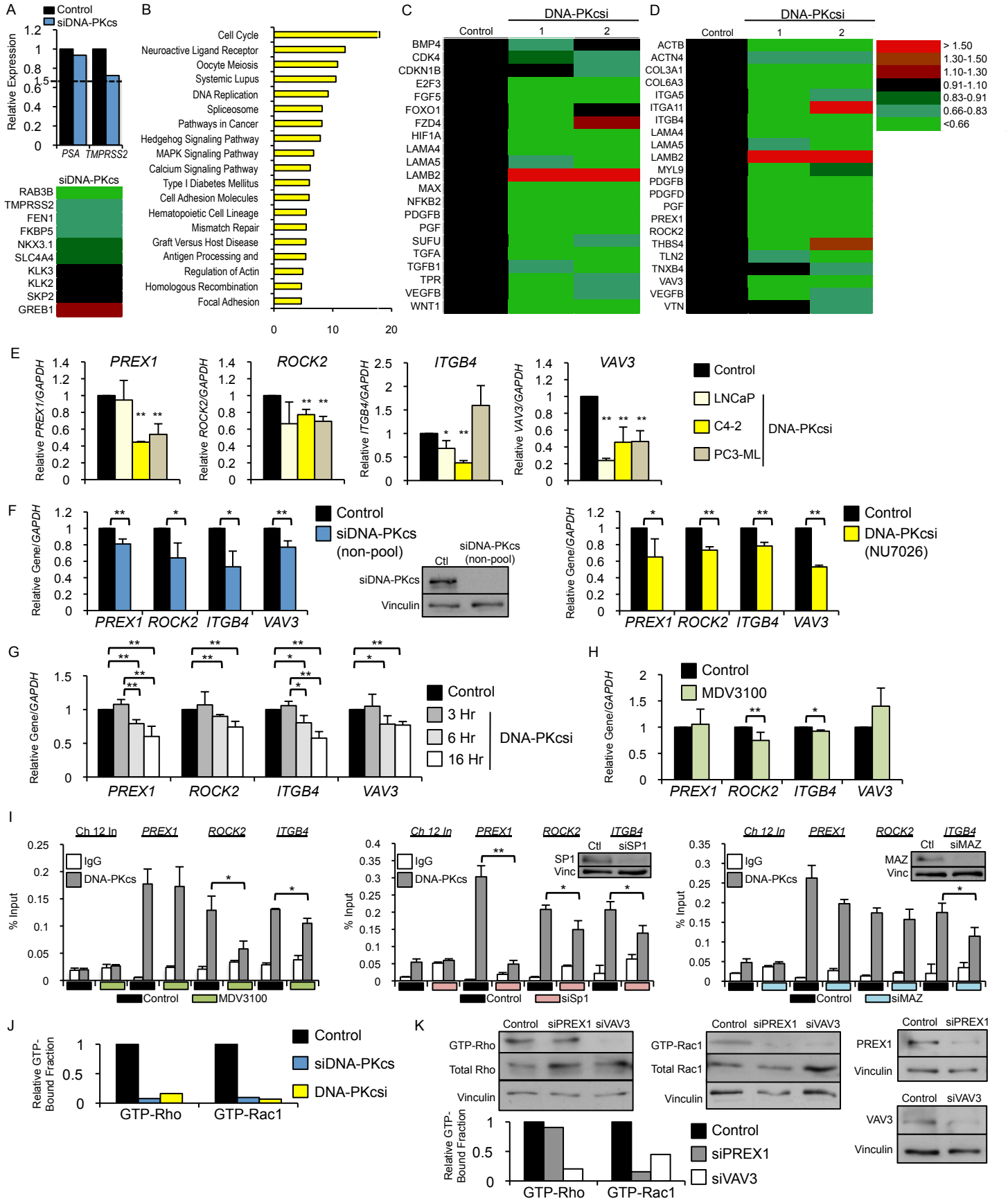

Figure S3, related to Figure 4: DNA-PKcs promotes pro-metastatic signaling. (A) Transcript expression of AR target genes from the microarray, 1.5 fold reduction for *PSA* and *TMPRRSS2* denoted. **(B)** GSEA KEGG pathway analysis of genes identified to be downregulated by ≥1.5 fold compared to control after DNA-PKcs inhibitor treatment. **(C,D)** Heat map of transcript change of pathways in cancer (C) or focal adhesion (D) pathway genes in the DNA-PKcs inhibitor treated groups. **(E)** CRPC or LNCaP cells in hormone deficient media treated with 1μM NU7441 or vehicle were subject to qPCR analysis with control data set to 1 for each cell line. **(F,G,H)** C4-2 cells (F) depleted of DNA-PKcs using a non-pool siRNA or treated with 1μM NU7026 for 24 hours, (G) treated with 1μM NU7441 for the indicated timepoints, or (H) treated with 10μM MDV3100 were harvested for qPCR analysis with indicated transcripts set relative to *GAPDH* mRNA. **(I)** C4-2 cells treated with 10μM MDV3100 or depleted of SP1 or MAZ were harvested for ChIP-qPCR analysis and percent (input) occupancy of DNA-PKcs reported. Black bar represents control, colored bar represents treatment. **(J)** Quantification of GTP-bound Rho and Rac1 in main text Figure 4G. **(K)** C4-2 cells depleted of PREX1 or VAV3 were analyzed for activated (GTP-bound) Rho and Rac1 by column binding followed by immunoblot. Data are reported as mean +/- SD. *p<0.05, **p<0.01 vs control.

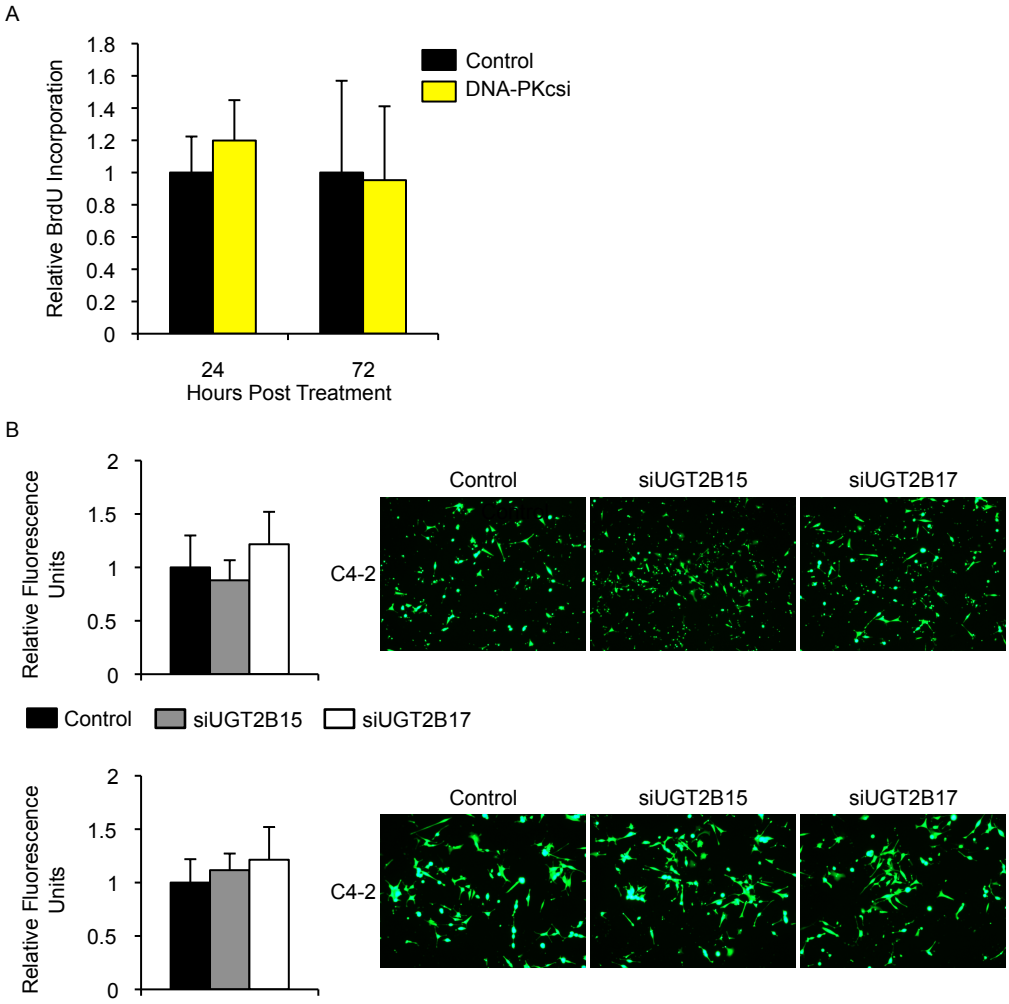

Figure S4, related to Figure 5: UGT2B15 and UGT2B17 do not directly alter metastatic phenotypes. (A) LNCaP cells seeded into hormone deficient media and treated with 1μM NU7441 for the indicated timepoints were subject to BrdU FACS analysis and set relative to control. **(B)** Cells depleted of UGT2B15 or UGT2B17 were seeded into hormone deficient media and allowed to migrate (top) for 24 hours or invade through matrigel (bottom) for 72 hours towards hormone proficient media.

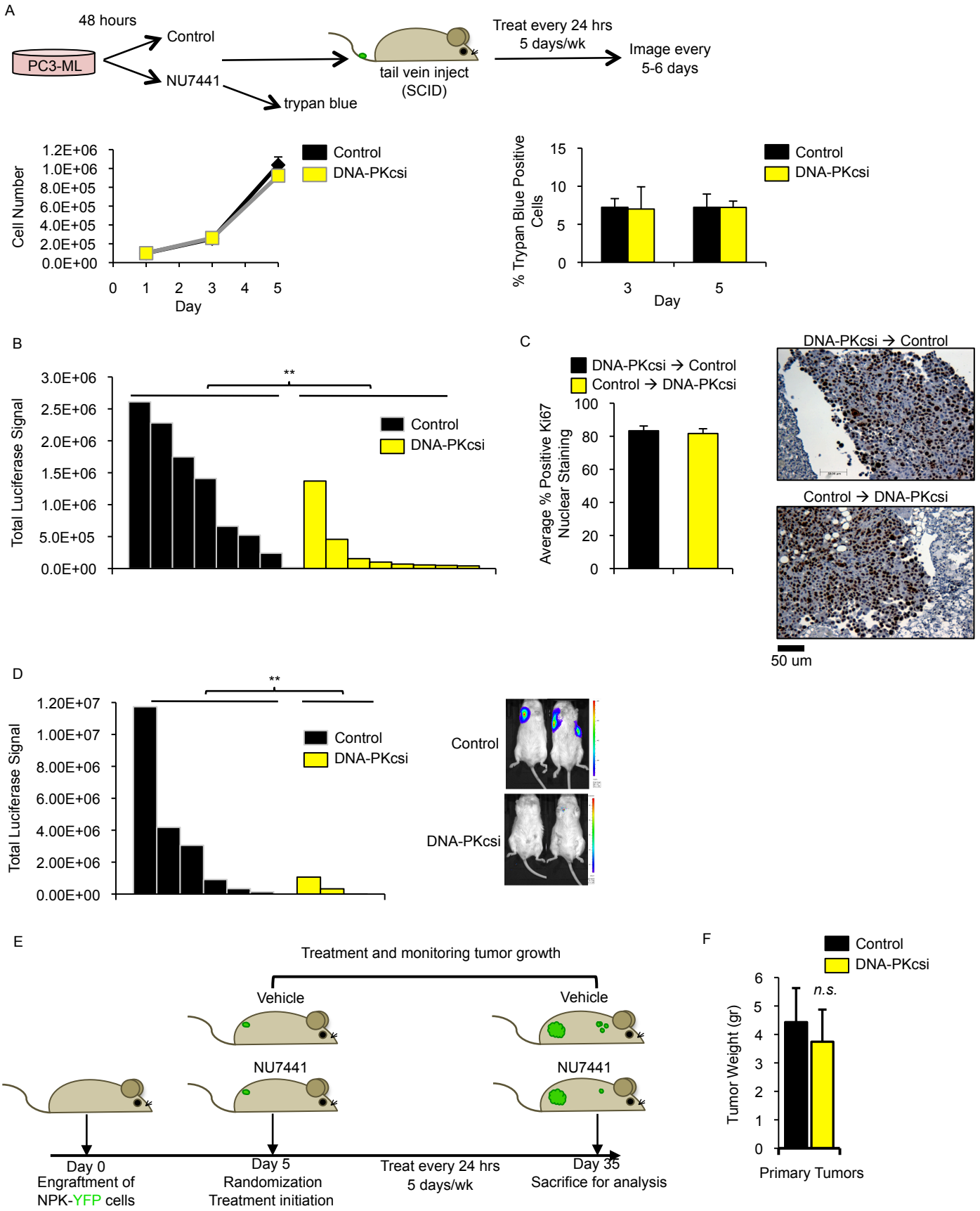

Figure S5, related to Figure 6: DNA-PKcs inhibitors delay formation of metastases *in vivo*. **(A)** Schematic of tail vein injection experimental design (top). Overall cell number (bottom left) and percentage of trypan blue positive cells (bottom right) were determined by trypan blue exclusion at the indicated time points for PC3-ML cells pretreated with 1μM NU7441 for 48 hours. **(B)** Mice not selected for crossover studies were injected with luciferin 38 days post tail vein injection of PC3-ML cells and imaged using the IVIS imaging system with total luciferase signal reported. **(C)** Lungs harvested from mice in the crossover studies were stained with Ki67 and metastatic lesions scored (left) with representative images shown (right). **(D)** Mice were injected with luciferin 45 days post tail vein injection of 22Rv1 cells and imaged using the IVIS imaging system with total luciferase signal reported (left) and representative images shown (right). **(E)** Schematic of *CASP-NPK-YFP* experimental design. **(F)** Primary subcutaneous tumors were harvested and weighed at time of sacrifice. **p<0.01.

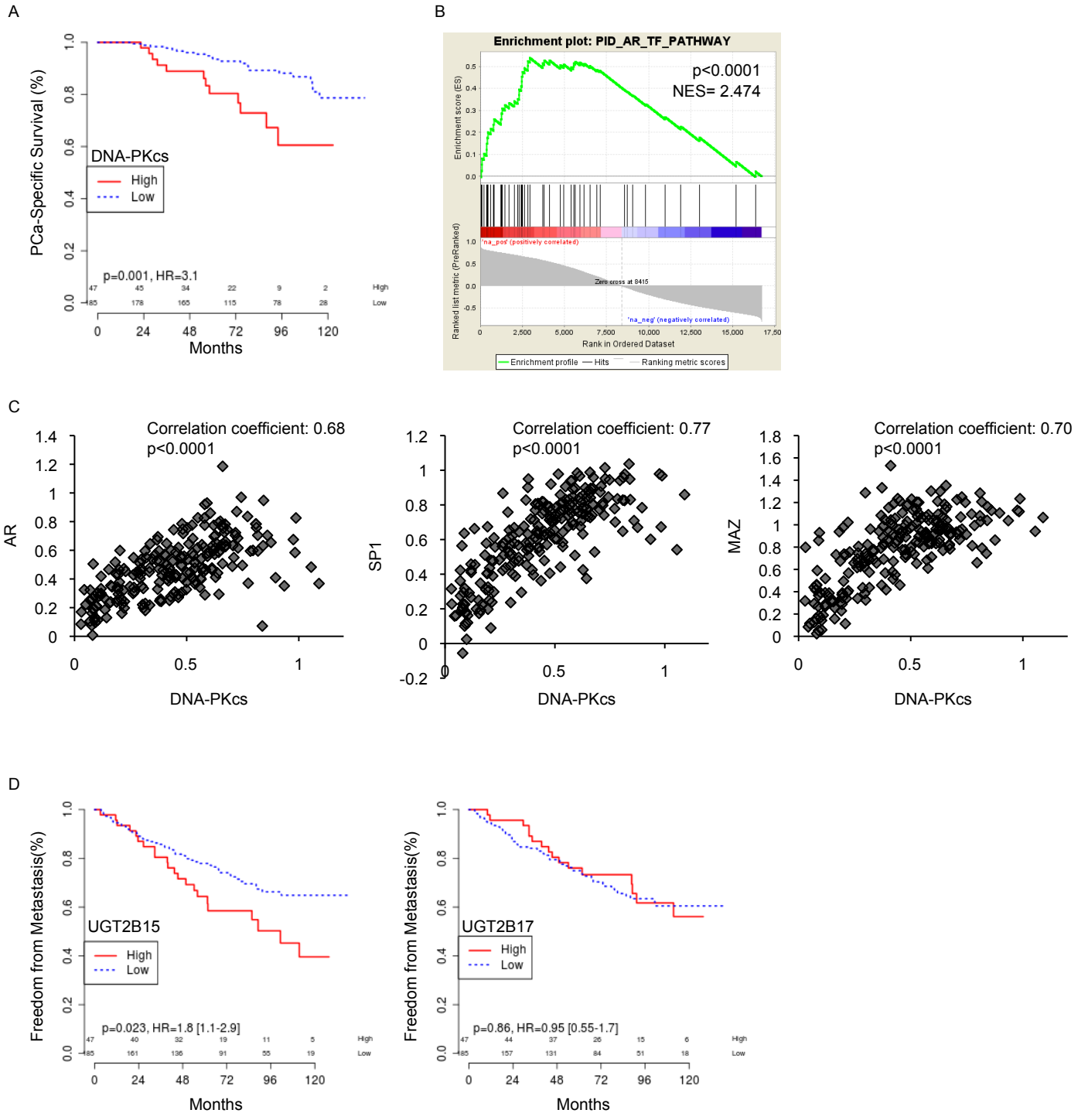

Figure S6, related to Figure 8: DNA-PKcs is associated clinically with disease recurrence and metastases. (A) Tumor samples were profiled for DNA-PKcs mRNA expression, split into high versus low by the 80th percentile of DNA-PKcs mRNA expression, and a Kaplan Meier curve generated. **(B)** GSEA analysis showed enrichment of the AR transcription factor pathway in genes correlated to DNA-PKcs in the tumor samples. **(C)** Tumor samples were profiled for mRNA expression of DNA-PKcs and AR (left), SP1 (middle), and MAZ (right) and correlation coefficients determined. **(D)** Tumor samples were profiled for UGT2B15 or UGT2B17 mRNA expression, which was split into high versus low by the 80th percentile of UGT2B15 (left) or UGT2B17 (right) mRNA expression for Kaplan Meier analysis.

Table S1, related to Figure 8. Provided as an excel file. List of pathways enriched in DNA-PKcs high tumors.

Table S2, related to Figure 8. Provided as an excel file. Mass spec peptide fragment data.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell Culture and Treatment

LNCaP and C4-2 cells were maintained in improved minimum essential media (IMEM) supplemented with 5% ΔFBS (heat-inactivated FBS). 22Rv1 and PC3-ML cells were maintained in Dulbecco's modified Eagle's media supplemented with 10% FBS. Cell lines used were not cultured longer than 6 months after receipt from the original source of American Type Culture Collection. All media were supplemented with 2 mmol/L of L-glutamine and 100 units/mL penicillin-streptomycin. For hormone deficient conditions, media used was phenol red-free media supplemented with 5 or 10% charcoal dextran-treated serum (CDT). DHT was obtained from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in ethanol. NU7441 and NU7026 were obtained from Selleckchem (Houston, TX, USA) and SLx-2119 was obtained from MedChem Express (Monmouth Junction, NJ, USA), and both were dissolved in DMSO. All experiments were seeded onto poly-L-lysine coated plates and allowed to adhere for 24 hours before initiation of treatments.

Chromatin Immunoprecipitation

Cells were seeded in hormone-proficient media and treated as specified. Cells were fixed with 1% formaldehyde at indicated timepoints and ChIP analyses conducted as previously described [Goodwin et al., 2013]. Genomic DNA was purified and quantitative PCR conducted for indicated loci using primers described. Negative control primers (Chr12 Intergenic) were designed to target the gene desert region chr12:60913317-60913384. Data were analyzed as percentage of input of total samples.

Co-immunoprecipitation

Cells seeded in hormone-proficient conditions were treated with 1μM NU7441 or 10nM DHT and harvested 6 hours post treatment. Cell pellets were lysed in NETN buffer with protease and phosphatase inhibitors added fresh, treated for 30 minutes on ice with 50ug/mL ethidium bromide or control, and clarified. 1000-2000ug of lysates were added to protein A (polycolonal, rabbit) or protein G (monoclonal, mouse) Dynabeads pre-incubated for 2 hours with 5ug of appropriate antibody and incubated overnight, with remaining lysate being diluted 1:1 with 2x SDS running buffer and retained as

input. Dynabeads were washed 3 times in NETN buffer, resuspended in 1:1 NETN: 2x SDS running buffer, boiled for 15 minutes, and analyzed by immunoblot analysis using antibodies described.

Immunoblotting

Cells were seeded in hormone-proficient or hormone-deficient conditions and treated as described. Cell lysates were generated and analyzed as previously described [Goodwin et al., 2013] using antibodies described.

Gene Expression

Cells were seeded in hormone-proficient media and treated as specified. RNA was isolated using TRIzol (Life Technologies #15596018) and quantitative PCR conducted as previously described [Goodwin et al., 2013] using primers described.

RNA Interference

Cells were seeded at a density of 1 x 10^5 in hormone-proficient conditions (complete media) for 24 hours. Cells were then transfected (6-8) hours in serum-free conditions with either control, *PRKDC, ATM, Sp1, MAZ, PREX1, VAV3, UGT2B15,* or *UGT2B17* siRNA pools (Thermo Scientific, D-001810-10-20, L-005084-00-005, L-003201-00-0010, L-026959000-0010, L-012588-00-0010, L-010063-01-0010, L-010178-00-0010, L-020194-02-005, or L-020195-00-005, respectively) or control or *PRKDC* non-pool siRNA (Life Technologies, AM4611 or AM51331, respectively) according to the manufacturer's specifications. Cells were maintained in complete media for an additional 96 hours then seeded in hormone-proficient or hormone-deficient conditions and harvested at indicated timepoints.

Microarray

mRNA was harvested from cells depleted of DNA-PKcs or treated with 1μM NU7441 or control for 24 hours (minimum of 2 biological replicates per condition) and cDNA generated using the Ovation Pico WTA-system V2 RNA amplification system (NuGen Technologies, Inc.). cDNA was fragmented and chemically labeled with biotin using the FL-Ovation cDNA biotin module (NuGen Technologies, Inc.). cDNA in 200 uL hybridization cocktail was hybridized on the Affymetrix Human Gene 2.0 ST Array (Affymetrix #902112) in a GeneChip Hybridization Oven 645. Chips were scanned on an Affymetrix Gene Chip Scanner 3000 and data processed using GeneSpring V11.5 software with an Interative

Plier16 summarization algorithm, quantile normalization, baseline transformation of median of all samples, and filtering to remove low expressing genes. A 1.5-fold differentially expressed gene list was generated. Gene lists were analyzed using Gene Set Enrichment Analysis (GSEA) [Mootha et al., 2003; Subramanian et al., 2005].

Rac and Rho Activity

Cells were seeded at a density of 5 x 10^5 in hormone-proficient media and treated as specified. Cells were lysed and assayed using Active Rho or Active Rac1 Detection Kits (Cell Signaling, #8820 or #8815, respectively) per manufacturer's instructions. Briefly, 600ug of lysate was incubated with GST-Rhotekin-RBD fusion protein or GST-PAK1-PBD fusion protein to bind GTP-bound Rho or Rac1, respectively, which were immunoprecipitated with glutathione resin. Additional lysates were used as control inputs to determine total Rho or Rac1 levels. Input and immunoprecipitated samples were subject to immunoblot analysis using anti-Rho or anti-Rac antibodies provided in kits.

DHT Measurement

Performed as previously described [Chang et al., 2013], with minor deviations. Briefly, 1 x 10^6 C4-2 cells were transfected with siControl or si*PRKDC*. After 96 hours of transfection, 1ml serum free RPMI1640 was changed and treated with [^{3}H]-labeled (500,000 – 1,000,000 cpm, PerkinElmer) and non-radiolabeled DHT (100nM). Media was collected at 3, 6, 12 or 24 hours, with the results of 24 hours shown. The non-glucuronidated DHT was extracted by isooctane, evaporated, and analyzed using HPLC. To eliminate the remaining non-glucuronidated DHT contamination, the media was extracted three times. Post extraction, the water phase, which contained glucuronidated DHT, was treated with β-glucuronidase (1,000 units; Sigma-Aldrich) at 65^{O}C for 4 hr followed by extraction, as described previously.

Migration and Invasion

2.5 x 10^4 cells depleted of DNA-PKcs or pre-treated with 1μM NU7441 or SLx-2119 for 24 hours were seeded into 0.5 mL serum-free media, added to the wells of Fluoroblok multiwell migration plates (Corning #351157) or Fluoroblok biocoat tumor invasion plates (Corning #354165) plates, and allowed to migrate or invade through a matrix towards 0.75 mL serum-containing media for 24 or 72 hours,

respectively. At the indicated timepoints, cell inserts were transferred to a 24 well plate containing 1 mL of 4ug/mL Calcein AM (Corning #354217) per well and incubated for 1 hour at 37° C. Fluorescent signal was determined using a Biotek Synergy HT plate reader and images obtained on a Leica DFC310 FX fluorescent microscope.

Flow Cytometry

5 x 10^5 cells were seeded into serum-free media for 24 hours prior to treatment with 1μM NU7441. Cells were labeled with BrdUrd (1:1,000, GE Healthcare Life Sciences #RPN201) 1 hour prior to harvest, then fixed, stained, and processed as previously described [Goodwin et al., 2013].

Immunohistochemistry

Tissues for immunohistochemical analysis were fixed and formalin and embedded into paraffin blocks. All immunohistochemical staining was performed by the Thomas Jefferson University Histology/Immunohistochemistry core facility, with Ki67 scored by pathologist. Briefly, tissue sections were deparaffinized in xylene and rehydrated through an ethanol gradient. For histological analysis, sections were stained with hematoxylin and eosin (H&E) using standard techniques. For analysis of Ki67, sections were initially boiled in Antigen Retrieval Solution (Dako North America, Inc.) using a microwave (5 minutes at 100% power followed by 20 minutes at 30% power). Sections were then cooled to room temperature, and endogenous peroxidase activity was quenched by treatment in 0.3% H_2O_2 in methanol (Dako North America, Inc.). Samples were blocked for 30 minutes in 5% goat serum in PBS. Primary Ki67 (MIB-1, Dako North America, Inc.) antibody was diluted 1:250 in blocking buffer and incubated for 1 hour at room temperature. Following washing with PBS, biotinylated secondary antibody and streptavidin-peroxidase conjugate were applied according to kit instructions (Vector Laboratories) for 1-2 minutes and quenched with H_2O. Slides were counterstained with hematoxylin, dehydrated through graded ethanols and xylene, and mounted using permount (Surgipath Medical Industries, Inc.).

CASP-NPK-YFP In Vivo Metastasis Model

For *in vivo* studies, 3x10^6 *CASP-NPK-YFP* cells [Aytes et al., 2013] were mixed with Matrigel (1:1 v/v) and injected into the right flank of immunodeficient NCr *nude* mice (Taconic). Five days after engraftement, mice were randomized to be enrolled into vehicle or NU7441 treatment (25 mg/kg). Briefly,

vehicle or NU-7441 was administered intraperitoneally five days a week (Monday to Friday); tumors were measured using calipers twice a week and volumes calculated using the formula [Volume = $(width)^2$ x length/2]. Tumor growth and body weights were monitored for 30 days after initiation of treatment. At the time of sacrifice, subcutaneous tumors were harvested and weighted, and metastases were documented in the lungs and livers by visualizing fluorescence using an Olympus SZX16 microscope equipped with epi-fluorescence capabilities. The total number of metastatic nodules for the lungs and livers of each mouse was assessed and the *p*-value of the difference calculated using the Mann-Whitney U test. RNA from was extracted from the subcutaneous primary tumors using the MagMAX Total RNA Isolation Kit (Life Technologies, #AM1830) per manufacturer's instructions.

Human Prostate Tumor Explants

Human prostate *ex vivo* explant cultures were conducted as previously described [Centenera et al., 2013]. Briefly, fresh primary tumor tissue was obtained from a clinical pathologist immediately following radical prostatectomy at Thomas Jefferson University Hospital in accordance with Institutional Review Board standards and in compliance with federal regulations governing research on deidentified specimens and/or clinical data (45 CFR 46.102(f)). The deidentified specimen was processed under laminar flow hood using sterile technique and transported in culture media (IMEM, 5% FBS, 0.01 mg/mL insulin (Invitrogen #12585-014), 30 umol/L hydrocortisone (Sigma #H-0888), and penicillin/streptomycin) on ice. Tissue was subdivided into approximately $1mm^3$ pieces and placed in a 24-well plate on presoaked dental sponges (Novartis #96002) (2-3 pieces per sponge) placed into 0.5 mL culture media in presence or absence of drug (control or 1μM NU7441). Plates were placed in an incubator at 37°C and 5% CO_2. Media were replaced every 48 hours with appropriate treatment, and explants were harvested on day 6. Tissue was either formalin fixed for immunohistochemical analysis or placed in 1 mL RNAlater (Ambion #AM7020) (kept at 4°C for 24 hours, then stored at -80°C until processing) for RNA analysis. RNA extraction was performed using Trizol (Life Technologies #15596018) per manufacturer's instructions.

Antibodies

Antibodies used for ChIP experiments were as follows: AR (N-20, directed against amino acids 1-20 by Bethyl Laboratories), DNA-PKcs (Thermo Scientific, #423-PABX), p300 (Active Motif, #61401), RNA Polymerase II (Santa Cruz Biotechnology, #SC-899X), NCoR (Abcam, #24552), and SMRT (Abcam, #24551). Antibodies used for immunoblot were as follows: DNA-PKcs (phospho S2056) (Abcam, #18192), DNA-PKcs (Thermo Scientific, #423-PABX), Lamin B (Santa Cruz, #6217), AR (N-20, directed against amino acids 1-20 by Bethyl Laboratories), Vinculin (Sigma-Aldrich, #V9264-200UL), UGT2B15 (Abcam, #83468), UGT2B17 (Abcam, #92610), PREX1 (EMD Millipore, #MABC178), ROCK2 (Abcam, #71598), ITGB4 (Abcam, #29042), VAV3 (EMD Millipore, #07-464), Ku70 (Thermo Scientific, #329-P0), ATM (Cell Signaling, #2873), Sp1 (Abcam, #77441), and MAZ (Abcam, #85725).

Statisical Analysis

All pooled data are represented as mean +/- standard deviation. P-values were calculated using Student's t test.

Primers

ChIP Primers	**Forward**	**Reverse**
KLK3/*PSA* Enhancer	TGGGACAACTTGCAAACCTG	CCAGAGTAGGTCTGTTTTCAATCCA
TMPRSS2 Enhancer V	TGGTCCTGGATGATAAAAAAAGTTT	GACATACGCCCCACAACAGA
Chr12 Intergenic	GGGATGATGTGTGGGTTTTACC	CAATATCCAGCGAAAAGGAAGCT
UGT2B15 Promoter	TCATGACCCCTCTGAACAAGC	CTCTGGAAGCTGTGGAAAGGT
UGT2B17 Promoter	TGAGCTGCATCAGCAGAAAGA	AAGCACTGCATAAGACCAGGA
PREX1 Promoter [Wong et al., 2011]	GGCACTTGGCAGACGGTCCCTGGCG	TCAGCGGCGGGCCGGGCTCCC
ROCK2 Promoter	AATGGTGGCCTGTGGATGAG	TCTGCTACTGTCGCGCTATG
ITGB4 Promoter [Drake et al., 2010]	CTGCAGCCCCATCTCCTA	CCCGTCCTGGACCTACCT
PLA1A Promoter [Brenner et al., 2011]	TGGCCACCCAGAGATGCAGGA	ACACACTGTCCCTCTTTGAGCCA
mRNA Primers	**Forward**	**Reverse**
KLK3/*PSA*	TGTGTGCTGGACGCTGGA	CACTGCCCCATGACGTGAT
TMPRSS2	GGACAGTGTGCACCTCAAAGAC	TCCCACGAGGAAGGTCCC
GAPDH	AACAGCCTCAAGATCATCAG	CATGAGTCCTTCCACGATAC

UGT2B15	GTGTTGGGAATATTATGACTACAGTAAC	GGGTATGTTAAATAGTTCAGCCAGT
UGT2B17	TTTTGTCGCAGGAAAAAGGAAA	AAGCCTGAAGTGGAATGACCAA
PREX1[Wong et al., 2011]	CCTTCTTCCTCTTCGACAAC	GTGGAGAATGTGGAAGATGG
ROCK2	TCCCCCATCAACGTGGAGAGCT	TGCCTTGTGACGAACCAACTGCA
ITGB4	AGGGTCAGTTCTGCGAGTATG	GTCCATTACAGATGCCCCCAT
VAV3	AGCTAAGGTTGCTAACCAAACA	TCCATTGGTCCGTTTCTCTGG
PLA1A	CTGAGATTTCCAGCTCAGCGA	CACCCAGCACCAGGAGTTTAT
Gapdh	AATGAGAGAGGCCCAGCTACT	CCAATACGGCCAAATCCGTTC
Prex1	TACGACGACGGCACTTACAAA	CAGTAGAGTCTCACGCCCTTG
Rock2	GTCTGCTGGATGGCTTAAATTCC	TTGGCCCACTTTTCAGGTACA
Itgb4	GCGTTTCTGATGACACTGAGC	CACGGGAATCTTGGGAGGAAAG
Vav3	AGTCGGAAGCCAGTGCTCA	TCCACGCGGGTTTCATCTATT

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