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DNA-PKcs-Mediated Transcriptional Regulation Drives Prostate Cancer Progression and Metastasis.

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DNA-PKcs mediated transcriptional regulation drives prostate cancer progression and metastasis

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SUMMARY

Emerging evidence demonstrates that the DNA repair kinase DNA-PKcs exerts divergent roles in transcriptional regulation of unsolved consequence. Here, *in vitro* and *in vivo* interrogation demonstrate that DNA-PKcs functions as a selective modulator of transcriptional networks that induce cell migration, invasion, and metastasis. Accordingly, suppression of DNA-PKcs inhibits tumor metastases. Clinical assessment revealed that DNA-PKcs is significantly elevated in advanced disease, and independently predicts for metastases, recurrence, and reduced overall survival. Further investigation demonstrated that DNA-PKcs in advanced tumors is highly activated, independent of DNA damage indicators. Combined, these findings reveal unexpected DNA-PKcs functions, identify DNA-PKcs as a potent driver of tumor progression and metastases, and nominate DNA-PKcs as a therapeutic target for advanced malignancies.

INTRODUCTION

The DNA-dependent protein kinase (DNA-PK) is a serine/threonine protein kinase complex composed of a Ku heterodimer (Ku70/Ku80) and a catalytic subunit (DNA-PKcs) that plays an important role in the DNA damage response (DDR) and maintenance of genomic stability. In this context, DNA-PK primarily mediates ligation of DNA double-strand breaks (DSBs) through nonhomologous end joining (NHEJ), wherein the Ku heterodimer recognizes and binds broken DNA ends, facilitating recruitment and activation of DNA-PKcs (Yoo and Dynan, 1999). Activated DNA-PKcs phosphorylates and alters the function of factors that mediate NHEJ, including DNA-PKcs itself and histone H2AX (γH2AX) (An et al., 2010; Chan et al., 2002). While mechanisms governing DNA-PKcs activity are incompletely defined, it is clear that DNA-PKcs activation is critical for DNA DSB repair (Kurimasa et al., 1999; Zhao et al., 2006).

DNA-PKcs expression has been shown to correlate with decreased therapeutic response to DNA-damaging agents in multiple cancers, implicating DNA-PKcs-mediated DNA repair as a mechanism for tumor cell survival (Beskow et al., 2009; Bouchaert et al., 2012). However, DNA-PKcs has also been linked to poor prognosis in the absence of DNA damaging therapies (Evert et al., 2013; Willmore et al., 2008), suggesting a DDR-independent role for

DNA-PKcs in human malignancies. Studies further identified DNA-PKcs as a modulator of cancer-associated pathways distinct from DNA repair, including hypoxia, metabolism, inflammatory response, and transcriptional regulation (Goodwin and Knudsen, 2014). Notably, DNA-PKcs was originally discovered and characterized as part of Sp1 transcriptional complexes (Jackson et al., 1990) and as a regulatory component of transcriptionally poised RNA polymerase II (RNAPII) (Dvir et al., 1992); accordingly, recent studies revealed that DNA-PKcs is recruited to active sites of transcription (Ju et al., 2006). DNA-PKcs can interact with the basal transcriptional machinery (Maldonado et al., 1996) and both binds and modulates the function of multiple sequence specific transcription factors (e.g. AIRE, p53, and ERG) as well as select nuclear receptors (including the glucocorticoid (GR), progesterone (PR), estrogen (ER), and androgen receptors (AR)) (Goodwin and Knudsen, 2014). Recently, a critical link was identified between AR signaling and DNA-PKcs that underlies the capacity of this steroid hormone receptor to promote DSB repair (Goodwin et al., 2013; Polkinghorn et al., 2013). Briefly, it was shown that AR binds to the regulatory locus of *PRKDC* (the gene encoding DNA-PKcs) in response to androgen stimulation and DNA damage, thereby inducing *PRKDC* expression and subsequent DNA-PKcs activity. This induction proved essential for AR-mediated DSB repair and cell survival in the presence of genomic insult, and elevated levels of DNA-PKcs were shown to create a positive feedback loop by virtue of the established ability of DNA-PKcs to serve as an AR comodulator. These findings provided the mechanistic basis for clinical observations demonstrating that suppression of AR activity enhances the response to radiotherapy (Al-Ubaidi et al., 2013; Warde et al., 2011), concordant with reports showing that AR suppression dampens expression of repair factors in prostatic adenocarcinoma (PCa) (Al-Ubaidi et al., 2013; Warde et al., 2011), and illustrated the significance of AR-DNA-PKcs interplay in PCa. Given the potential implications of DNA-PKcs-mediated transcriptional activity in human malignancies, it was imperative to discern the molecular basis of DNA-PKcs function and the contribution of DNA-PKcs-mediated transcriptional regulation on tumor phenotypes.

RESULTS

DNA-PKcs interacts with AR and is recruited to sites of AR action

Since DNA-PKcs is induced by AR activity and functions as an AR coactivator in advanced PCa that can bypass anti-androgen therapy (castration-resistant PCa, CRPC), CRPC models were selected to interrogate DNA-PKcs-mediated transcriptional regulation. PCa is dependent on AR activity for growth and progression, and therapies that suppress AR activity through ligand deprivation are the first line of intervention for metastatic disease. While effective, tumors ultimately recur, almost invariably through restoration of AR activity (Knudsen and Scher, 2009). Thus, discerning the impact of DNA-PKcs on AR function in CRPC is of translational relevance. Consistent with identification of *PRKDC* and *XRCC6* as androgen-regulated genes in CRPC (Al-Ubaidi et al., 2013; Goodwin et al., 2013), hormone deprivation decreased DNA-PKcs S2056 phosphorylation (indicative of decreased activity (Chen et al., 2005)) along with total DNA-PKcs and Ku70 levels (Fig 1A). As such, studies assessing the function of DNA-PKcs as a transcriptional regulator were performed in hormone-proficient conditions. Loci explored initially focused on gene

regulatory elements governed by AR and ERG in PCa cells, as DNA-PKcs was implicated as a modulator of both factors. As predicted, chromatin immunoprecipitation (ChIP) analysis revealed AR occupancy at two well-characterized loci (*KLK3*/PSA and *TMPRSS2* enhancers), but not at the promoter of the ERG-regulated gene *PLA1A* (Fig 1B, left). DNA-PKcs was detected at all three regions (Fig 1B, right), but not in the control region, showing specificity of DNA-PKcs binding (Brenner et al., 2011). In response to DHT, AR was recruited to each AR regulatory site within 30 min, with maximum occupancy at 16 hrs post-treatment (Fig 1C, top left, Fig S1A). In contrast, DNA-PKcs recruitment was delayed (6 hrs post-treatment) at AR regulatory regions with maximum occupancy at 16 hrs (Fig 1C, top right, Fig S1A), and unchanged at *PLA1A*, demonstrating specificity of the DNA-PKcs response to hormone stimulation (Fig 1C, top). The AR coregulator p300 was enriched 30 min post-DHT, followed by RNAPII binding (3-6 hrs) at the AR regulatory loci, while neither was enriched at the *PLA1A* promoter in response to DHT (Fig 1C, bottom, Fig S1A), suggesting that DNA-PKcs binding facilitates coactivator function and potentiates transcriptional activation. DNA-PKcs levels were not significantly enriched after DHT treatment at these early timepoints (Fig S1B). Notably, DNA-PKcs detection was abrogated at all 3 loci by siRNA-mediated depletion (Fig S1C), but was specifically undetected at AR-regulated loci after treatment with the AR antagonist MDV3100 (Fig S1D). Combined, these findings suggest that DNA-PKcs is recruited to sites of AR function in response to AR and initiating p300 occupancy, facilitating active transcription. The impact of DNA-PKcs recruitment was determined in parallel. *PLA1A* was not induced in response to DHT, and while significant induction of both *KLK3*/PSA and *TMPRSS2* was observed 3 hrs post-DHT (Fig 1D), maximum induction was not observed until after peak recruitment of AR and DNA-PKcs. Further analyses revealed that AR and DNA-PKcs are found in complex, and that the interaction is not further enriched by exogenous DHT (Fig 1E). The AR-DNA-PKcs interaction is not dependent on DNA binding, as pre-addition of ethidium bromide did not disrupt the complex (Fig 1F), but did result in dismissal of Ku70, as expected (Brenner et al., 2011) (Fig S1E). Further, coimmunoprecipitation in 22Rv1 cells (which contain full length AR and an AR splice variant, AR-V7, lacking the ligand binding domain (LBD) (Guo et al., 2009)) revealed DNA-PKcs interaction with AR-V7 (Fig S1F), suggesting that DNA-PKcs can bind AR-V7 containing complexes; by contrast, *in vitro* interaction between Ku70 and AR was mapped to the AR LBD (Mayeur et al., 2005). Finally, DNA-PKcs activity was not required for AR interaction but is important for AR function, as a highly selective DNA-PKcs inhibitor, NU7441 (Zhao et al., 2006) (Fig S1G,H) did not suppress complex formation but decreased DHT-stimulated AR activity (Fig S1I). In sum, these findings reveal that DNA-PKcs is found in complex with AR and facilitates AR-dependent transcriptional transactivation.

DNA-PKcs is a selective effector of transcriptional networks

Given the impact of DNA-PKcs on AR, subsequent studies were directed at identifying the totality of DNA-PKcs mediated transcriptional networks. Initial gene expression analyses were performed in CRPC cells either depleted of DNA-PKcs or treated with NU7441 (Fig 2A, left); as shown, the si*PRKDC* pool suppressed DNA-PKcs expression, whereas NU7441 had no effect on DNA-PKcs levels, and neither impacted Ku70 expression (Fig 2A, right). Genes up- or downregulated by >1.5 fold were selected for further analysis (Fig 2B). For

both manipulations, the number of genes downregulated far exceeded those that were upregulated, suggesting that DNA-PKcs primarily positively regulates transcriptional events. Comparison between groups demonstrated that DNA-PKcs depletion results in overlapping but distinct effects as compared to enzymatic inhibition. To minimize potential off-target effects of NU7441, subsequent analyses primarily focused on transcriptional alterations induced by DNA-PKcs knockdown. Gene Set Enrichment Analysis (GSEA) and associated motif analysis revealed significant enrichment of genes regulated by MAZ, MYC and the known DNA-PKcs-interacting partner Sp1, validating the concept that DNA-PKcs modulates a select subset of transcriptional networks (Fig 2C). Gene ontology (GO) analysis demonstrated that genes sensitive to DNA-PKcs associate with distinct biological processes including transcription and regulation of gene expression, further supporting a role for DNA-PKcs in gene regulation (Fig 2D). Combined, these findings begin to define the cellular consequence of DNA-PKcs mediated transcriptional regulation, and demonstrate that DNA-PKcs selectively governs transcriptional networks.

DNA-PKcs and AR cooperate to suppress UGT enzyme expression in CRPC

Numerous metabolic and hormone pathways of potential clinical impact in PCa were upregulated by DNA-PKcs depletion (Fig 3A), including steroid hormone biosynthesis, which exhibited upregulation of UGT glycosyltransferases (Fig 3B). UGT enzymes catalyze transfer of glucuronic acid to small molecules (including androgens), facilitating metabolism and excretion (Rowland et al., 2013). In the prostate, local androgen inactivation occurs when DHT is directly modified by glucuronidation or is metabolized to 5α-androstane-3α-diol (3α-diol) and androsterone (AST), which are then glucuronidated by UGT2B15 and UGT2B17. Consistent with previous reports suggesting that these genes are also AR regulated (Bao et al., 2008), AR occupied the proximal promoters of both *UGT2B15* and *2B17*, with a modest but significant increase observed upon DNA-PKcs depletion (Fig 3C, D left). DNA-PKcs co-occupied these sites (Fig 3D, right), suggesting that negative regulation by DNA-PKcs is direct. DNA-PKcs depletion resulted in increased *UGT2B15* and *2B17* expression, underscoring the impact of DNA-PKcs on this pathway (Fig 3E). Previous studies showed that DNA-PKcs negative transcriptional regulation can be mediated through NCoR and SMRT (Jeyakumar et al., 2007; Yu et al., 2006), and both were both enriched at the *UGT2B15* and *2B17* promoters. Corepressor binding was significantly reduced by DNA-PKcs depletion (Fig S2A) but not after kinase inhibition (Fig S2B), suggesting that DNA-PKcs occupancy (but not activity) is needed for NCoR and SMRT residence. As expected, DHT stimulation decreased *UGT2B15* and *2B17*, which was partially reversed by DNA-PKcs depletion (Fig S2C), consistent with a role for DNA-PKcs in negative regulation. As UGT2B15 and 2B17 protein accumulation was also enhanced after DNA-PKcs depletion (Fig 3F), the impact of DNA-PKcs depletion on DHT metabolites was quantified by HPLC (Fig S2D). Cells depleted of DNA-PKcs trended towards decreased overall levels of free DHT, but did not reach statistical significance (Fig 3G, left) and there was no impact on G-DHT (Fig 3G, right) or G-AST (Fig S2E), suggesting that elevated UGT2B15 and 2B17 is not sufficient to independently alter hormone metabolism. Similar regulation of other UGT enzymes after DNA-PKcs depletion (Fig 3B) argues against functional redundancy impacting DHT levels. The overall findings are of translational significance, as UGT2B15 and 2B17 are being developed as prognostic

markers and therapeutic targets in PCa (Grosse et al., 2013), and the mechanisms of regulation are not well understood. To assess clinical relevance, a cohort of 232 patients with high-risk localized PCa was examined, wherein it was observed that both UGT2B15 (correlation coefficient −0.28, $p<0.0001$) and UGT2B17 (correlation coefficient −0.38, $p<0.0001$) expression strongly negatively correlated with DNA-PKcs (Fig 3H), supporting the concept that DNA-PKcs suppresses expression of UGT enzymes in human tumors. Further analysis in response to NU7441 confirmed the function of DNA-PKcs as a selective negative regulator of transcription (Fig S2F). On balance, these findings identify gene networks that are negatively regulated by DNA-PKcs, and identify DNA-PKcs as a key modulator of the UGT enzyme cancer-associated pathway.

DNA-PKcs promotes pro-metastatic signaling

Whereas DNA-PKcs negatively regulates steroid regulated pathways, the majority of DNA-PKcs mediated transcriptional effects support coactivator functions. *KLK3*/PSA, *TMPRSS2*, and other well-characterized PCa-relevant AR-regulated genes (Goodwin et al., 2013; Mayeur et al., 2005) were generally reduced after DNA-PKcs depletion (Fig S3A), as expected. Analysis of genes downregulated after DNA-PKcs depletion (Fig 4A) or NU7441 (Fig S3B) revealed enrichment in pathways associated with cancer progression (Fig 4B, Fig S3C), prominently associated with cell migration and invasion. The focal adhesion gene signature was markedly suppressed by DNA-PKcs depletion (Fig 4C) or NU7441 (Fig S3D). Factors in the focal adhesion signature have previously been implicated in PCa progression and metastasis, including PREX1 (GEF for Rac1) (Qin et al., 2009), ROCK2 (effector of Rho signaling) (Kroiss et al., 2014), Integrin β4 (ITGB4, which regulates matrix organization through the Rac1 pathway) (Yoshioka et al., 2013), and VAV3 (GEF for Rho and Rac1) (Lyons and Burnstein, 2006). Expression of each was significantly reduced in hormone-therapy (HT)-sensitive cells (LNCaP), CRPC cells with limited metastatic potential (C4-2), and AR-negative CRPC cells with high metastatic potential (PC3-ML) after DNA-PKcs depletion (Fig 4D) or 24hr inhibitor treatment (Fig S3E), with the exception of *ITGB4* in PC3-ML cells, which was not significantly altered, suggesting a possible role for AR in regulation. As expected, ATM levels were diminished after DNA-PKcs depletion but not after DNA-PKcs inhibition (Goodwin et al., 2013; Peng et al., 2005) (Fig 4E). ATM depletion did not significantly alter expression of the identified genes (Fig 4F), suggesting that these transcriptional events are not ATM mediated. Observations were confirmed using alternative strategies to deplete DNA-PKcs or a second highly selective DNA-PKcs inhibitor NU7026 (the lead compound in generation of NU7441) (Veuger et al., 2003) (Fig S3F). Kinetic analysis revealed a time dependent decrease in target gene expression 6 hrs after treatment (Fig S3G), suggesting direct impact of DNA-PKcs on transcriptional regulation. Consonantly, DNA-PKcs binds to the proximal promoter regions containing motifs of known DNA-PKcs associated transcription factors for *PREX1* (Wong et al., 2011), *ROCK2*, and *ITGB4* (Drake et al., 2010) (Fig 4G). Treatment with MDV3100 modestly decreased expression of *ROCK2* and *ITGB4* (Fig S3H), suggesting that AR is not universally required for DNA-PKcs mediated regulation of genes in this pathway. DNA-PKcs occupancy was further examined at the proximal promoter regions of *PREX1*, *ROCK2*, and *ITGB4* after MDV3100 treatment or depletion of Sp1 or MAZ, the top motifs identified. MDV3100 decreased DNA-PKcs occupancy at the *ROCK2* and *ITGB4* promoters but not at

the *PREX1* promoter, consistent with the transcript data (Fig S3I, left). Sp1 depletion resulted in remarkable reduction in DNA-PKcs occupancy at the *PREX1* promoter (consistent with PREX1 being regulated by Sp1 (Wong et al., 2011)) and modest but significant reduction in occupancy at both the *ROCK2* and *ITGB4* promoters (Fig S3I, middle), while MAZ depletion produced a significant reduction in DNA-PKcs occupancy at the *ITGB4* promoter, modest reduction in occupancy at the *PREX1* promoter (not statistically significant), and no change at the *ROCK2* promoter (Fig S3I, right). These studies reveal that DNA-PKcs mediated expression is differentially regulated by transcription factors whose activities are modulated by DNA-PKcs. Decreased transcript expression resulted in reduced protein levels for the factors analyzed (Fig 4H), identifying DNA-PKcs as a positive regulator of metastatic signaling. PREX1, ROCK2, ITGB4, and VAV3 all interact with Rho GTPases that influence cell motility and invasion (Cook et al., 2014). DNA-PKcs depletion or inhibition decreased Rho and Rac1 activity (Fig 4I, Fig S3J). Depletion of VAV3 strongly reduced activated Rho and moderately suppressed Rac1 activity, while depletion of PREX1 diminished activated Rac1 with minimal effects on Rho (Fig S3K), demonstrating importance in DNA-PKcs mediated regulation of Rac/Rho signaling pathways, though other GEFs may be involved. Combined, these findings identify DNA-PKcs as a direct and positive regulator of Rac/Rho function and pro-metastatic pathways.

DNA-PKcs promotes metastatic phenotypes

Given the impact of DNA-PKcs on pro-metastatic signaling, the consequence for metastatic potential was determined. Depletion of DNA-PKcs resulted in decreased migration in all models (Fig 5A, left) and invasion in the CRPC models (Fig 5A, right). Consonantly, DNA-PKcs inhibition suppressed migration (Fig 5B, top) and invasion (Fig 5B, bottom) in all models. Both C4-2 and PC3-ML are CRPC lines capable of proliferating in the absence of hormone, and proliferation of LNCaP cells in hormone-deficient media was not significantly altered after DNA-PKcs inhibitor treatment (Fig S4A). The ROCK2 inhibitor reduced migration and invasion similar to that observed with NU7441 (Fig 5B). Combination of the DNA-PKcs and ROCK2 inhibitors resulted in modest but significant decreases in migration in C4-2 and PC3-ML cells, and further suppressed invasion in all models compared to either inhibitor alone, suggesting that DNA-PKcs regulates migration and invasion through pathways in addition to Rho signaling. Further, cells depleted of UGT2B15 or 2B17 failed to demonstrate significant changes in migratory or invasive potential (Fig S4B), suggesting that DNA-PKcs impact on metastatic phenotypes are independent from effects on metabolism. In sum, these findings establish DNA-PKcs as a positive regulator of gene expression events that induce migration and invasion.

DNA-PKcs inhibition delays formation of metastases in vivo

To determine the impact of DNA-PKcs on metastatic development *in vivo*, PC3-ML cells expressing luciferase were pre-treated for 48 hrs with NU7441 or vehicle and injected into the tail veins of SCID mice. Mice were treated every 24 hrs (5 days/week) with 25mg/kg NU7441 or vehicle, and tumor formation monitored by live imaging (Fig S5A). Parallel studies wherein cells were maintained in culture revealed no significant differences in cell number or viability between the cohorts (Fig S5A). Whereas robust metastases were

observed in the control arm, total tumor burden observed in the DNA-PKcs inhibitor treated cohort was significantly reduced, demonstrating that DNA-PKcs inhibition delays formation of productive metastases *in vivo* (Fig 6A). These findings provide evidence linking DNA-PKcs enzymatic activity to development of metastases.

To further investigate the impact of DNA-PKcs, crossover studies were performed wherein animals in the control arm with the greatest tumor burden (denoted 1, 2, and 3) were switched to the NU7441 arm; conversely, 3 mice randomly selected from the NU7441 arm (denoted 4, 5, and 6) were removed from treatment. After 2 weeks, animals moved from control to NU7441 failed to show reductions in tumor burden at established sites of metastases, consistent with the concept that DNA-PKcs inhibitors block development of productive metastases rather than suppressing tumor growth (Fig 6B). Conversely, animals released from NU7441 incurred dramatic induction of metastatic burden, with the tumor-doubling time reduced by ~50-90% (Fig 6C), suggesting that resurgent DNA-PKcs activity drives metastatic development. Mice not selected for crossover were continued on study, and total tumor burden remained suppressed in the NU7441 cohort but not the control arm (Fig S5B). Proliferation rates of the metastatic lesions in the lungs were similar in both crossover cohorts (Fig S5C) again suggesting that tumor changes in animals released from inhibitor illustrate the impact of DNA-PKcs on metastases and not proliferation, though it is possible that NU7441 treatment of large tumors in the crossover may be less effective due to tumor size. Experiments utilizing AR-positive 22Rv1 cells also demonstrated a significant decrease in overall metastatic tumor burden (Fig S5D), though this model is less aggressive in developing metastatic lesions. Combined, these findings clearly reveal that DNA-PKcs induces tumor metastases *in vivo*, confirming the importance of DNA-PKcs regulated pathways in metastatic development.

To further characterize the impact of DNA-PKcs on metastatic development in AR-positive but aggressive models of spontaneous metastasis, *CASP-NPK-YFP* tumor cells (Aytes et al., 2013) were engrafted into nude mice. Post-engraftment (5 days), mice were randomized for treatment with 25mg/kg NU7441 or vehicle (5 days/week for 30 days) (Fig S5E). DNA-PKcs suppression decreased overall tumor burden (Fig 6D), though primary tumor weight was not significantly altered between the treatment groups (Fig S5F); by contrast, significant reduction of metastatic lung lesions was observed in the inhibitor treated cohort, with a less pronounced but similar trend in liver metastases (Fig 6E). Finally, analysis of tumors harvested at sacrifice revealed significant decrease in transcript expression of *Prex1*, *Rock2*, *Itgb4*, and *Vav3* (Fig 6F), demonstrating that DNA-PKcs modulates expression of these four metastatic genes and promotes development of metastatic lesions *in vivo*. Thus, DNA-PK promotes metastatic signaling and tumor metastases in both AR-positive and AR-negative cancers.

DNA-PKcs inhibition modulates expression of pro-metastatic factors in primary human tumors

Transcriptional regulatory functions of DNA-PKcs on pro-metastatic factors were further assessed using an *ex vivo* culture system of primary human PCa, in which tissue obtained immediately upon surgical resection can be subdivided, cultured, and subjected to targeted

therapy as previously described (Centenera et al., 2013) (Fig 7A). Explant specimens retain the complex 3D structure and microenvironment of the original tumor, and can be used for clinical assessment of targeted agents (Centenera et al., 2013; Schiewer et al., 2012). While major alterations in histoarchitecture were not observed after exposure to NU7441 (Fig 7B), DNA-PKcs inhibition effectively suppressed expression of *PREX1*, *ROCK2*, *ITGB4*, and *VAV3* (Fig 7C). In sum, these findings confirm that DNA-PKcs inhibition regulates expression of pro-metastatic factors in primary human tumors.

DNA-PKcs expression and activity predicts clinical disease recurrence and metastatic development

Finally, the prognostic impact of DNA-PKcs in the clinical setting was investigated. A cohort of 232 patients with high-risk localized PCa was examined to assess the relevance of DNA-PKcs expression on outcomes following prostatectomy. As shown, elevated DNA-PKcs conferred reduced freedom from biochemical recurrence (Fig 8A, p=0.050, HR=1.5), and dramatically worse freedom from metastatic progression (Fig 8B, p=0.0004, HR=2.4), PCa-specific survival (Fig S6A, p=0.001, HR=2.8), and overall survival (Fig 8C, p<0.0002, HR=3.1). These results were comparable to the hazard ratios of high Gleason score for these same outcomes (BCR: HR=1.3, p=0.1; metastasis: HR=2.2, p=0.0007; PCSS: HR=4.4, p<0.0001; OS: HR=2.2, p=0.003). As Gleason score is one of the strongest known predictors for aggressive disease (Van der Kwast, 2014), these data illustrate the potent role of DNA-PKcs in promoting lethal PCa. Further, analysis of DNA-PKcs correlated genes showed significant enrichment in the AR pathway (Fig 8D, p<0.0001, normalized enrichment score (NES)= 2.673), the AR transcription factor pathway (Fig S6B, p<0.0001, NES= 2.474), MAZ targets (Fig 8E, p<0.0001, NES= 1.689), Sp1 targets (Fig 8F, p<0.0001, NES= 1.758), and the focal adhesion pathway (Fig 8G, p<0.0001, NES= 1.635), thus validating the preclinical findings. As expected, multiple pathways associated with DDR were also enriched (Table S1). DNA-PKcs was significantly positively correlated with AR, Sp1, and MAZ expression in the clinical samples (Fig S6C, correlation coefficients of 0.68, 0.77, and 0.70, respectively, all p<0.0001), further supporting the functional connectivity. Finally, elevated UGT2B15, but not 2B17, was associated with decreased freedom from metastases (Fig S6D). These findings, compared with previous reports (Mitsiades et al., 2012; Paquet et al., 2012), provide the basis for future studies directed at discerning the potentially divergent roles of UGT2B15 and 2B17 in CRPC progression. These observations identify DNA-PKcs as markedly upregulated in advanced disease, confirm the link between DNA-PKcs and metastatic signaling, and strongly support the contention that DNA-PKcs mediated transcriptional regulation is a major effector of lethal tumor phenotypes.

To further interrogate the link between DNA-PKcs and metastasis, an independent cohort was analyzed wherein DNA-PKcs phosphorylation was quantified by phospho-proteomic analyses of fresh clinical specimens from organ confined, treatment naïve PCa vs. metastatic CRPC. Multiple DNA-PKcs residues were hyper-phosphorylated in metastatic CRPC, including Thr2609, an autophosphorylation residue also reported to be phosphorylated by ATM (Chen et al., 2007) and indicative of enzymatic activation (Chan et al., 2002) (Fig 8H, Table S2). These findings reveal that DNA-PKcs is not only present, but highly active in late stage, metastatic CRPC. By contrast, analysis of γH2AX, a marker of DNA DSBs, in

metastatic tissues demonstrated no detectable change in phosphorylation levels compared to treatment naïve tissues (Fig 8H), suggesting that the heightened DNA-PKcs activation is not the result (or readout) of elevated DNA damage in metastatic tissues. Combined, these clinical analyses reveal that DNA-PKcs expression predicts for disease recurrence and DNA-PKcs phosphorylation suggests significant activation in metastatic tissues independent of heightened damage response, validating the preclinical evidence that DNA-PKcs is a master regulator of transcriptional events driving disease progression and development of metastatic lesions (Fig 8I).

DISCUSSION

Understanding mechanisms contributing to tumor progression and metastatic development is crucial for development of effective therapeutic strategies targeting advanced cancers. This study identifies DNA-PKcs as a key contributor to metastatic progression, mediated through transcriptional regulation. Key findings reveal that *i.* DNA-PKcs interacts with AR and is recruited to regulatory loci of AR target genes upon DHT stimulation, facilitating transcriptional activation; *ii.* DNA-PKcs selectively modifies transcriptional networks associated with tumor progression, and is recruited to loci regulated by DNA-PKcs-associated transcription factors; *iii.* UGT enzymes are negatively regulated by DNA-PKcs, implicating DNA-PKcs in pathways associated with therapeutic relapse; *iv.* DNA-PKcs positively regulates a transcriptional network that promotes pro-metastatic signaling, resulting in DNA-PKcs-induced tumor cell migration and invasion; *v.* pharmacological DNA-PKcs inhibition prevents formation of metastases *in vivo*; *vi.* analyses of clinical specimens reveal that DNA-PKcs is elevated and highly active in advanced disease, distinct from marks of DNA damage; and *vii.* DNA-PKcs dysregulation is strongly associated with development of distant metastases and reduced survival. In sum, these findings strongly support a model wherein the transcriptional regulatory functions of DNA-PKcs induce a pro-metastatic signaling program that drives tumor metastases and lethal disease. These studies not only define DNA-PKcs as a metastatic driver and a putative biomarker of disease progression, but nominate DNA-PKcs as a therapeutic target.

Data here are consistent with literature identifying DNA-PKcs as associated with sequence-specific transcription factors. Recent studies identified DNA-PKcs in ER/coregulator complexes (Foulds et al., 2013) and as an AR coactivator (Goodwin et al., 2013; Mayeur et al., 2005). This study provides direct insight into the mechanism of coordinated transcriptional regulation between AR and DNA-PKcs, wherein DNA-PKcs is recruited with delayed kinetics to sites of AR function, and is required for maximum AR activity. Amongst the AR target genes sensitive to DNA-PKcs regulation, *TMPRSS2* was recently shown to promote metastasis (Lucas et al., 2014), providing another mechanism by which DNA-PKcs may modulate metastatic development. Ongoing investigation is directed at discerning the impact of DNA-PKcs on the chromatin microenvironment surrounding AR and DNA-PKcs binding. The studies herein identify DNA-PKcs as an AR coregulator, supporting a role for DNA-PKcs in cancer-relevant transcriptional events.

Consistent with these findings, emerging evidence links DNA repair factors to transcriptional regulation. Initial studies reported that recruitment of DDR machinery was

primarily the result of transient, site-specific DSBs required for transcriptional activation (Ju et al., 2006). Further, the gene rearrangements observed in PCa can result from fusion events in transcriptional hubs bringing together distant chromosomal regions (Tomlins et al., 2005), suggesting that DNA repair capacity is needed at sites of active transcription. However, recent findings suggest that repair factors hold transcriptional regulatory functions independent of damage response, as exemplified by PARP1, a DNA repair factor with roles in transcriptional regulation whose functions can be segregated (Steffen et al., 2014). While the effects of DNA-PKcs on transcriptional activation reported here occurred in the absence of exogenous damage, it is possible that transcription-associated DNA breaks may contribute to observed DNA-PKcs activation. Irrespective of the means of activation, the findings herein demonstrate that DNA-PKcs interacts with known transcriptional modulators, binds to sites of transcriptional activation, and selectively engages a transcriptional network of strong cancer relevance.

The concept that DNA-PKcs suppresses UGT2B15 and 2B17 enzyme expression at least partially through NCoR and SMRT provides insight into how this PCa-relevant pathway is governed (Chouinard et al., 2006). Deregulation of androgen metabolism contributes to PCa progression (Chang et al., 2013) and may contribute to metastatic development (Mitsiades et al., 2012). Gene suppressive roles for DNA-PKcs have previously been reported (Hill et al., 2011; Jeyakumar et al., 2007; Yu et al., 2006), suggesting that DNA-PKcs-mediated transcriptional repression is not unique. While AR is required for basal expression of both *UGT2B15* and *2B17*, stimulation with androgen results in gene downregulation (Bao et al., 2008), suggesting that resurgent AR signaling in CRPC may have a role in DNA-PKcs-mediated transcriptional repression of UGT enzyme expression. Factors influencing UGT expression in non-prostatic tissues include NRF and Sp1 (Mackenzie et al., 2010), and influence on these factors may contribute to the impact of DNA-PKcs. Since UGT2B15 and 2B17 are being evaluated as pharmacologic targets for PCa management (Grosse et al., 2013), the identified link to DNA-PKcs may prove important in designing therapeutic regimens.

Identification of DNA-PKcs as a master regulator of pro-metastatic signaling complements previous studies linking the kinase to cancer-associated transcription factors (Brenner et al., 2011). The top scoring pathway for positively regulated DNA-PKcs genes is focal adhesion, hallmarked by factors that contribute to progression of multiple malignancies. Though the mechanisms regulating *ITGB4*, *PREX1*, *ROCK2*, and *VAV3* expression are not well defined, previous reports identified binding sites for DNA-PKcs interacting transcription factors within regulatory regions. Moreover, promoter motif analysis of genes sensitive to DNA-PKcs depletion revealed enrichment for binding sites of DNA-PKcs interacting transcription factors (*e.g.* Sp1, LEF1, and MYC). The AR binding sequence was not among the top motifs identified, likely influenced by the fact that androgen response elements (AREs) are present at only ~40% of known AR-binding sites, and AR primarily regulates transcription from enhancers (Yu et al., 2010). However, one of the top motifs identified was ELK1, an ETS domain factor required for expression of a major subset of AR target genes (Patki et al., 2013), supporting the finding that DNA-PKcs modulates AR-dependent transcription. Characterization of genome-wide DNA-PKcs occupancy combined with identification of the

DNA-PKcs-associated proteome is a focus of current studies, and will help to completely define partners of DNA-PKcs used to selectively modulate transcription.

A major consequence of DNA-PKcs mediated transcriptional regulation is tumor metastasis, and the Rho/Rac pathway was identified as a critical effector of DNA-PKcs activity. Previous studies established a role for Rho/Rac signaling in metastases (Matsuoka and Yashiro, 2014). The finding that ROCK2 and DNA-PKcs inhibitors functioned cooperatively to suppress migration and invasion suggests that pathways in addition to Rho/Rac signaling may contribute to DNA-PKcs induced metastasis (eg Wnt-β-catenin, TGFβ), and it is intriguing to speculate that DNA-PKcs forms a central signaling point modulating metastatic networks. The importance of DNA-PKcs in metastatic formation was confirmed in multiple *in vivo* models, as inhibition of DNA-PKcs activity strongly delayed formation of metastases, and crossover studies suggest that DNA-PKcs functions early in establishment of metastatic lesions. Combined, these findings provide comprehensive analysis of cancer-associated factors regulated by DNA-PKcs, and identify DNA-PKcs mediated transcriptional regulation as a driver of metastasis.

Finally, findings herein provide robust clinical evidence of DNA-PKcs as promoting metastasis in human disease and as a candidate biomarker to predict poor outcome. Despite recent advances (Mitsiades et al., 2012), clinical biomarkers predicting progression or therapeutic response in PCa are lacking. Analyses of clinical samples demonstrated that high DNA-PKcs expression strongly correlates with decreased freedom from recurrence, freedom from metastases, and survival, implicating DNA-PKcs as a major driver of lethal cancer development. Strikingly, DNA-PKcs held similar prognostic value to Gleason score, underscoring its importance in disease progression. Additionally, a second independent analysis revealed that DNA-PKcs phosphorylation on residues associated with activation (Thr2609) and chromatin binding (Thr2609, Ser2612) is highly enriched in metastatic vs. treatment naïve tissues, indicating that DNA-PKcs is highly active in metastatic PCa, independent of DNA damage markers. While it was previously thought that DNA-PKcs activation occurs only through Ku-mediated binding to broken DNA, recent studies identified additional mechanisms that contribute to DNA-PKcs activation, such as interaction with factors including AKT, EGFR, CK2, and multiple protein phosphatases (Douglas et al., 2001; Goodwin and Knudsen, 2014). While future studies are required to determine which (if any) of these mechanisms contribute to DNA-PKcs activation in the context of transcription, the kinase activity of DNA-PKcs is targetable, and DNA-PKcs inhibitors are currently in clinical trials for advanced solid tumors, hematologic malignancies, and metastases (clinicaltrials.gov, NCT01353625). As development of metastases is nearly universally lethal in solid tumors, the clinical value in targeting DNA-PKcs for prevention of metastatic development in multiple malignancies should be evaluated.

In sum, the studies herein reveal paradigms for DNA-PKcs activity, unveil definitive transcriptional regulatory functions that promote the development of lethal tumor phenotypes, and nominate DNA-PKcs as a therapeutic target.

EXPERIMENTAL PROCEDURES

Tail Vein Assays

Mouse studies were performed with Thomas Jefferson University IACUC approval. PC3-ML or 22Rv1 cells expressing luciferase were pre-treated for 48 hrs with 1 μM NU7441 or DMSO. After 48 hrs, 1×10^5 cells were seeded in hormone proficient media for viability studies and 5×10^5 cells in 100uL PBS were injected into the tail vein of 6 week old SCID mice. Cell number and viability were determined via trypan blue exclusion. Mice were treated every 24 hrs 5 days/week with 25mg/kg NU7441 or control through IP injection. Tumor volume was monitored by IP injection of 150uL RediJect D-Luciferin followed by IVIS imaging, with tumor volume quantified by Living Image Software. At day 31 of the PC3-ML study, 3 mice per cohort were selected for crossover studies. Mice not selected continued original treatment for an additional week. Crossover mice received new treatment for 2 weeks prior to sacrifice. Average doubling time pre- and post-crossover was determined using $T_d= (t_2-t_1)*((\ln2)/ (\ln(q_2/q_1)))$.

Clinical Analyses

DNA-PKcs expression—Tumor samples were obtained from Mayo Clinic utilizing a case-cohort study design to randomly sample 20% of patients for analysis, in addition to all who developed metastases, from a cohort of 1,010 high-risk men who underwent radical prostatectomy between 2000-2006, for a total cohort of 232 patients as described (Karnes et al., 2013). Studies were approved by the Mayo Clinic IRB and informed consent obtained from all subjects. DNA-PKcs expression was profiled using Affymetrix Human Exon 1.0 ST arrays. Expression data was normalized and summarized using the SCAN algorithm (Karnes et al., 2013). Expression was split into high versus low by the 80th percentile of DNA-PKcs expression. Gleason was split into high (8-10) versus intermediate/low (≤7). Kaplan Meier curves and p-values were generated using the log-rank test. Expression of other genes was correlated with DNA-PKcs using Spearman's correlation. Pre-ranked GSEA analyses were run using spearman's rho and indicated pathways analyzed.

DNA-PKcs phosphorylation—DNA-PKcs and H2AX phosphorylation were measured in organ confined, treatment naïve PCa and metastatic CRPC tissues (Drake et al., 2013). Studies were approved by the UCLA IRB and informed consent obtained from all subjects. Phosphopeptide enrichment was performed as previously described (Zimman et al., 2010) with minor modifications. LC-MS/MS was performed using a Q-Exactive mass spectrometer. MS/MS fragmentation spectra were searched using Andromeda (Cox et al., 2011) against the Uniprot human reference proteome database with canonical and isoform sequences (downloaded January 2012 from uniprot.org) and a reversed decoy database with an FDR <0.01. Search parameters included N-terminal acetylation and oxidized methionine as variable modifications and carbamidomethyl cysteine as a fixed modification. Variable modifications included phosphorylated serine, threonine, or tyrosine [phospho (STY)]. In addition, group-specific parameters included max missed cleavages of 2. Search scores are reported in Table S2. Quantitation was performed using Skyline 2.6.0.6851 (Schilling et al., 2012). Prior to analysis, redundant spectral libraries were generated from Proteome Discoverer search results of the raw data files using the same Uniprot human reference

proteome database. Retention time filtering was used so that only scans within 2 minutes of an MS/MS id were included. The precursor isotopic import filter was set to include only the first isotopomer (M0) at a Skyline resolution setting of 70,000. Reintegration of the peaks was performed with mProphet to improve peak picking, with a scoring model based on precursor mass error, identification and co-elution count. Results were reported as areas under the curve (AUC) for each peptide. AUC values were compared across the treatment naïve PCa and metastatic CRPC for DNA-PKcs and H2AX phosphopeptides. Relative fold changes for each phosphoresidue as determined by the average of treatment naïve and metastatic CRPC tissues were plotted. To calculate significance, two-tailed t-tests or Mann-Whitney U tests were used for normally and non-normally distributed phosphopeptide data.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Identification of DNA-PKcs-modulated transcriptional networks and consequence
- DNA-PKcs-mediated gene regulation promotes migration, invasion, and metastases
- DNA-PKcs is upregulated and highly activated in aggressive human tumors
- DNA-PKcs independently predicts for metastases, recurrence and poor survival

SIGNIFICANCE

Mechanisms underlying metastatic development remain incompletely defined, and few therapeutic regimens effectively target the metastatic process. Studies here identify DNA-PKcs as a master driver of pro-metastatic signaling and tumor metastasis through transcriptional regulation, thus shifting paradigms with regard to DNA-PKcs activity and illuminating critical functions in human malignancy. Preclinical findings are strongly supported by clinical observations which demonstrate that DNA-PKcs is significantly upregulated in advanced disease, and predicts for tumor metastases, recurrence, and poor survival. Moreover, DNA-PKcs was shown to be highly activated in metastatic tumors, independent of DNA damage indicators. These collective findings transform understanding of DNA-PKcs function, establish clinical relevance, and nominate DNA-PKcs as a therapeutic target to suppress metastases.

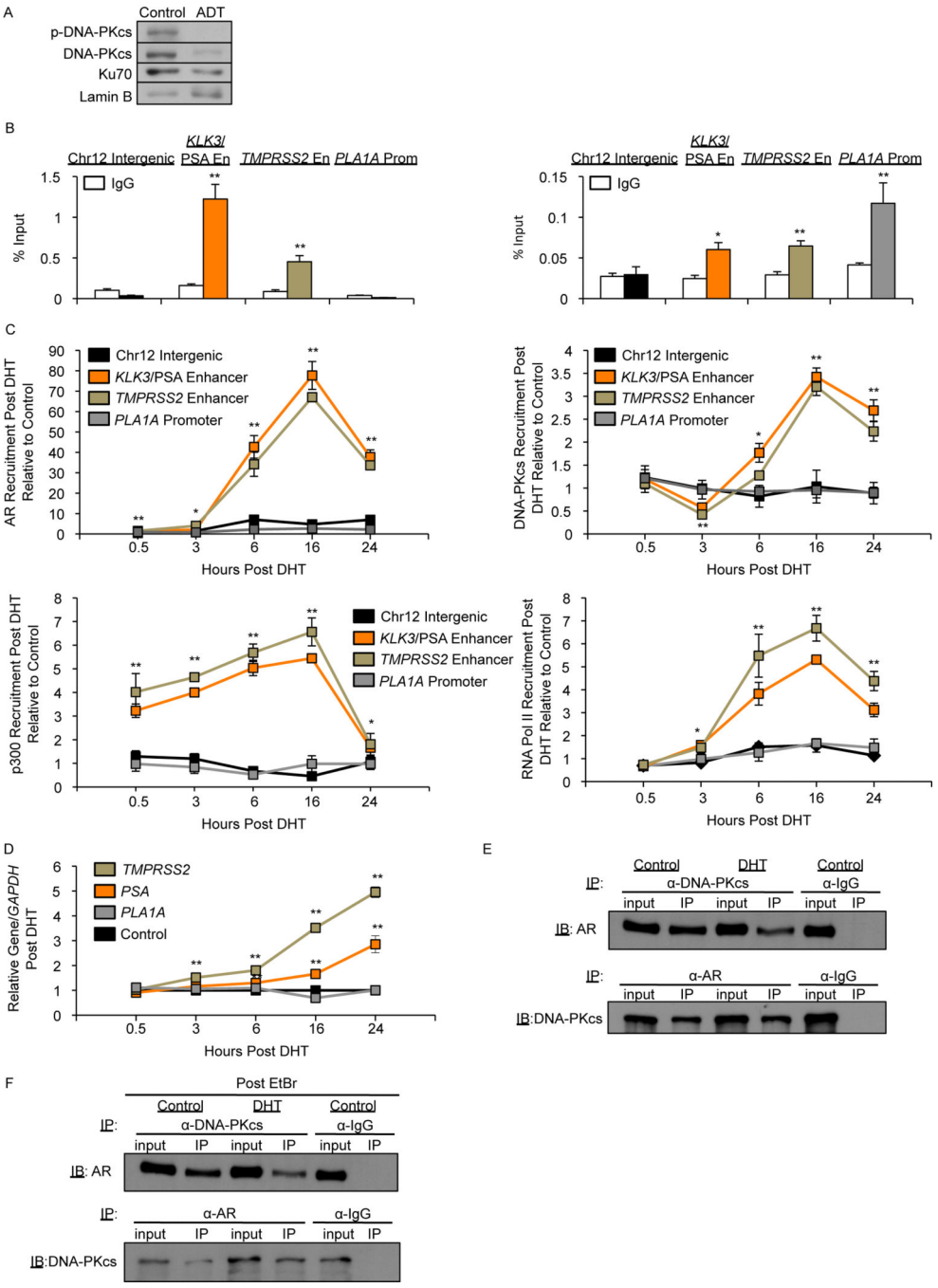

Figure 1. DNA-PKcs binds AR and is recruited to sites of AR action

(A) C4-2 cells were treated with ADT (CSS) for 24 hrs and immunoblot analysis for phospho-S2056 DNA-PKcs, total DNA-PKcs, and Ku70, performed. (B,C) C4-2 cells in hormone proficient media were (B) harvested for ChIP-qPCR analysis and percent (input) occupancy of AR (left) or DNA-PKcs (right) reported or (C) treated with 10nM DHT and harvested for ChIP-qPCR analysis with percent (input) occupancy of AR, DNA-PKcs, p300, or RNPII set relative to control at each time point. (D) C4-2 cells were treated with 10nM DHT and relative transcript expression analyzed as normalized to *GAPDH* mRNA at each

timepoint. (E,F) C4-2 cells were treated with 10nM DHT for 6 hrs and co-immunoprecipitation performed in the absence (E) or presence (F) of 50μg/mL ethidium bromide. Data are reported as mean +/− SD. *p<0.05 **p<0.01. See also Fig S1.

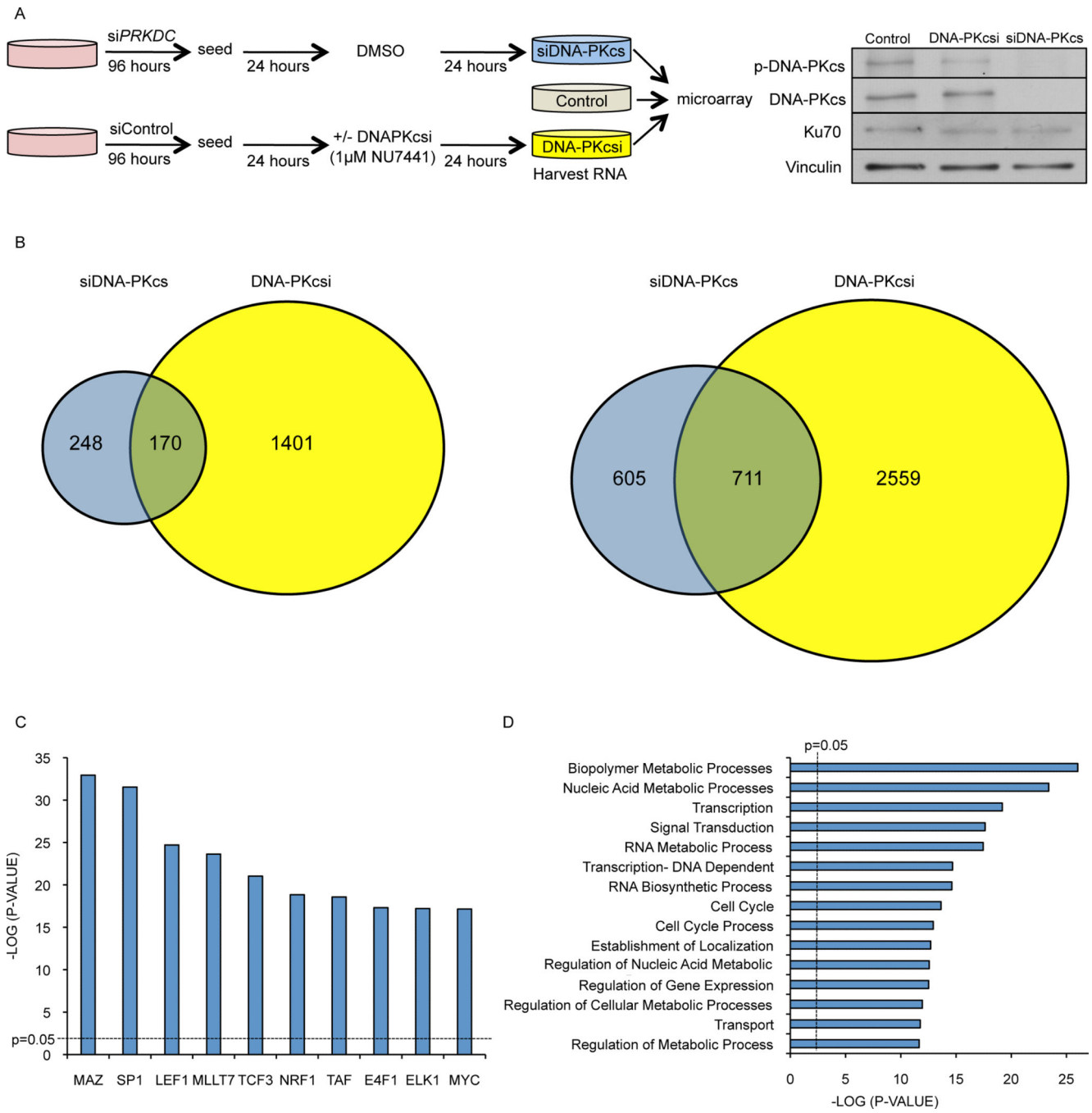

Figure 2. DNA-PKcs selectively impacts gene expression in CRPC

(A) RNA harvested from C4-2 cells depleted of DNA-PKcs or treated with 1μM NU7441 (DNA-PKcsi) for 24 hrs was analyzed by microarray analysis (left). Immunoblot of phospho-S2056 DNA-PKcs, total DNA-PKcs, and Ku70 after knockdown or NU7441 treatment (right). (B) Genes identified as upregulated (left) or downregulated (right) by ≥1.5 fold compared to untreated. (C,D) GSEA motif (left) or gene ontology (right) analyses of all genes altered at least 1.5-fold after DNA-PKcs knockdown.

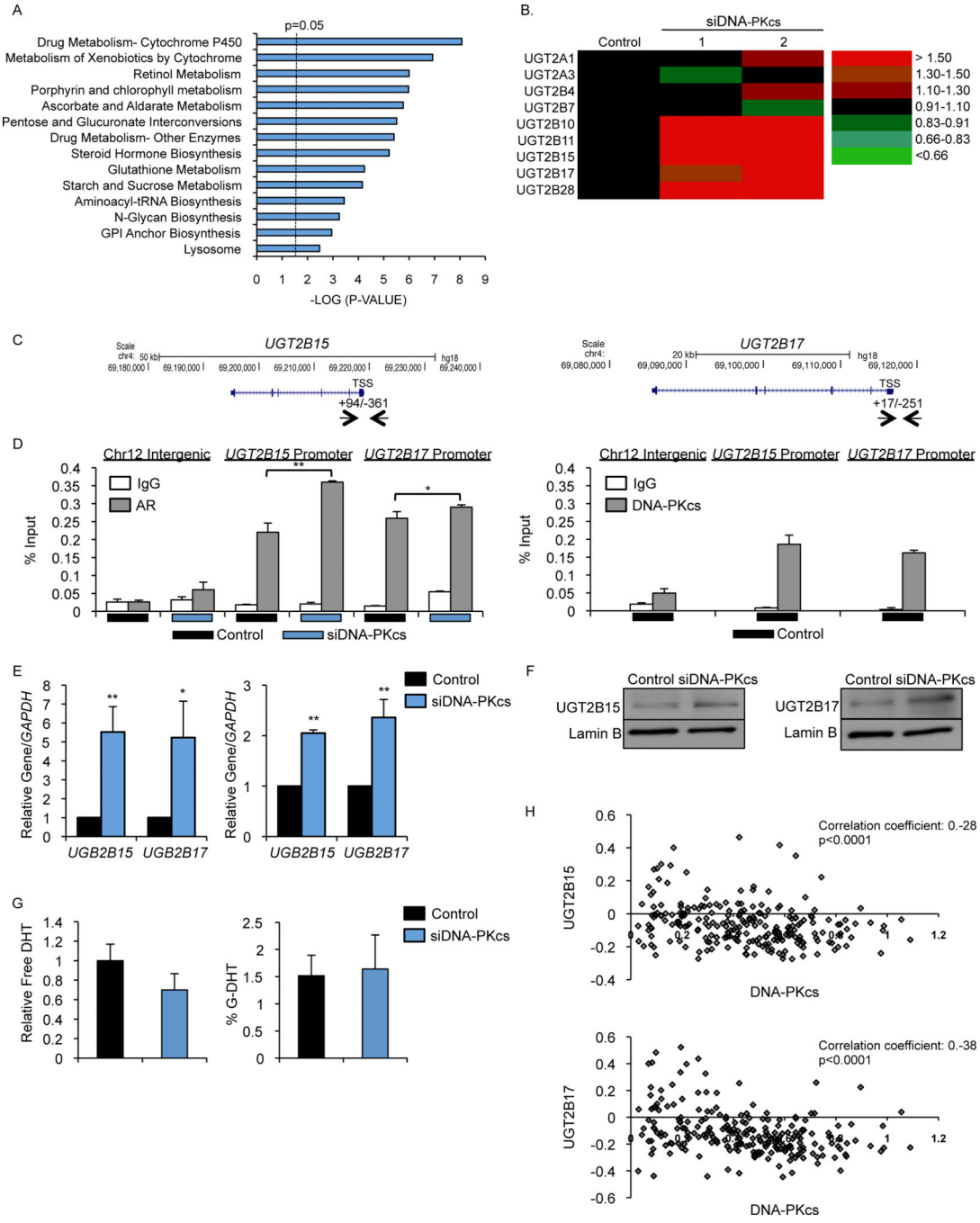

Figure 3. DNA-PKcs and AR cooperate to suppress UGT enzyme expression in CRPC

(A) GSEA KEGG pathway analysis of genes upregulated by ≥1.5 fold compared to control after DNA-PKcs knockdown. (B) Heat map of transcript change of UGT enzymes in the DNA-PKcs knockdown groups. (C,D) C4-2 cells depleted of DNA-PKcs were harvested for ChIP-qPCR analysis and percent (input) occupancy of AR (D, left) or DNA-PKcs (D, right) at indicated loci reported, TSS= transcriptional start site. (E,F) CRPC cells depleted of DNA-PKcs were subject to either qPCR (E, C4-2 left, 22Rv1 right) or immunoblot (F, C4-2) analysis. (G) Free (left) and G-DHT (right) levels in C4-2 cells depleted of DNA-PKcs were

determined by HPLC. (H) Tumor samples were profiled for mRNA expression of DNA-PKcs, UGT2B15, and UGT2B17 and correlation coefficients determined. Data are reported as mean +/− SD. *p<0.05 **p<0.01. See also Fig S2.

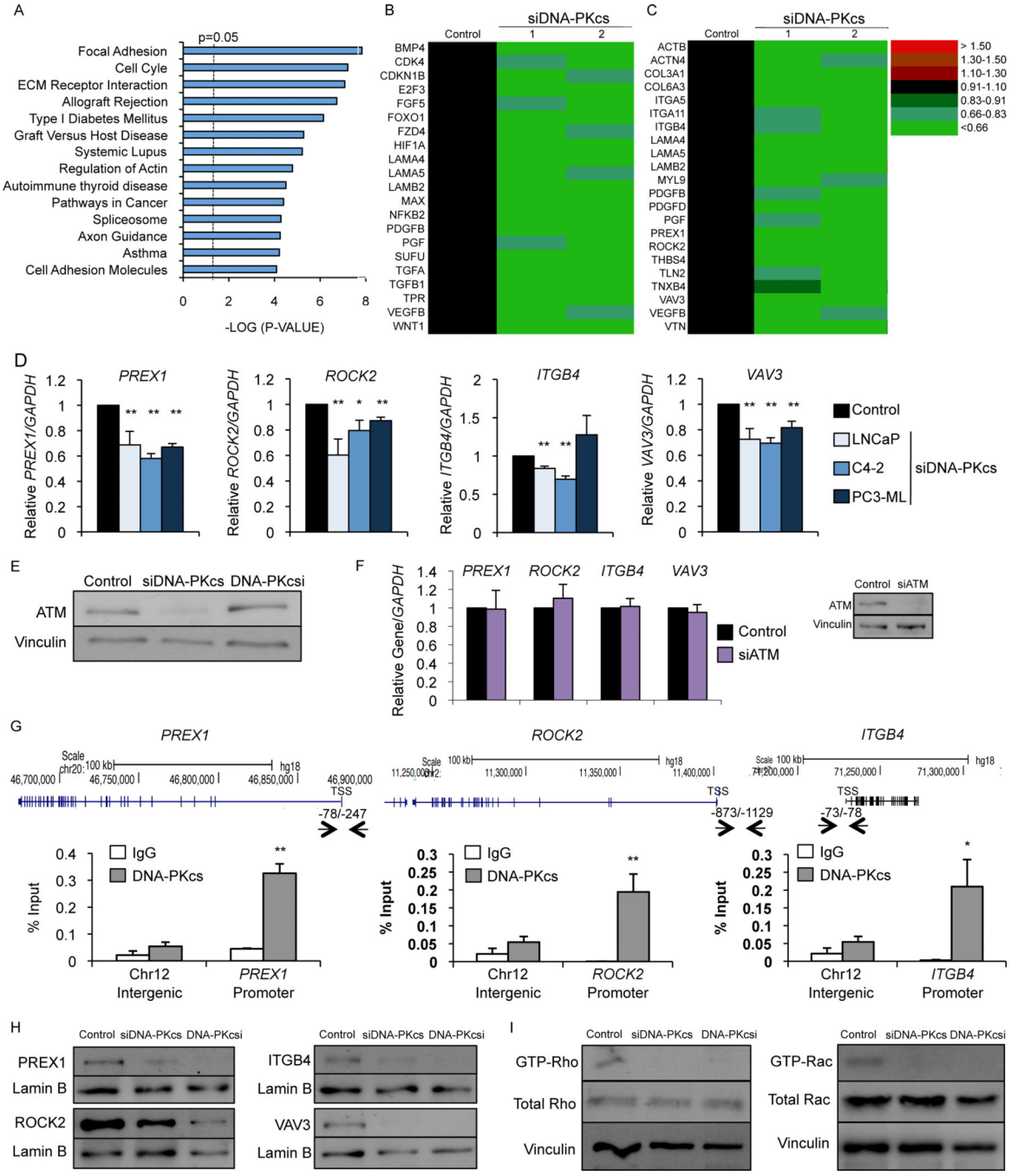

Figure 4. DNA-PKcs promotes pro-metastatic signaling

(A) GSEA KEGG pathway analysis of genes downregulated by ≥1.5 fold compared to control after DNA-PKcs knockdown. (B,C) Heat map of transcript change of pathways in cancer (B) or focal adhesion (C) pathway genes in the DNA-PKcs knockdown groups. (D) C4-2 and PC3-ML cells in hormone proficient or LNCaP cells in hormone deficient media treated with siDNA-PKcs or siControl were subject to qPCR analysis with control data set to 1 for each cell line. (E) Immunoblot analyses of C4-2 cells depleted of DNA-PKcs or treated with 1μM NU7441. (F) C4-2 cells depleted of ATM were harvested for qPCR analysis with

relative expression of indicated transcripts analyzed and normalized to *GAPDH*. (G) C4-2 cells harvested for ChIP-qPCR analysis and percent (input) occupancy of DNA-PKcs at the indicated regulatory regions. (H) C4-2 cells depleted of DNA-PKcs or treated with 1μM NU7441 for 48 hrs were subject to immunoblot analysis. (I) C4-2 cells depleted of DNA-PKcs or treated with 1μM NU7441 for 48 hrs 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. See also Fig S3.

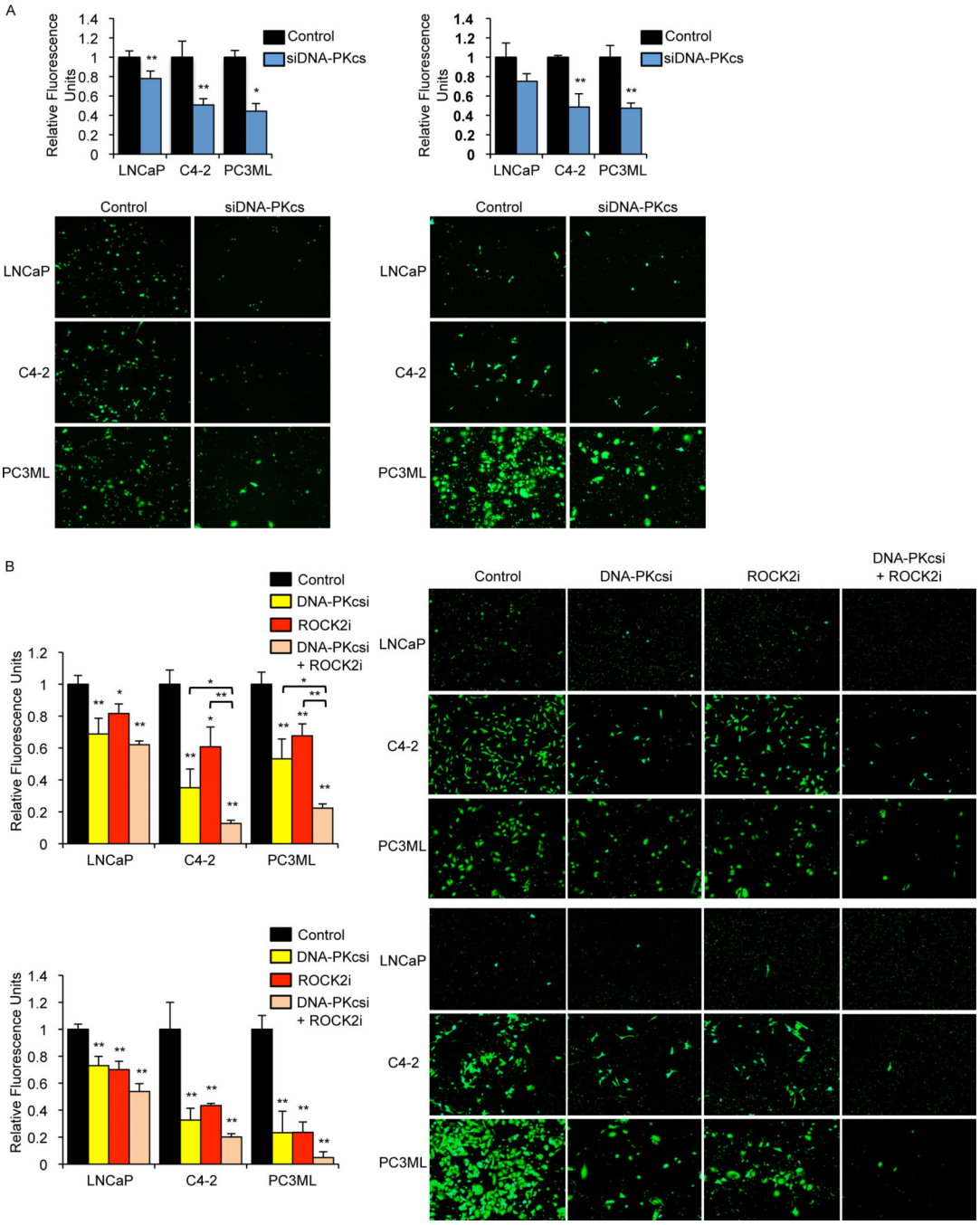

Figure 5. DNA-PKcs induces metastatic phenotypes

(A) Cells depleted of DNA-PKcs were seeded into hormone deficient media and allowed to migrate (left) or invade through matrigel (right) towards hormone proficient media. (B) Cells pretreated with 1μM NU7441, SLx-2119 or combination of both for 24 hrs were seeded into hormone deficient media and allowed to migrate for 24 hrs (top) or invade through matrigel for 72 hrs (bottom) towards hormone proficient media. Data are reported as mean +/− SD. *p<0.05, **p<0.01 compared to control unless otherwise indicated. See also Fig S4.

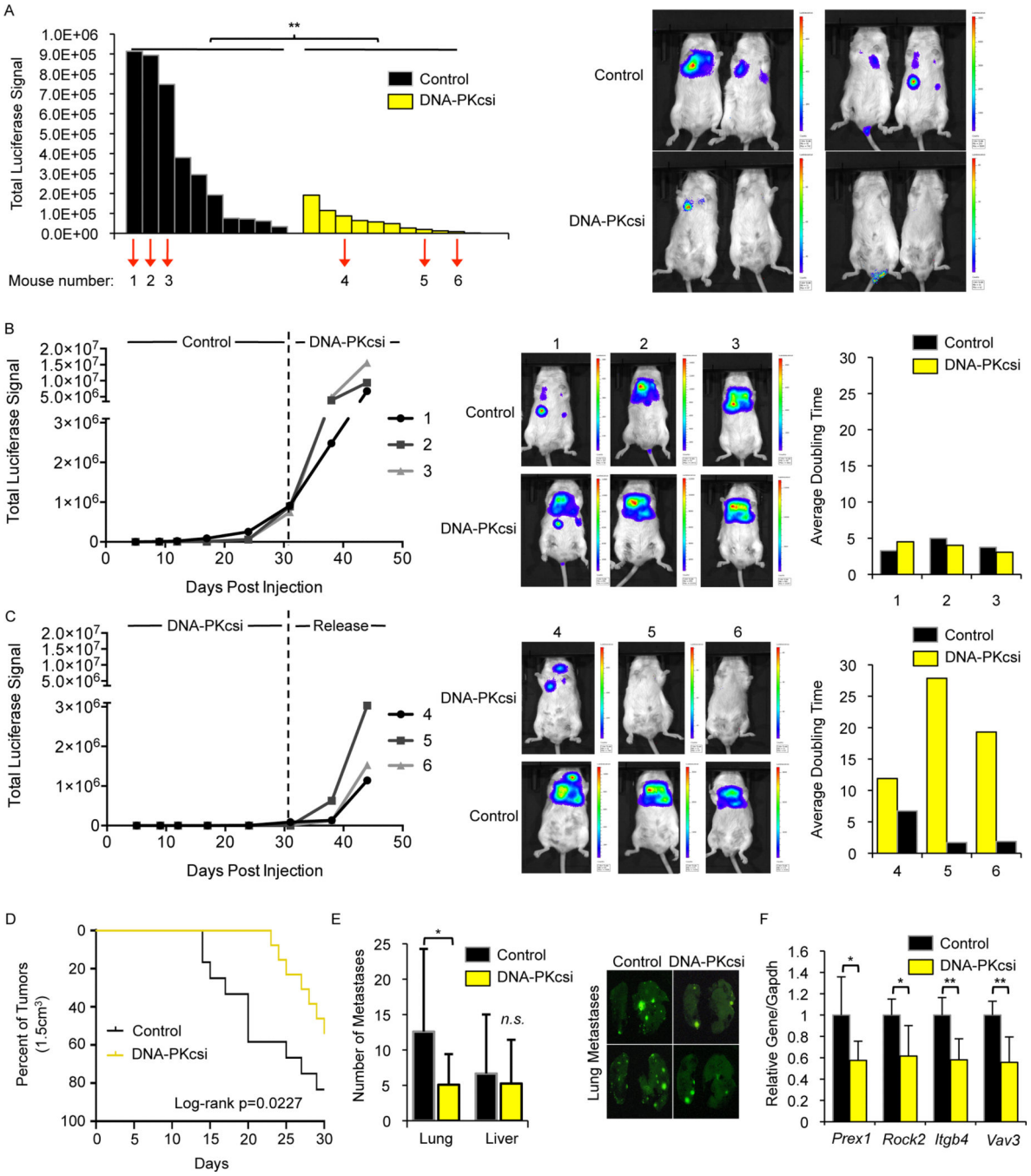

Figure 6. DNA-PKcs inhibitors delay formation of metastases *in vivo*

(A) Mice were injected with luciferin 31 days post tail vein injection of PC3-ML cells and imaged using the IVIS imaging system with total luciferase signal reported (left) and representative images shown (right). Indicated mice were selected for crossover studies. (B,C) Mice were injected with luciferin and imaged for 2 weeks after initiation of crossover studies with total luciferase signal reported (left), representative images shown (middle), and average doubling times pre and post crossover calculated. (D) *CASP-NPK-YFP* tumors were measured twice weekly for 30 days after initiation of treatment (end point for survival was

the predefined tumor volume of 1.5cm^3) with volumes calculated using the formula volume=(width)^{2}xlength/2. (E) At time of sacrifice, metastases were documented *ex vivo* in the lungs and livers by visualizing fluorescence with the total number of metastatic nodules for the lungs and livers assessed. (F) *CASPNPK-YFP* tumors were harvested for qPCR analysis with the indicated transcripts set relative to *Gapdh* mRNA. Data are reported as mean +/− SD. **p<0.01. See also Fig S5.

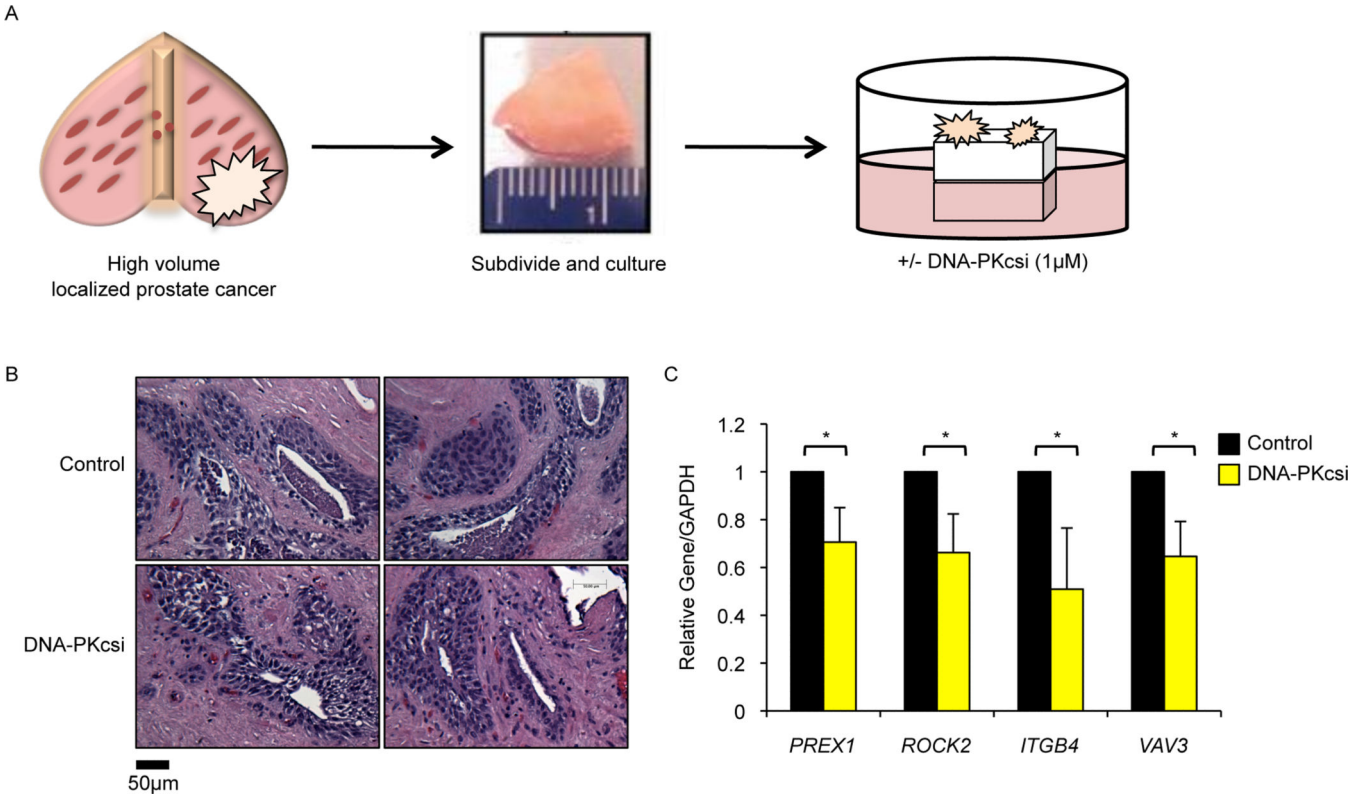

Figure 7. DNA-PKcs inhibition modulates expression of pro-metastatic factors in primary human disease

(A) Schematic of explant assay, adapted from (Schiewer et al., 2012). (B) Representative images of explant tissues treated with control or 1μM NU7441 and stained with hematoxylin & eosin. (C) Explant tissues were harvested on day 6 for qPCR analysis with indicated transcripts set relative to *GAPDH*. Data are reported as mean +/− SD. *p<0.05.

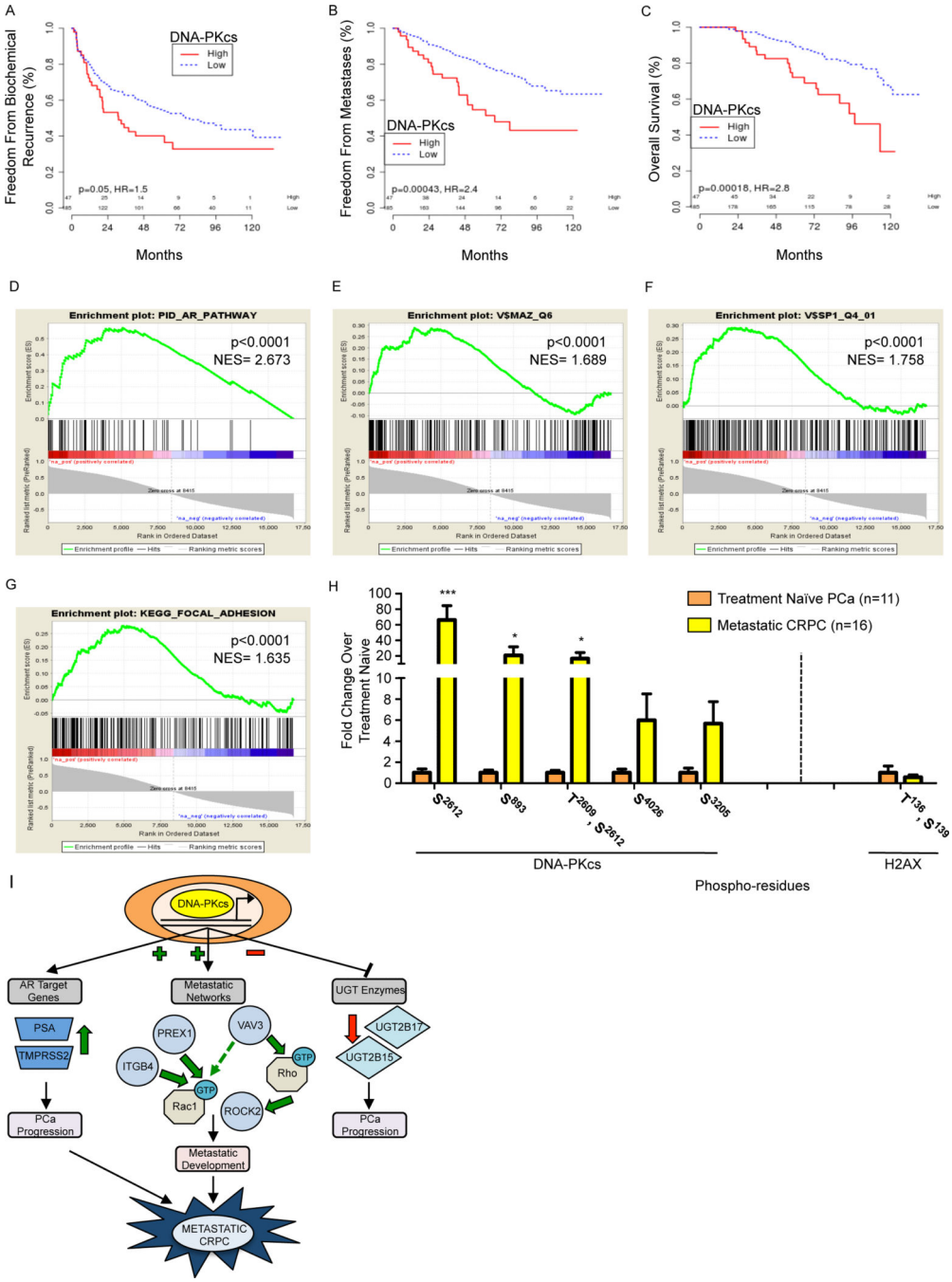

Figure 8. DNA-PKcs is associated clinically with disease recurrence and metastases

(A-C) Tumor samples were profiled for DNA-PKcs mRNA, which was split into high vs. low by the 80th percentile for Kaplan Meier analysis. (D-G) GSEA analyses showed enrichment of the AR pathway (D), MAZ (E) and SP1 (F) targets, and the focal adhesion pathway (G) in genes correlated to DNA-PKcs. (H) DNA-PKcs and histone H2AX phosphorylation were measured by mass spectrometry in organ confined, treatment naïve PCa and metastatic CRPC tissues. (I) DNA-PKcs modulates cancer-associated transcriptional networks, inducing expression of AR targets and genes that regulate pro-

metastatic Rho/Rac signaling pathways and suppressing expression of UGT enzymes known to impact DHT metabolism, identifying DNA-PKcs as a clinically actionable driver of metastatic CRPC. ***p<0.001, *p<0.05. See also Fig S6, Table S1, S2.