Cellular and molecular effects of PNCK, a non-canonical kinase target in renal cell carcinoma

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Highlights
PNCK overexpression correlates with poor prognosis in human RCC
PNCK overexpression leads to increased pCREB, cell cycle progression, and cell growth
PNCK k/d causes cell-cycle arrest, apoptosis, and decreased ANGPT1 and ANGPT2 levels

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**SUMMARY**

Renal cell carcinoma (RCC) is a fatal disease when advanced. While immunotherapy and tyrosine kinase inhibitor-based combinations are associated with improved survival, the majority of patients eventually succumb to the disease. Through a comprehensive pan-cancer, pan-kinome analysis of the Cancer Genome Atlas (TCGA), pregnancy-upregulated non-ubiquitous calcium-calmodulin-dependent kinase (PNCK), was identified as the most differentially overexpressed kinase in RCC. PNCK overexpression correlated with tumor stage, grade and poor survival. PNCK overexpression in RCC cells was associated with increased CREB phosphorylation, increased cell proliferation, and cell cycle progression. PNCK down-regulation, conversely, was associated with the opposite, in addition to increased apoptosis. Pathway analyses in PNCK knockdown cells showed significant down-regulation of hypoxia and angiogenesis pathways, as well as the modulation of the cell cycle, DNA damage, and apoptosis pathways. These results demonstrate for the first time the biological role of PNCK, an understudied kinase, in RCC and validate PNCK as a druggable target.

**INTRODUCTION**

Renal Cell Carcinoma (RCC) is an aggressive malignancy expected to affect 79,000 new Americans in 2022 of which an estimated 13,920 are expected to succumb to this disease. Advances in the understanding of RCC pathogenesis, including VHL silencing, HIF accumulation, increased angiogenesis and the RCC immune microenvironment have led to the development of new treatment options, including Tyrosine Kinase Inhibitors (TKIs), mTOR inhibitors and more recently, immune checkpoint inhibitors (CPIs), given alone or in combination. While these agents have significantly improved progression-free and overall survival in advanced clear cell RCC, a large number of patients eventually develop treatment resistance, leaving patients with few treatment options and a poor prognosis. Therefore, identifying novel targets for RCC drug development is an urgent, unmet need.

The calcium-calmodulin kinase (CAMK) family as a whole is understudied with currently no approved drugs or clinical trials targeting these kinases. PNCK (CAMK1B), an isoform of CAMK1, is among the least characterized CAMK members, despite emerging studies demonstrating its prognostic and potential therapeutic relevance. PNCK is a 38-kDa protein whose catalytic domain shares 45-70% sequence identity with other members of the multifunctional CAMK family. PNCK is temporally expressed and restricted to very few tissues through development and into adulthood, with highest expression in the dentate gyrus in the hippocampus. The in vivo substrates of PNCK are unknown. However, in vitro studies suggest that PNCK phosphorylates CAMK1A, CREB, ATF1, and Synapsin. PNCK has been linked to aggressive phenotypes and poor outcomes in limited studies in breast (BC), renal cell (RCC), nasopharyngeal, and hepatocellular carcinomas. In clear cell RCC, PNCK expression (by immunohistochemistry) was shown to be significantly higher in tumor compared to adjacent normal tissues and was established as an independent predictor of poor survival, tumor size, and histological grade. Studies in breast cancer suggest that PNCK stimulates proliferation, clonogenicity, cell cycle progression and treatment resistance, and that it may play a role in modulating the tumor microenvironment. To date, however, there have been no comprehensive studies on PNCK, CAMKs and the CAMK pathway in renal cell carcinoma.
Toward our goal to find new RCC targets, we performed an integrative analysis of available cancer databases (TCGA and Pharos) to identify understudied kinases that are overexpressed in RCC. Our analysis identified pregnancy-upregulated non-ubiquitous calcium/calmodulin-dependent kinase (PNCK), as the most differentially overexpressed kinase in RCC, and was significantly associated with poor clinical outcomes. The current report aims to characterize the in vitro biological effects and mechanisms of PNCK up and down-regulation in renal cell carcinoma models. Our results suggest that PNCK is a promising target for novel drug development efforts in this fatal disease.

RESULTS

Pregnancy-upregulated non-ubiquitous calcium-calmodulin-dependent kinase multi-omics analysis

A comprehensive and integrative analysis of the cancer genome atlas (TCGA) was performed to identify understudied kinases that were differentially expressed in RCC versus normal (non-cancer) tissues as described in our previous work. Understudied kinases, as defined by the NIH common fund project “Illuminating the Druggable Genome”, represent kinases that have few annotations (GeneRIF, GO Molecular Function), validated antibodies, experimentally resolved crystal structures, and small molecule compounds with sufficient bioactivity. Results from the analysis demonstrated that several understudied calcium-calmodulin-dependent kinases (CAMK) were overexpressed in RCC tumors (Figure S1A). Among CAMK members, pregnancy-upregulated non-ubiquitous calcium/calmodulin-dependent kinase (PNCK or CAMK1B) was found to be the most differentially overexpressed kinase in clear cell renal cell carcinoma (KIRC), with a difference of 6 log₂-fold between median expression of tumor samples and adjacent normal kidney tissue (Figure 1A). We have previously demonstrated that a broader examination of the RNAseq data found PNCK to be in the top 1% of all differentially overexpressed genes in clear cell renal carcinoma. Additionally, PNCK was shown to be significantly overexpressed in papillary (KIRP) (Figure 1B) and chromophobe RCCs (Figure S1D). Other tumors that significantly overexpressed PNCK include lung squamous carcinoma (LUSC), breast carcinoma (BRCA) and hepatocellular carcinoma (LIHC) (Figures S1B and S1C). Merging all kidney cancer cohorts, including papillary, clear cell, and chromophobe tumors, to a combined kidney cancer dataset (KIPAN), PNCK was shown to be significantly overexpressed (Figure 1C). PNCK expression in all cohorts correlated significantly with AJCC stage in all cohorts (Figures 1D–1F), and with T staging and histologic grade (Fuhrman Grade) in clear cell RCC cohorts (Figures S1E and S1F). Kaplan-Meier survival analyses demonstrated a significant correlation between elevated PNCK expression and disease-specific survival (DSS) in Clear Cell (KIRC), Papillary (KIRP), and KIPAN RCC cohorts (Figures 1G–1I). PNCK expression quantified via immunohistochemistry of human kidney tumor samples has been correlated with negative survival outcomes in one small study (n = 248). Overall, our analysis is in congruence with previous data that demonstrating PNCK expression in RCC is linked to worse clinical outcomes.

Pregnancy-upregulated non-ubiquitous calcium-calmodulin-dependent kinase overexpression leads to increased levels of phosphorylated cAMP response element binding protein (CREB) with nuclear translocation

PNCK mRNA expression was quantified in a panel of kidney cancer cell lines and 3 control cell lines (HEK293, HUVEC, and HREC). Consistent with data from the Cancer Cell Line Encyclopedia (CCLE), PNCK is significantly overexpressed in kidney cancer cell lines compared to normal human renal epithelial cells (HREC) (Figure S2A). To evaluate the effects of PNCK overexpression to the scale observed in human tumor samples on in vitro biological functions, renal cell carcinoma (786-O, A498, ACHN & Caki-1) cells were engineered to stably express PNCK (See STAR methods). Overexpression (OE) was confirmed via qPCR (Figures S2B–S2E), western blot, and immunofluorescence (Figures 2A–2L). PNCK, unlike other isoforms of CAMK1, lacks the conserved nuclear exclusion signal (NES). Therefore, we hypothesized that PNCK would be localized to both nuclear and cytoplasmic compartments. Indeed, IF signal of PNCK was intensely nuclear in 786-O (Figure 2B) and ACHN (Figure 2H) cells while in A498 (Figure 2E) and Caki-1 cells (Figure 2K), the signal was diffusely cytoplasmic and intensely peri-nuclear. Previous studies have shown that calcium calmodulin-dependent kinases (CAMK1 and CAMK2) phosphorylate and activate the transcription factor (Cyclic AMP Response element binding protein) CREB at Ser133 in vitro. However, this has never been shown for PNCK. We observed that PNCK overexpression led to increased pCREB signal, as measured by immunofluorescence (Figures 2C, 2F, 2I, and 2L). While the pCREB signal was present both in the control and PNCK OE cells, a more prominent nuclear localization of pCREB was observed in PNCK OE cells while control cells displayed a diffuse (cytoplasmic) signal. CREB localization to the cytoplasm, mitochondria, or nucleus is determined by many factors such as pH, hypoxia, and other post-translational modifications.
including phosphorylation. Signal intensity in the nucleus in PNCK overexpression suggests PNCK may alter the sub-cellular location of CREB and the expression of downstream CREB effector genes.

**Effects of pregnancy-upregulated non-ubiquitous calcium-calmodulin-dependent kinase overexpression on renal cell carcinoma proliferation and cell cycle progression**

To assess the effects of PNCK overexpression on RCC cell growth in vitro, rates of cellular proliferation in PNCK OE RCC cells were examined using the real-time xCelligence assay for up to 120 h (See STAR methods). As predicted, PNCK overexpression led to significant increases in cell growth in all 4 RCC cell lines tested, compared to GFP-transfected controls (Figures 3A–3D). Notably, PNCK overexpression in HEK-293 cells led to no appreciable difference in proliferation rate or cell index compared to control (Figure S3A). No appreciable morphological differences were noted in PNCK overexpression cells versus GFP controls thus differences in cell indices and electrical impedance were likely due to differences in cell count.
as opposed to cell shape. Additionally, growth curves displayed a characteristic "left" shift, with OE cells displaying rapid acceleration of growth in the first 24-48 h of seeding and reach confluence (As quantified by normalized cell index) faster than GFP controls. After 120 h, the cell indices for both OE and GFP control generally reach the same height and begin to decrease as cells reach confluence and detach from the sensor (data not shown). Of note, PNCK overexpression also did not have any significant effects on migration or invasion \textit{in vitro} (data not shown).

To further explore the role of PNCK expression in cellular proliferation, flow cytometry was utilized to quantify the population of cells in each stage of the cell cycle (See STAR methods). Previous studies have shown that CAMK1 and CAMK2 partly control G1/S and S/G2 transitions through various mechanisms including the activation of CDK/Cyclin complexes. In cells that were engineered to overexpress PNCK, there was a significantly higher population of cells in the S and G2 phases and a significantly lower number of cells in the G1 phase compared to GFP controls in 786-O, A498, ACHN, and Caki-1 cells (Figures 3E–3H). In congruence with our proliferation studies, HEK293 cells overexpressing PNCK did not show any changes in cell cycle populations compared to GFP controls (Figure S3B). Collectively, these data suggest that PNCK activity likely increases cellular proliferation through effects exerted on cell cycle progression at either G1/S or S/G2 transition.
Effects of pregnancy-upregulated non-ubiquitous calcium-calmodulin-dependent kinase down-regulation on renal cell carcinoma CREB phosphorylation, proliferation and cell cycle

As overexpression of PNCK led to significant increases in cellular proliferation via progression through cell cycle checkpoints, likely through the upregulation of downstream CREB target oncogenes, we hypothesized that knockdown of PNCK would lead to growth arrest. Targeted PNCK inhibition was achieved using double-stranded siRNA (dsiRNA) technology (See STAR methods). RCC cells (786-O, A498, ACHN, Caki-1) were transfected with dsiRNA targeted against the canonical PNCK sequence or Cy3-labeled scramble control. PNCK knockdown was confirmed via qPCR (Figure S4), western blot, and immunofluorescence, (Figures 4A–4P). No significant morphological changes were observed between control and PNCK k/d cells. Compared to Cy3 control, PNCK dsiRNA treated cells had markedly diminished pCREB signal in all RCC cell lines (Figures 4A–4P). PNCK knockdown, compared to the dsiRNA scramble control, led to statistically significant inhibition of RCC cell growth in vitro (Figures 5A–5D). The degree and dynamics of growth inhibition differed among cell lines, with ACHN and Caki-1 showing a marked inhibition in proliferation at all time points. Conversely, 786-O cells showed a decrease in proliferation at earlier time points (before 72 h post-transfection), and A498 PNCK k/d was associated with a delayed inhibition in proliferation (beyond 72 h post-transfection) with a noted decrease in normalized cell index between 72 and 120 h, suggestive of increased cell death. PNCK k/d did not significantly interfere with RCC migration or invasion in vitro (Data not shown).

Next, the effects of PNCK k/d on cell cycle progression were assessed. PNCK knockdown in 786-O, ACHN, and Caki-1 cells was associated with G1 cell-cycle arrest (with a large population of cells accumulating in G1 and smaller populations in the proliferative S phase and G2 phases) compared to scramble-treated controls (Figures 5E–5H). In A498 cells, cell-cycle arrest appeared to occur at the S/G2 transition, with a significant accumulation of cells in the S phase with a very small population observed in G2. Knockdown of PNCK in HEK293 cells was not associated with significant changes in cellular proliferation or cell cycle population (Figures S3C and S3D). To glean insight into the mechanisms of cell-cycle arrest, signals of key cell cycle control proteins were analyzed via immunofluorescence. IF studies determined that all k/d cells had increased p21 and p27 signals with decreased cyclin D1 and ki67 (Figures 6A–6P). Differential effects were observed in 786-O cells with decreased cyclin B1 and CDK4/CDK6 signals while no appreciable

**Figure 3. Effects of PNCK overexpression on RCC cell proliferation and cell cycle progression**

(A–D) in vitro cell growth of PNCK OE cells and GFP control cells was determined via xCelligence real-time analysis (up to 120 h) as described in STAR methods (n = 6 per cell line). Cell index was normalized to that of GFP-transfected control cells at each time point. Data are displayed as bar graphs of the average cell index at each time point with the error bars displayed as SD. Experiments were performed in sextuplicate and repeated at least 3 times. *p < 0.05, **p < 0.005, ***p < 0.0001 as determined by Student’s t-test.

(E–H) Flow cytometry analysis of PNCK overexpressing and GFP control cells was performed as in STAR methods. Data are displayed as bar graphs of the average percent population in each cell cycle phase with error bars displayed as SD. Experiments were performed in triplicate and repeated 3 times. *p < 0.05, **p < 0.005, ***p < 0.0001 as determined by Student’s t-test.
differences were noted in A 498, ACHN, or Caki-1 cells (Figure S4). These data support the hypothesis that PNCK plays a significant role in cell cycle progression at various checkpoints and that the inhibition of PNCK and CAMK signaling in kidney cancer cells is cytostatic (as opposed to cytotoxic) via cell-cycle arrest.

Pregnancy-upregulated non-ubiquitous calcium-calmodulin-dependent kinase knockdown disrupts angiogenesis, apoptosis, cell cycle, DNA-damage response related pathways

To gain a further understanding of the biological functions of PNCK and the pathways through which it exerts its effects, a targeted cancer-pathway gene array analysis was conducted (See STAR methods and Table S1). The expression of 84 diverse cancer-related genes representing effector genes in key pathways was assessed upon knockdown of PNCK with dsiRNA.

First, PNCK knockdown was associated with significant changes in the expression of angiogenesis and HIF-1α related pathway genes across all cell lines tested (Figure 7A). Specifically, angiopoietin-1 and angiopoietin-2 were downregulated with the largest magnitudes in each cell line in addition to decreases in FLT1 (VEGFR1), TEK1 (Angiopoietin-1 Receptor), MAP2K1 (MEK1) and PGF (Placental growth factor). PNCK k/d significantly decreased expression of FGF2 and CCL2 in the majority (3 out of 4 cell lines), while

Figure 4. Characterization of PNCK k/d RCC cell lines

(A–C 786-O, D–F A498, G–I ACHN, J–L Caki-1) PNCK was transiently knocked down in 786-O (A–C), A498 (D–F), ACHN (G–I), and Caki-1 (J–L) as described in STAR methods. PNCK k/d was confirmed by qPCR (Figure S4), western blot (A, D, G, and J), and immunofluorescence (B, E, H, and K). Changes in phospho-CREB expression were analyzed by immunofluorescence (C, F, I, and L). I: GFP control cells, II: PNCK overexpressing cells. Scale bar represents 10 μm. qPCR Data are represented as a bar plot of mean ± SD.
expression of VEGFC and KDR were increased in 3 of 4 cell lines. ARNT (HIF2-B) was significantly downregulated in VHL wild-type cells, Caki-1 and ACHN. The effects of PNCK k/d on ANGPT1 and ANGPT2 expression were analyzed at the protein level (by immunofluorescence), showing a marked decrease in angipoietin-1 and angiopoietin-2 expression in PNCK k/d vs control cells (Figures 8 A–8H).

Second, PNCK knockdown resulted in significant modulation in apoptosis-related genes, independent of VHL mutational status (Figure 7B). For example, PNCK knockdown led to significantly increased expression of the pro-apoptotic genes CASP7 and CASP9 in all cell lines and increase in the pro-apoptotic APAF-1 in 3 out of 4 cell lines. Significant down-regulation in the expression of the anti-apoptotic genes BIRC3, BCL2L11, and XIAP was observed in all cell lines, while a decrease in the anti-apoptotic NOL-3 was observed in 3 of 4 cell lines. The above gene expression changes were associated with increased RCC apoptosis, as demonstrated by a significant increase in caspase 3 and 7 activity in vitro in 786-0, A498, ACHN, and Caki-1 upon PNCK down-regulation (Figures 9 A–9D).

Third, in congruence with previous studies linking PNCK to the DNA damage-response, we found that PNCK k/d led to significant perturbations in DNA damage response and chromosomal and teleomeric integrity pathway genes (Figure 7D). Across all cell lines tested, TEP1, LIG4 and DDR2, and PINX1 were all significantly downregulated, with Caki-1 showing >-65-fold difference in the expression of DDR2. Conversely, all cell lines showed significant overexpression of DDIT3 (DNA Damage Inducible Transcript 3) also known as CHOP, as well as TNKS.

Cell-cycle-related genes demonstrated variability across the 4-cell lines tested with some consensus around significant downregulation of WEE131 and AURKA,32 involved in cell cycle regulation (Figure 7C). Expression of MKI67, CDC20, and STMN1 was significantly decreased in 3 of 4 cell lines upon PNCK k/d. 786-O, a cell line with p53 mutation, was a clear outlier with the upregulation of many cell-cycle-related genes in response to PNCK knockdown. Interestingly, common changes in gene expression were observed only in VHL mutant cell lines (786-O and A498), including significant upregulation of the tumor suppressors GADD45α and TERF1, as well as POLB and ERCC5. Our previously mentioned experiments confirmed the k/d of PNCK leads to cell-cycle arrest at G1 though the distinct mechanisms are likely different between...
p53-mutant and p53 wild-type cell lines. However, decreases in cyclin D1 and increase in p21/p27 were consistent across all cell lines (Figures 6A–6P).

DISCUSSION

The human genome encodes approximately 634 kinases, of which only 49 are current targets of FDA-approved oncological drugs. Renal cell carcinoma (RCC) is an example of a human malignancy where (7 FDA-approved) kinase inhibitors have led to improved outcomes, but not cures. During the last decade, kinase inhibitor research in RCC has been limited to developing more potent or selective antiangiogenic TKIs in combination with checkpoint inhibitors, while overlooking potentially promising non-TK targets. This status quo has prevented progress in the development of novel kinase inhibitor in RCC. Despite several studies associating Calcium/Calmodulin kinase activity with cancer disease progression, there has never been a small molecule inhibitor in clinical trials that targets this pathway. There are no small molecule inhibitors and no experimentally derived crystal structure for PNCK; and its substrate is not known. Yet, as most kinases, PNCK and other calcium/calmodulin-dependent protein kinases are likely targetable by small molecules and therefore potential drug targets for the development of novel first in class cancer therapeutics.

The current study demonstrates the clinical and biological relevance of PNCK, an understudied kinase, in renal cell carcinoma. Using data from TCGA, PNCK was shown to be the most overexpressed kinase in RCC, at higher levels than well-established kinases currently targeted by FDA-approved anti-RCC agents. PNCK was significantly overexpressed in both clear cell and importantly, papillary RCC, an RCC subtype lacking highly effective therapies. Not only PNCK was associated with tumor grade and stage, but importantly, its overexpression was associated with poor disease-specific survival (Figure 1). The above results validate a
prior study with smaller number of patients, where overexpression of PNCK (by IHC determination) was found to be an independent negative prognostic factor. Moreover, our observations support other reports demonstrating the prognostic significance of PNCK in other cancers, such as breast, hepatocellular and nasopharyngeal carcinoma.

In this report, we demonstrate that PNCK plays an important role in RCC proliferation in vitro, as demonstrated by the promotion of cell growth when PNCK was overexpressed, while growth was significantly inhibited upon PNCK knockdown. Inhibition of cell growth by PNCK knockdown was associated with cell-cycle arrest and apoptosis. These effects were observed across the 4 cell lines studied, including VHL mutant (786-O, A498) and VHL wild type (Caki-1) clear cell RCC lines, as well as in the papillary RCC cell line, ACHN, underscoring the biological relevance, and therapeutic potential of PNCK inhibition across different RCC subtypes. Interestingly, the effects of PNCK overexpression and knockdown were absent in control HEK293 cells, suggesting that cancer-promoting PNCK activity may require an oncogenic milieu.

Our findings strongly suggest that PNCK regulates CREB phosphorylation in RCC cell lines. This was demonstrated by increased pCREB signal (by IF) in PNCK overexpressing (Figure 1) and a marked decrease in pCREB levels when PNCK is knocked down (Figure 4). CREB is a transcription factor known to play a critical role in oncogenesis and progression in multiple cancers, including RCC. Wang et al. reported that targeting CREB is associated with the inhibition of RCC growth and metastatic abilities. CREB regulates the expression of several cell cycle checkpoints, such as cyclin A and D1, and is crucial in cell cycle progression in cancer. CREB inhibition induces cell-cycle arrest in the S phase, followed by apoptosis in esophageal cancer cells, while treatment with a CREB inhibitor caused arrest at the G2/M transition in non-small cell lung cancer. Decreased pCREB expression upon PNCK knockdown was associated with cell-cycle arrest in RCC cell in vitro, with G1 arrest in 786-O, ACHN, and Caki-1 cells, and arrest in the S-phase in A498 cells (Figure 5). Moreover, PNCK k/d decreased cyclin D1 and increased p21 and p27 expression (Figure 6). Our gene expression studies (Figures 7A–7H) showed that PNCK down-regulates WEE-1 and AURKA, genes that play a critical role in cell cycle regulation. To further support the above findings, additional IF studies showed that PNCK k/d led to decreased levels if ki67 (Figure 6), cyclin A, and cyclin B (Figure S5).

Figure 7. Transcriptional perturbation of cancer pathways upon PNCK knockdown
Cy3-control and PNCK knockdown RCC cell lines were generated as in STAR methods. At 48 h post-transfection, RNA was extracted from cells and was used to assay 84-cancer pathway-related genes in a qPCR array (See Table S1, RT² Caner Profiler Array Data). (A–D) HeatMap of differentially expressed Angiogenesis (A), Apoptosis (B), Cell cycle (C), and DNA damage (D) pathway genes in 4 RCC cell lines. Colors represent Log2fold change compared to control with white representing 0. Exact values for each gene are found in Table S1. (E–H) Bar plot representation of differentially expressed Angiogenesis (A), Apoptosis (B), Cell cycle (C), and DNA damage (D) pathway genes in 4 RCC cell lines.
PNCK down-regulation induced RCC apoptosis in vitro, as evidenced by significant upregulation of pro-apoptotic and down-regulation of anti-apoptotic genes upon PNCK knockdown (Figure 7B). Gene expression findings were further validated by the demonstration of increased caspase 3 and 7 activity in PNCK k/d in all RCC cell lines tested (Figure 9). PNCK knockdown and knockout have been shown to induce apoptosis in vitro and vivo in nasopharyngeal carcinoma.\(^\text{10}\) Transcriptomic analyses found significant alterations to the PI3K/Akt/mTOR signaling pathways, offering a clue to the mechanism of apoptosis. Our western blot analyses only saw decreased pAkt signal in VHL-mutant cell line 786-O (data not shown). The mechanism by which PNCK downregulation causes apoptosis is likely multifactorial, though centered around the downregulation of CREB-mediated genes, prolonged cell-cycle arrest, and increased DNA damage burden.

Prior work suggests that PNCK plays a role in the DNA damage pathway, as knockout of PNCK sensitized cells to DNA-damaging chemotherapies such as carmustine and temozolomide\(^\text{42}\) and also led to increased chromosomal instability.\(^\text{43}\) The present study provides evidence that PNCK knockdown leads to significant decreases in DDB2 and LIG4 and in all 4 RCC cell lines (Figure 7). DDB2 encodes for DNA Damage Binding Protein 2 while LIG4 encodes for DNA Ligase 4, which plays an important role in DNA damage responses, apoptosis inhibition, and chemoresistance.\(^\text{44}\) In triple-negative breast cancer, DDB2 inhibition induces cell-cycle arrest and apoptosis. LIG4 is involved in double-stranded DNA breaks and NHEJ and is frequently overexpressed in tumors.\(^\text{45}\) Expression of DDIT3 (DNA Damage Inducible Transcript 3), the gene that encodes for CHOP, whose overexpression is linked to cell-cycle arrest and apoptosis,\(^\text{46}\) was significantly increased in all 4 cell lines in response to PNCK knockdown. The above results suggest that PNCK may be involved in DNA damage response pathways and that PNCK knockdown, by disrupting these pathways, triggers cell-cycle arrest, apoptosis, and may sensitize cells to DNA-damaging agents. This is of particular interest in the treatment of chemo- and radio-insensitive tumors (such as RCC), as pharmacological PNCK inhibition may sensitize tumors to DNA-damaging agents or cell cycle inhibitors.

Tumor angiogenesis is a predominant feature in renal cell carcinoma,\(^\text{47}\) therefore, the identification of novel antiangiogenic strategies in RCC is an urgent need.\(^\text{4}\) We provide evidence that PNCK targeting downregulates hypoxia and angiogenesis signaling pathways, including CA9, ANRT, LDH ANGPT1, ANGPT2, TEK, Pgf, MAP2K1, and FLT1 (Figure 7A). Inhibition of Angiopoietin-1 and 2 expressions was marked and was confirmed in all PNCK k/d RCC lines tested, by immunofluorescence (Figures 8A–8H). The prominent effects of PNCK k/d on these 2 angiogenic cytokines suggest that PNCK may regulate tumor angiogenesis via angiopoietin signaling. The mechanisms of PNCK regulation of angiogenesis are not well understood. Sang et al. reported that a long-noncoding RNA (called CamK-A) activates PNCK, leading to calcium-dependent NFKB activation, and subsequent remodeling of the tumor microenvironment, angiogenesis, and tumor progression. CamK-A knockdown led to decreased tumor angiogenesis and growth in vivo.\(^\text{48}\) Our results support the concept of PNCK targeting may represent an alternative, non-canonical antiangiogenic strategy, which could be useful in the setting of advanced RCC refractory

**Figure 8.** PNCK knockdown significantly alters angiogenesis pathways in all 3 kidney cancer cell lines
(A–H) (A–D) angiopoietin 1, (E–H) angiopoietin 2: I: Cy3 control, II: PNCK K/d. (Left panel = Primary antibody, right panel = DAPI merged image). PNCK was knocked down in all 4 RCC Cell lines as described in STAR methods. IF images were taken at 72 h post-dsRNA transfection. Scale bar represents 10 µm.
to currently approved anti-VEGF receptor tyrosine kinase inhibitors. All the above findings suggest that PNCK is a potential target for renal cell carcinoma biotherapies. These results will require further validation using \textit{in vivo} models of PNCK blockade in human and murine RCC, which are not currently available, but are the focus of our current research efforts.

In summary, this is the first report to our knowledge characterizing the cellular and molecular effects of PNCK and PNCK inhibition in renal cell carcinoma \textit{in vitro}. We showed that PNCK inhibition exerts direct antitumor effects, by the inhibition of cell growth, induction of RCC cell-cycle arrest and apoptosis, as well as indirect effects, by regulating the expression of angiogenesis and DNA damage response pathways. The above data strongly suggest that PNCK represents a potentially promising target for novel, urgently needed RCC biotherapies, to improve outcomes and bring hope to patients with advanced renal cell carcinoma. Studies aimed at the development of small molecule PNCK inhibitors, as well as \textit{in vivo} and preclinical studies aimed at genetic and pharmacological targeting of PNCK in RCC and other cancers are underway.

**Limitations of the study**

Our results demonstrate that PNCK overexpression is associated with poor clinical outcomes in RCC and that targeting PNCK may represent a promising therapeutic strategy for this disease. Limitations of the study include the lack of a PNCK-targeted small molecule inhibitor, which will further validate our findings. In addition, our \textit{in vitro} findings require \textit{in vivo} validation using appropriate human RCC models. Results from analysis of the TCGA RCC cohort, which demonstrate a statistically significant correlation between PNCK expression, TNM stage, and disease-specific survival Studies aimed at developing \textit{in vivo} genetic models of PNCK silencing and at identifying novel small molecule chemical inhibitors of PNCK are currently underway.\textsuperscript{48}

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
  - Lead contact
  - Materials availability
  - Data and code availability
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - Cell lines
- **METHOD DETAIL**
  - TCGA transcriptomics analysis
  - Real-time quantitative PCR and analysis
- SDS protein gel and Western blot analysis
- Generation of PNCK overexpressing cell lines
- PNCK knock-down
- Immunofluorescence (IF)
- XCelligence real time cell analysis (RTCA) cell proliferation assay
- Cell cycle analysis by flow cytometry
- RT-q profiler cancer pathwayfinder array
- Caspase Glo 3/7 apoptosis assay

QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105621.

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AUTHOR CONTRIBUTIONS

DE, VC, SC, and JM were involved in the design of all experiments and analyses. DE performed TCGA analysis. DE, VC, and FB performed in vitro cell experiments and DE and VC analyzed all data. DE and VC wrote the article while all authors contributed to editing and formatting of the article.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Phospho-CREB (Ser133) rabbit monoclonal antibody | Cell Signaling | RRID:AB_2561044 |
| PNCK rabbit polyclonal antibody | Invitrogen | RRID:AB_2818534 |
| Angiopoetin-1 goat polyclonal antibody | Abcam | RRID:AB_11156692 |
| Angiopoetin-2 rabbit polyclonal antibody | Abcam | RRID:AB_2226229 |
| Cyclin D1 rabbit monoclonal antibody | Abcam | RRID:AB_443423 |
| p21 rabbit monoclonal antibody | Abcam | RRID:AB_10860537 |
| p27 Kip1 rabbit monoclonal antibody | Cell Signaling | RRID:AB_2077836 |
| Cyclin A2 rabbit monoclonal antibody | Cell Signaling | RRID:AB_2909603 |
| Cyclin B2 rabbit monoclonal antibody | Abcam | RRID:AB_297514 |
| ki67 rabbit polyclonal antibody | Abcam | RRID:AB_443209 |
| GAPDH rabbit monoclonal antibody | Abcam | RRID:AB_2630358 |
| Bacterial and virus strains |        |            |
| PNCK Lenti-ORF Particles | Origene | RC22921SL4 |
| Critical commercial assays |        |            |
| RNAeasy Mini Kit | Qiagen | 74104 |
| Quantitect Reverse Transcriptase Kit | Qiagen | 205311 |
| PrimeTime Gene expression Master Mix | IDT | 1055772 |
| RT2 First Strand Kit | Qiagen | 330401 |
| RT2 Profiler Human Cancer PathwayFinder PCR Array | Qiagen | PAHS-033Z |
| Caspase Glo 3/7 Apoptosis Assay | Promega | G8090 |
| Experimental models: Cell lines |        |            |
| A-704 | ATCC | ATCC® HTB-45 |
| 786-O | ATCC | ATCC® CRL-1932 |
| ACHN | ATCC | ATCC® CRL-1611 |
| Caki-1 | ATCC | ATCC® HTB-46 |
| HEK293 | ATCC | ATCC® CRL-1573 |
| A498 | ATCC | ATCC® HTB-44 |
| HREC | ATCC | ATCC® PCS-400-012 |
| HUVEC | ATCC | ATCC® CRL-1730 |
| Oligonucleotides |        |            |
| dsiRNA: rArArG rArUrC rArUrG rGrUrC rUrCrU rGrArC rUrUrU rGrG A C | IDT | PNCK 13.1 |
| dsiRNA: rArCrA rUrCrA rGrCrA rGrCrG rUrCrU rArCrG rArGrA rUrCC G | IDT | PNCK 13.2 |
| dsiRNA: rGrGrG rArUrC rUrCrG rUrArG rArCrG rGrUrG rArUrG rUrCrC | IDT | PNCK 13.3 |
| PNCK Primers Forward: TTTGACTCT CCTTCTGGGATG. Reverse: GTT GGCAGTGAACCTCCTT. | IDT | NM_001039582(2) |
| PNCK Primers Reverse: GTT GGCAGTGAACCTCCTT. | IDT | NM_001039582(2) |

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RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jaime R. Merchan (jmerchan2@med.miami.edu).

Materials availability
This study did not generate any new, unique reagents, cell lines or animal models.

Data and code availability
- All generated data from RNA-array analysis is available in the supplement.
- No other data generated is necessary for repository deposition.
- No new code has been generated in this manuscript.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines
A-704 (ATCC® HTB-45™), 786-O (ATCC® CRL-1932™), ACHN (ATCC® CRL-1611™), Caki-1 (ATCC® HTB-46™), HEK293 (ATCC® CRL-1573™), A498 (ATCC® HTB-44™), HREC (ATCC® PCS-400-012) and HUVEC (ATCC® CRL-1730™) cells were all obtained from ATCC. ACHN, A704 and HEK293 cells were cultured using EMEM (Eagle’s minimal essential medium). HUVEC cells were grown with F-12K Medium with Heparin and Endothelial cell growth supplement. 786-O and A498 cells were cultured with RPMI 1640 Media. Caki-1 cells were cultured with McCoy’s 5A Modified Medium. All cell media was supplemented with 10% Fetal Bovine Serum and 1X penicillin/streptomycin. For all transfections, antibiotics were excluded from media. Cells were grown and maintained at 37°C, 5% CO2. Frozen cultures were stored at −80°C or long-term in liquid nitrogen.

METHOD DETAILS

TCGA transcriptomics analysis
Analysis of Pharos and TCGA data was executed as described by Essegian et al. Understudied kinases were identified in Pharos and IDG (https://commonfund.nih.gov/idg/understudiedproteins) and were queried in TCGA for differential expression. Transcriptomic data was normalized using the R package LIMMA and differential expression was calculated using the TCGAbiolinks R package with RSEM converted to Log2 expression. Only primary tumor samples were included in the analyses- excluding secondary or metastatic tumor samples. KIPAN (Pan-Kidney) analyses were done by combining KICH, KIRC and KIRP datasets. Kaplan-Meier (KM) curves were generated using Prism and Xena. Cohorts for KM analyses were split into high and low expressers based on the median expression PNCK. Statistical analyses were calculated using Prism- including students t-test and ANOVA to determine difference in expression between normal and tumor tissues and amongst tumor grades and stages.

Real-time quantitative PCR and analysis
Total RNA was extracted from cells grown to 80% confluence using the Qiagen (Hilden, Germany) RNAeasy Mini Kit (Cat No: 74104). Quantity and purity of RNA was assessed using the ThermoFisher Scientific (Waltham, MA, USA) NanoDrop OneC Microvolume UV-Vis Spectrophotometer. RNA was reverse transcribed into cDNA using Qiagen Quantitect Reverse Transcriptase kit (Cat No: 205311). cDNA was diluted to 50 ng/µL and stored at −20°C until further use. qPCR primers were designed from IDT (Coralville, IA, USA) against
ref seq NM_001039582(2) which targets exons 9–11 and detects all variants and isoforms of PNCK. Forward: TTTGACTCT CCTTTCTGGGATG. Reverse: GTT GGCAGGTGAACCTCTT. cDNA was amplified using 10 ng of cDNA and IDT PrimeTime Gene expression Master Mix (#1055772) and a final reaction volume of 20uL. qPCR experiments were run on the Biorad CFX96 and gene expressions were measured relative to GAPDH. No reverse transcriptase (NRT) and no template control (NTC) were used to evaluate the signal contamination of genomic DNA or background from primers. The amplification conditions for the quantitative PCR (qPCR) reactions were as follows: 1 cycle of initial denaturation at 95°C for 3 min followed by 40 cycles of denaturation at 95°C for 30s and annealing at 60°C for 1 min. All reactions were performed in quadruplicate. qPCR data was analyzed by the comparative threshold (ΔΔCt) method. The derived ΔΔCt values were converted into fold-difference values.

**SDS protein gel and Western blot analysis**
Protein concentration was quantified using the Bradford protein assay (#23236, ThermoFisher Scientific, Waltham, MA, USA) following manufacturer’s recommendations. Western blots were run using 25–50ug of protein per well with pre-cast gradient gels. Gels were treated with 8M Urea and transferred to nitrocellulose film overnight at RT. Membranes were blocked for two hours with 5% BSA or 5%/milk (depending on manufacturers suggestions for antibody). For PNCK (# ab235093, Abcam Cambridge, MA, USA), membranes were blocked with BSA and incubated overnight with primary antibody at a 1:500 dilution. Membranes were then washed 3x with TBS/Tween and incubated at RT for 1 h with secondary antibody. Chemiluminescence peroxidase activity was revealed with the Thermo Scientific SuperSignal West kit. Bands were visualized using ChemiDoc™ MP Imaging System (Biorad, Hercules, CA, USA). Protein loading was determined by GAPDH analysis (# ab181602, 1:10000 dilution; Abcam Cambridge, MA, USA).

**Generation of PNCK overexpressing cell lines**
Stable PNCK overexpressing cells were generated via lentiviral transduction Lenti ORF particles of PNCK (transcript variant 1) obtained from Origene (Rockville, MD, USA) (Cat #RC229215L4) at a titer of >1 × 10^7 TU/mL. PNCK ORF and control particles were built with the pLenti-P2A-Puro vector. Cells were plated in a 48-well plate to 50% confluency and were infected with the particles at an MOI of 25 as per manufacturers recommendations. At 48 h post-infection, GFP positivity was detected via fluorescence microscopy. Cells were then split and single colonies were isolated and selected with puromycin (1-2 ug/mL) for 7–10 days. After the first round of single colony selection, cells were sorted using flow cytometry selecting for the top 1% of GFP expression. A second round of single colony selection was done on the sorted cells. Overexpression was confirmed via qPCR and western blot and colonies with the highest level of expression were chosen for further experiments.

**PNCK knock-down**
Double stranded siRNAs (dsiRNA) were obtained from IDT DNA Technologies (Coralville, IA, USA). The predesigned sequences were from the TriFECTa Kit which consisted of 3 PNCK dsiRNAs (13.1 rArArG rAr UrC rArUrG rGrUrC rUrCrU rGrArC rGrUrU rGrGA C, 13.2 rArCrA rUrCrA rGrCrA rGrCrG rUrCrU rArCrG rAr GrA rUrCC G, 13.3, rCrGrG rArUrC rUrCrG rUrArG rArCrG rCrUrG rCrUrG rArUrG rUrCrC), Negative controls and resuspension buffer. To determine the most potent and selective dsiRNA, all three were tested (13.1, 13.2 and 13.3) at concentrations from 1 nM to 20 nM. Cells were grown in 10 cm plates to 60–70% confluence and dsiRNA was added using a mixture of Optimem Serum-Free Media and Lipofectamine RNAiMax (#13778030, ThermoFisher Scientific, Waltham, MA, USA). At 24 h, Cy3-labeled scrambled transfection controls were used to confirm >90% transfection efficiency via microscopy. PNCK knock down was confirmed at different time-points post-transfection via qPCR, western blot and immunofluorescence. Dose response curves determined that siRNA #2 delivered the greatest levels of knockdown with low toxicity at 10 nM. For A498 cells, the greatest knockdown was achieved using siRNA #1 at 20 nM. Increasing the concentration >25 nM to 50 nM did not result in increased levels of knockdown.

**Immunofluorescence (IF)**
40,000 cells/well were seeded in Labtek slides, followed by fixation in Formalin after overnight incubation. After fixing, the background produced from formalin was quenched with NH4Cl for 15 min and then samples were blocked and permeabilized with 0.5% Goat serum + Triton-X 0.3% in PBS for 2 h, prior to overnight incubation with primary antibodies: (p-CREB: 1:1000 (Cell Signaling 9198), PNCK 1:250 (Invitrogen PAS-99601) Angiopoietin 1 (Abcam 133425) 2.5μ/mL, Angiopoietin 2 (Abcam 153934) 1:500, Cyclin D1
XCelligence real time cell analysis (RTCA) cell proliferation assay
Cells were grown in T75 flasks to 60–80% confluency. The xCelligence RTCA (Agilent and ACEA, Santa Clara, CA, USA) 96-well electronic microtiter plate (E-plate) was first “blanked” with 100µL of cell culture media in every well to test background electrical impedance. Cells were then trypsinized and counted using a hemocytometer to plate 6,000–10,000 cells per well in a 96-well xCelligence E-plate in an additional 100µl of culture media (for a final well volume of 200µL). Growth was monitored in real-time for up to 120 hours with impedance assessed at 15-30-minute intervals using the xCelligence Real-time Cell Analyzer (RTCA). Data was exported from the xCelligence system and analyzed per individual well. Cell growth results were shown as bar graphs showing the averages (+/− SD) of experiments done at least in sextuplicate.

Cell cycle analysis by flow cytometry
To assess the effect of PNCK activity on cell cycle progression, flow cytometry was utilized to quantify the relative populations of cells in each stage of the cell cycle. For overexpression experiments, Cells were grown in 10 cm plates to 60–80% confluence and were trypsinized and washed 3x with ice cold PBS. Cells were then fixed and permeabilized with 70% ethanol overnight at −20°C. For knockdown experiments, cells were first synchronized by serum starvation overnight using 1% FBS media before being transfected with dsiRNA. Cells were then fixed and permeabilized with 70% ethanol overnight at −20°C at desired post-transfection time-points. Samples were then treated with a mixture of RNase-H and Propidium Iodine and incubated at 37°C for 1 hour. Using the LSR-II Cytometer, samples were analyzed. 50–100K events were recorded at a low-voltage setting with a slow flow rate. Side scatter and forward scatter gating was employed to eliminate both apoptotic cells and clumps of cells so that only single cells were used in the final analysis. FCS files were exported and analyzed using FlowJo v.10.6.2 (https://www.flowjo.com/solutions/flowjo). Experiments were run in sextuplicate and repeated at least 3 times. Student’s T-test was used to determine significant changes in cell cycle population ratios in treated and control cells.

RT² profiler cancer pathwayfinder array
Cell lines were treated with siRNA or control siRNA as described above with 3 biological replicates and 3 technical replicates. At 72 h post transfection, RNA was extracted and purified as described above. RNA was reverse transcribed using the RT2 First Strand Kit (Qiagen, 330401), and cDNA was then added with SYBR green mastermix (Qiagen, 330504) to 96-well RT2 Profiler Human Cancer PathwayFinder PCR Array plates (Qiagen, PAHS-0332), according to the manufacturer’s instructions. Quantitative PCR was run as described previously and data was analyzed using Qiagen’s GeneGlobe Data Analysis center, using both ACTB and GAPDH as housekeeping genes. Input genes for each cell line studied were either significantly up-regulated or downregulated genes from the RT2 Profiler Cancer PathwayFinder array with a p-value <0.05.

Caspase Glo 3/7 apoptosis assay
Cell lines were treated with siRNA or Cy3-control siRNA at 10 nM as described above in replicates of 6 and were transferred to 96-well plates. For A498 cells, siRNA #1 was used at a concentration of 20 nM. Cell count was normalized to 8,000 cells per well prior to adding Glo reagent at 72 h post transfection, caspase activity was detected using Promega Caspase Glo 3/7 Apoptosis Assay and Promega Luminometer. Luminescence signal was corrected with blank and untreated cells.

QUANTIFICATION AND STATISTICAL ANALYSIS
In vitro data are presented as means ± SD. All in vitro experiments were performed at least in triplicate unless otherwise specified and repeated at least twice. Statistical analysis among groups was performed by ANOVA, and sub-group comparisons were made with the student’s T-test, as appropriate. Disease Specific survival was analyzed by the Kaplan–Meier method and differences were analyzed by the log-rank test. Statistical significance was displayed in figures at *p < 0.05, **p < 0.01, ***p < 0.001, with adjustments for multiple comparisons as appropriate. p value < 0.05 were considered statistically significant. All statistical tests were two-sided.