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PDK4-deficiency results in expedited cellular proliferation through E2F1-mediated increase of cyclins

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Running title: PDK4 and arsenic crosstalk in HCC cell proliferation

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Non-standard abbreviations:

HCC, Hepatocellular carcinoma; PDK4, pyruvate dehydrogenase kinase 4; CDK, cyclin dependent kinases; PDC, pyruvate dehydrogenase complex; NR, Nuclear receptors; SHP, Small heterodimer partner; DNMT, DNA methyltransferase; MeDIP, Methylated DNA-IP-on-ChIP; LD, light/dark cycle

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Abstract

Hepatocellular carcinoma (HCC) is a common form of cancer with prevalence worldwide. There are many factors that lead to the development and progression of HCC. The aim of this study was to identify potential new tumor suppressors and examine their function as cell cycle modulators and investigate their impact on the cyclin family of proteins and cyclin dependent kinases. In this study the pyruvate dehydrogenase kinase 4 (PDK4) gene was shown to have potential tumor suppressor characteristics. PDK4 expression was significantly downregulated in human HCC. *Pdk4*$^{-/-}$ mouse liver exhibited a consistent increase in cell cycle regulator proteins including Cyclin D1, Cyclin E1, Cyclin A2, some associated cyclin dependent kinases (CDKs), and transcription factor E2F1. *PDK4*-knockdown HCC cells also progressed faster through the cell cycle, which concurrently expressed high levels of cyclins and E2F1 as seen in the *Pdk4*$^{-/-}$ mice. Interestingly, the induced Cyclin E1 and Cyclin A2 caused by *Pdk4*-deficiency was repressed by arsenic treatment in mouse liver and in HCC cells. E2f1-deficiency in *E2f1*$^{-/-}$ mouse liver or knockdown E2F1 using shRNAs in HCC cells significantly decreased CYCLIN E1, A2, and E2F1 proteins. In contrast, inhibition of PDK4 activity in HCC cells increased CYCLIN E1, A2, and E2F1 proteins. These findings demonstrate that PDK4 is a critical regulator of hepatocyte proliferation via E2F1-mediated regulation of cyclins.

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Introduction

Liver cancer is the second leading cause of cancer related death worldwide with an estimated 745,500 deaths yearly. The majority of liver cancer deaths are identified as hepatocellular carcinoma (HCC). The mechanisms behind HCC progression are continually studied. Many factors have been identified to increase the risk of HCC including environmental factors, viral infection, alcohol consumption, and smoking (Kanda et al 2015). Cyclins are vital cell cycle regulators that normally function to assure the control of cellular proliferation. The cyclin family of proteins and associated cyclin dependent kinases have been shown to be significantly elevated in HCC tissues (Masaki et al 2003). Identification of cyclin modulators will aid in the understanding of HCC development and progression.

Pyruvate dehydrogenase kinase 4 (PDK4) is a mitochondrial protein with a histidine kinase domain which inhibits the pyruvate dehydrogenase complex (PDC). Inhibition of the PDC results in reduced conversion of pyruvate to acetyl-CoA. Acetyl-CoA is used in the citric acid cycle in order to carry out cellular respiration (Sugden and Holness 2003). PDK4 is a major factor in cellular respiration since it works to inhibit the progression from glycolysis to the citric acid cycle. Cellular respiration is a major physiological factor that has been shown to be altered in cancer cells (Scatena 2012), therefore PDK4 is a prime molecular suspect in crosstalk between cellular respiration and cell cycle progression. Because the cells of the liver are highly aerobic and metabolically active, liver tissue and associated cells serve as excellent test subjects.

Identified as a group 1 carcinogen; Arsenic is the 20th most common element in the earth crust (Humans 2012). Over 100 million people worldwide rely on arsenic contaminated drinking water making it a global health concern (Polya and Charlet 2009). Arsenic has been reported to induce epigenetic alterations related to HCC progression (Liu et al 2014) and silence hepatic PDK4 expression through activation of histone H3K9 methylatransferase G9a (Zhang et al 2016).

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Nuclear receptors (NRs) are a class of proteins that mediate the activity of hormones and have been implicated in various diseases including HCC (Rudraiah et al 2016). Small heterodimer partner (SHP) is a unique transcriptional repressor (Zhou et al 2010) and has been implicated as a critical inhibitor of HCC progression (Zhang et al 2011b) by regulating multiple pathways involved in tumor growth, including cell proliferation (Zhang et al 2008), apoptosis (Farhana et al 2007, Zhang et al 2010), and migration and invasion (Yang et al 2016). Intriguingly, SHP represses DNA methylation via suppressing DNA methyltransferase (DNMT) expression (Zhang and Wang 2011, Zhang et al 2012), suggesting that modulating SHP function by its agonist may be useful to develop epigenetic based therapeutic treatment for HCC.

In this current study PDK4 was identified as a potential tumor suppressor gene using an unbiased approach, i.e. a combination of Methylated DNA-IP-on-ChIP (MeDIP) and RNA-sequencing (RNA-seq) methods in *Shp*$^{-/-}$ mice. The expression of PDK4 was markedly reduced in human HCC specimens and in mouse liver tumors. The *Pdk4*$^{-/-}$ liver had a significant induction in multiple cyclin proteins with the most striking elevation of CyclinE1, CyclinA2, and E2F1, while Pdk4 knockdown HCC cells exhibited a similar activation of cyclin proteins and progressed faster through the G2/M phase of cell cycle compared to control cells. Interestingly, Arsenic decreased the levels of cyclin proteins in both mouse livers and cultured HCC cells that were induced by *Pdk4*-deficiency. Overall, our study revealed a novel function of PDK4 in cell cycle control, suggesting a critical role of PDK4 in HCC progression.

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Materials and Methods

Mouse study and Treatment

Wild-type (WT) and *Shp*$^{-/-}$ mice were described previously (Lee et al 2015, Wang et al 2003). *Pdk4*$^{-/-}$ mice were kindly provided by Dr. Robert Harris (Hwang et al 2009). All mice were of C57BL/6J background and fed a standard rodent chow with free access to water and maintained in a 12 hours light/dark (LD) cycle (light on 6 AM to 6 PM), temperature-controlled (23°C), and virus-free facility. Experiments were performed on male mice at the age of 8 to 12 weeks unless stated otherwise (n=5/group). Experimental groups included WT control (WT Con), WT with arsenic exposure (WT As), *Pdk4*$^{-/-}$ control (*Pdk4*$^{-/-}$ Con), and *Pdk4*$^{-/-}$ with arsenic treatment (*Pdk4*$^{-/-}$ As). For arsenic treatment, mice were fed with 50 ppm $NaAsO_2$ (Sigma) via distilled drinking water, which was replaced every other day for 4 weeks (Zhang et al 2016). RNA-seq and MeDIP were conducted at the Microarray and Genomic Analysis Core Facility at the University of Utah as described previously (Smalling et al 2013). Immunohistochemistry (IHC) staining was performed on paraffin embedded liver section slides with PCNA antibody (Cell Signaling 13110). ImmPACT DAB Peroxidase (Vector Laboratories SK-4105) was used for color detection with a hematoxylin counter stain. PCNA staining was conducted on five mice per group with three liver tissue sections per mouse. ImageJ software was used to quantify positive PCNA staining. Protocols for animal use were approved by the Institutional Animal Care Committee (IACUC) at the University of Connecticut.

Liver Specimens

The coded human liver specimens were obtained through the Liver Tissue Procurement and Distribution System (Minneapolis, Minnesota) and have been described previously (He et al 2008). Because we don't ascertain individual identities associated with the samples, the Institutional Review Board for human research committee at University of Connecticut determined that the project is not research involving human subjects.

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Cell culture experiments, FACS sorting, and luciferase promoter assay

Huh7 (Zhang and Wang 2011) and 293T (Yang and Wang 2012) cells were described previously. Cultured cells were maintained in DMEM (Gibco, NY) with 10% fetal bovine serum (Gibco) and 100 U/ml penicillin-streptomycin (Mediatech, VA). High (shPDK4 H) and low (shPDK4 L) efficiency PDK4 knockdown cell lines were generated by a lentiviral vector containing the shRNA along with packing vector and envelope vector. These components were co-transfected into 293T cells using X-tremeGENE™ HP DNA Transfection Reagent according to the manufacturer's protocol. The supernatant containing virus particles were collected and concentrated. Suspended virus was applied to target cells with 6 μg/ml polybrene. The shPDK4H cell line was generated using shRNA (TRCN0000006264; Clone ID: NM_002612.2-2954s1c) from Sigma-Aldrich and shPDK4 L was generated using shRNA (TRCN0000194917; Clone ID: NM_002612.2-1297s1c1) from Sigma-Aldrich. shPDK4L cells were treated with 15 μM arsenic for 24 hours. E2F1 shRNAs were from Sigma-Aldrich; shE2F1 #1: TRCN0000039659, Clone ID:NM_005225.1-502s1c1; shE2F1 #2: TRCN0000010328, Clone ID:NM_005225.x-1171s1c1. PDK4 inhibitor Diisopropylamine dichloroacetate (DADA) was from Fisher Scientific (Cat. AAH6134103).

Western blot, qPCR, Transient Transfection and Luciferase Assay

Western blot and qPCR were well established in our lab as described previously (Tsuchiya et al 2015, Yang et al 2013b). All antibodies used for western blotting are as follows, Cyclin D1 (Cell Signaling 2978), Cyclin E1 (Cell Signaling 20808), Cyclin A2 (Cell Signaling 4656), Cyclin D3 (Cell Signaling 2936), Cyclin H (cell signaling 2927), Cyclin E2 (cell signaling 4132), CDK2 (Santa Cruz 6248), CDK4 (Santa Cruz 260), β-Actin (Cell Signaling 3700) GAPDH (Cell Signaling 5174), p21 (Santa Cruz 6246), Rb (Santa Cruz 102), pRB (Cell Signaling 9307), MyB

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(Millipore 05-175), GSK3-Beata (abcam ab18893), SMAD4 (ThermoFisher MA5-14300), E2F1 (ThermoFisher 32-1400). All qPCR experiments were repeated three times with each sample run in triplicate. All western blot experiments were repeated three times. For mouse study, five mouse samples were pooled per group and the samples were loaded as singlets or duplicates. For transient transfection and luciferase reporter assay, Huh7 cells were transfected with the plasmids as indicated in the Figure legends. Transfection was carried out using Lipofectamine 2000 (Invitrogen). Luciferase activity was measured and normalized against Renilla activity (Promega). Experiments were done in three independent triplicate transfection assays (Yang et al 2013a).

Statistical analysis

Data are shown as the mean ± standard error of the mean (SEM). Statistical analysis was carried out using the Student's *t* test for unpaired data to compare the values between the two groups and one way ANOVA among multiple groups. $P<0.05$ was considered statistically significant.

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Results

An unbiased approach to identify new tumor suppressors using *Shp*$^{-/-}$ mice

In the past decade, our published results using both *in vivo* mouse and *in vitro* cell models, as well as human HCC specimens, revealed a tumor suppressive role of SHP in HCC (He et al 2008, Yang et al 2013b, Zhang et al 2008, Zhang et al 2010). More recently, our studies established a crucial inhibitory feedback loop between SHP and Dnmt1 (Zhang and Wang 2011, Zhang et al 2012). Tumor suppressors are generally downregulated in cancers by DNA hypermethylation via the activity of DNMTs (Jin and Robertson 2013). We propose that the upregulated Dnmts in *Shp*$^{-/-}$ mice due to the loss of SHP inhibition will in turn methylate other tumor suppressor genes (Fig. 1A). From this regulatory model we developed an unbiased approach to identify putative new tumor suppressors, as described below.

Two genome-wide high throughput analyses were conducted in *Shp*$^{-/-}$ mice. MeDIP was used to characterize alterations in DNA methylation of specific genes (Fig. 1B) and RNA-seq was used to determine changes in gene expression (Fig. 1C). By integrating both datasets using Bioinformatics tools and appropriate validation, we aimed at identifying genes that met the criteria by showing promoter hypermethylation and mRNA downregulation in SKO mice, the characteristics of tumor suppressors.

Integrating RNA-seq and MeDIP revealed PDK4 as a potential tumor suppressor

Shp$^{-/-}$ mice were originally generated by replacing its exon1 with the β-gal/pGKneo cassette (Wang et al 2002). The accuracy of RNA-seq was validated by the missing exon 1 of the Shp gene (Fig. 2A left, top) and the upregulation of a known SHP target gene early growth response 1 (Egr-1) in *Shp*$^{-/-}$ mice (Smalling et al 2013, Zhang et al 2011a) (Fig. 2A left, bottom). MeDIP analysis revealed enhanced DNA methylation in the Shp gene promoter in *Shp*$^{-/-}$ mice (Fig. 2A right), further supporting our proposed feedback regulatory loop between SHP and Dnmts (Fig. 1A). We integrated MeDIP and RNA-seq data and identified the top 10 genes that showed

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hypermethylation and downregulation in *Shp*$^{-/-}$ mice (Fig. 2B). We chose to focus on the pyruvate dehydrogenase kinase 4 (PDK4) gene due to its striking hypermethylated promoter and downregulated expression in *Shp*$^{-/-}$ mice (Fig. 2C). In agreement with the RNA-seq results, qPCR confirmed a marked reduction of Pdk4 mRNA in *Shp*$^{-/-}$ livers that were collected from both young (2 mon) and old (13 mon) mice (Fig. 2D). Pdk4 mRNA levels were also consistently low in *Shp*$^{-/-}$ mouse tumor tissue (*Shp*$^{-/-}$-T).

Importantly, PDK4 mRNA was significantly diminished in human HCC specimens relative to surrounding liver tissue (Fig. 2E), suggesting that a low level of PDK4 is associated with the development of HCC. Analysis of the Oncomine database revealed an overall diminished expression of PDK4 in a variety of human cancers (Supplementary Figure 1). It should be noted that other PDK isoenzymes (PDK1-3) did not show such universal downregulation (not shown), suggesting a specific regulatory role of PDK4 in HCC and other cancers.

Treatment of multiple HCC cell lines with the demethylating agent 5'-aza -2'-deoxycytidine (Aza) or histone deacetylase inhibitor trichostatin (TSA) revealed an induction of PDK4 mRNA in Hep3B and HepG2 cells by Aza and in Hep3B, MH97H and MH97L cells by TSA (Fig. 2F). In addition, Aza decreased PDK4 gene promoter methylation in HepG2 cells (Fig. 2G left), which was in agreement with the increased PDK4 mRNA (Fig. 2G right). Overall, the results suggest that PDK4 downregulation in HCC is associated with epigenetic silencing.

Arsenic repressed hepatocytes proliferation induced by *Pdk4*-deficiency

To examine the *in vivo* effect of Pdk4 in hepatocytes proliferation and the impact of arsenic, WT and *Pdk4*$^{-/-}$ mice were fed with arsenic for two weeks. Immunohistochemistry (IHC) staining for the proliferating cell nuclear antigen (PCNA) protein in arsenic treated and untreated mice showed that *Pdk4*$^{-/-}$ mice exhibited greater hepatocyte proliferation compared to WT control

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mice. The liver sections of arsenic treated mice showed decreased PCNA staining compared to clean water control mice in both WT and *Pdk4*$^{-/-}$ animals (Fig. 3A).

To further investigate the molecular components responsible for the cell proliferation changes among test groups, cell cycle proteins were analyzed via western blot. We first examined three major cyclins and observed that *Pdk4*$^{-/-}$ control livers had a drastic increase in CYCLIN E1 and A2 proteins compared to that of WT control livers (Fig. 3B). In contrast, arsenic treatment prevented the induction of CYCLIN E1 and A2 proteins in *Pdk4*$^{-/-}$ As mice compared to *Pdk4*$^{-/-}$ con mice (Fig. 3B).

We continued to analyze other cell cycle regulatory proteins by western blot in order to gain a better understanding of the overall changes. Interestingly, CYCLIN D2 and D3 levels were moderately increased in WT As vs WT Con (Fig. 3C). On the other hand, CDK2 and CDK4 were noticeably elevated in *Pdk4*$^{-/-}$ mice relative to WT mice. Among other cell cycle regulators we analyzed, transcription factor E2F1 protein was found to be highly induced in *Pdk4*$^{-/-}$ Con mice compared to WT Con mice, and its induction was prevented by arsenic treatment (Fig. 3D). Because E2F1 is a well established transcriptional activator of cyclins (Wu et al 2001), the similar expression pattern between E2F1 and CYCLIN E1 and A2 in *Pdk4*$^{-/-}$ mice suggesting that E2F1 may be responsible for the upregulation of both cyclins.

Knockdown of PDK4 expedited HCC cell progression

We next examined the effect of PDK4 in HCC cell proliferation using *in vitro* cell models. Huh7 cells were treated with Hydroxyurea in order to synchronize all cells at the G0/G1 phase (Fig. 4A). When released into cell cycle, PDK4 knockdown (shPDK4) cells progressed faster through all phases of cell cycle. At 6h shPDK4 cells had a higher percentage in S phase compared to shCon cells. By time points 9h and 12h shPDK4 cells showed a greater transition to the G2/M phase compared to shCon cells.

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Nocodazole was used to arrest Huh7 cells in the G2/M phase where a higher percent of shPDK4 cells accumulated in the G2/M phase compared to shCon cells (Fig. 4A). As time progressing shPDK4 cells appeared to maintain a constant high percent of G2/M cells compared to shCon cells (Fig. 4B).

Because CYCLIN E1 and A2 were the two most highly induced cyclins in *Pdk4*$^{-/-}$ liver (Fig. 3B), we examined their protein expression in PDK4 knockdown Huh7 cells. Two knockdown cell lines were established with moderate (Fig. 5A top) to strong knockdown efficiency (Fig. 5A bottom). In cells with moderate Pdk4 knockdown, small increases in CYCLIN A2, CDK2, and E2F1 were observed (Fig. 5B). In high Pdk4 knockdown cells, a more striking elevation of CYCLIN E1, CDK2 and E2F1 was observed (Fig. 5C). Therefore, the more efficient knockdown of PDK4 resulted in greater induction of cell cycle regulator proteins. As expected, E2F1 dose-dependently transactivated Cyclin E1 promoter activity (Fig. 5D).

The levels of CYCLIN E1 and A2 proteins were markedly diminished in *E2f1*$^{-/-}$ mouse liver compared with WT mice (Fig. 5E). In addition, knockdown E2F1 using shRNAs in Huh7 cells significantly decreased CYCLIN E1, A2, and E2F1 proteins (Fig. 5F). Furthermore, treatment of Huh7 cells with PDK4 inhibitor diisopropylamine dichloroacetate (DADA) increased CYCLIN E1, A2, and E2F1 proteins dose-dependently (Fig. 5G). Taken together, the results suggest an E2F1-dependent activation of CYCLIN E1 and A2 by PDK4.

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Discussion

The aim of this study was to identify potential new tumor suppressor genes using *Shp*$^{-/-}$ mice based on our previous findings that established a feedback regulatory loop between DNMTs and SHP (Zhang and Wang 2011, Zhang et al 2012). Tumor suppressors are often downregulated in cancers due to their promoter hypermethylation. PDK4 was identified as a putative tumor suppressor because its expression was largely diminished in human HCC specimens and other cancers.

One prime characteristic of tumor suppressor genes is their ability to inhibit tumor cell growth. Thus we examined the role of PDK4 in hepatic cell proliferation. Consistent with our hypothesis, *Pdk4*-deficient mouse liver showed increased hepatocytes proliferation, as evidenced by the activation and induction of cyclins. In addition, HCC cells with PDK4 knockdown exhibited a similar activation of cell cycle regulators, which was accompanied by the enhanced cell cycle progression. It was noted that results in HCC cells were less striking than in mouse livers, which was likely contributed by the different cellular environment in HCC cells vs mouse livers. Overall, these results strongly suggest PDK4 as an important new regulator of cell cycle.

Very little research has been done analyzing PDK4 outside of its known metabolic function. The Warburg effect suggests that cancer cells rely highly on glycolysis rather than oxidative phosphorylation (Liberti and Locasale 2016). Since PDK4 functions to inhibit PDC, which converts pyruvate into acetyl-CoA, therefore feeding the TCA cycle; PDK4 is expected to interact with other proteins leading to the subsequent inhibition in cellular proliferation. Because the levels of multiple cyclins were highly induced by *PDK4*-deficiency, it is postulated that PDK4 may repress the expression of cyclins via interaction with other transcription factors. E2F1 is a major transcriptional activator of cyclins that we observed to be strongly induced in *Pdk4*$^{-/-}$ liver

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and PDK4 knockdown HCC cells. Therefore we examined a direct protein-protein interaction between PDK4 and E2F1 by Co-IP and WB but failed to validate their interaction (not shown). As expected, E2F1 dose-dependently activated Cyclin E1 promoter activity (Fig. 5E). Unfortunately, co-expression of PDK4 with E2F1 did not alter E2F1 transactivation (not shown). These results suggest that PDK4, a mitochondria protein, does not seem to act as a transcriptional factor to enhance E2F1 activity. It is likely that there are intermediate players between PDK4 and E2F1. Future studies may focus on identifying the molecular intermediates leading to increased levels of Cyclin E1 caused by *Pdk4*-deficiency.

In this study arsenic was found to decrease Cyclin protein levels in both mouse and cultured HCC cells. This decrease in cyclin protein levels indicates a reduction in cellular proliferation. Previous studies have shown in different model organisms that inorganic arsenic treatment inhibits cell cycle progression and induces apoptosis (Sidhu et al 2006, Wang et al 2007). Our results were in agreement with the prior observations. The inhibitory effect of arsenic in HCC cell proliferation is in contrast to the general knowledge that arsenic is often considered having oncogenic property by predisposed cells for cancer development. We also noted that despite reduced hepatocyte proliferation by arsenic treatment, the overall cyclins levels were not decreased. Future studies are warranted to fully understand the action of arsenic. The results suggest that the final outcome of arsenic exposure would be largely depending on the cellular context.

In summary, the present study shows that *Pdk4*-deficiency results in increased hepatocytes proliferation as a consequence of induction of cyclins and E2F1. These results suggest that PDK4 may function as a potential tumor suppressor. Unfortunately, we are unable to establish an *in vivo* HCC model, because *Pdk4*$^{-/-}$ mice injected with a single dose of diethylnitrosamine died within a week. We are currently investigating the molecular mechanisms that may explain the causes of death. Nonetheless, identification of tumor suppressor genes and their molecular mechanisms will and has led to therapies and anticancer treatments. Future

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studies may focus on further elucidating the molecular pathway in which *Pdk4*-deficiency increases the level of Cyclin proteins, and the effects of arsenic and *Pdk4* on apoptosis in the liver.

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Authorship contributions:

Participated in research design: L.W, J.C, J.W

Conducted experiments: J.C, J.W

Performed data analysis: J.C, L.W

Wrote or contributed to the writing of the manuscript: J.C, L.W

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Footnotes

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Figure legends

Fig. 1. Regulatory model and unbiased approach to identify new tumor suppressor candidates in HCC**.** (A) A feedback inhibitory loop between small heterodimer partner (Shp) and DNA methyltransferase (Dnmts). SHP downregulation by loss of heterozygosity (LOH) in hepatocellular carcinoma (HCC) releases SHP repression of Dnmt and increases Dnmt activity. Upregulated Dnmts further methylate SHP and other tumor suppressor genes. (B) Methylated DNA-IP-on-ChIP (MeDIP) arrays. The Mouse Promoter 1.0R Array (Affymetrix) is a single array comprised of over 4.6 million probes tiled to interrogate over 28000 mouse promoter regions. (C) RNA-sequencing (RNA-seq). Pol II RNAs were isolated by a 5'-capped purification method and analyzed by RNA-seq. Mice: 2 mon male (n=5/group).

Fig. 2. Integrating MeDIP and RNA-seq results identified PDK4 as a putative tumor suppressor in HCC. (A) Left: Integrated Genome Browser visualization tracks from RNA-seq reads depicts complete loss of Shp exon1 in *Shp*$^{-/-}$ mice. Egr-1, a known target of Shp, is increased 8-fold in *Shp*$^{-/-}$ vs. WT livers. Right: Enhanced DNA methylation signal (blue lines) in the Shp gene promoter in *Shp*$^{-/-}$ vs. WT mice. (B) Genes showed good correlation between promoter methylation and gene expression in *Shp*$^{-/-}$ mice and Pdk4 was among the top 10 genes. (C) MeDIP and RNA-seq revealed Pdk4 promoter hypermethylation and gene downregulation in *Shp*$^{-/-}$ livers. (D) qPCR of Pdk4 mRNA in 2 and 13 month-old WT, *Shp*$^{-/-}$, and *Shp*$^{-/-}$-T (tumor) livers (n=5/group). (E) qPCR of PDK4 mRNA in twenty one pairs of human HCC specimens and the corresponding controls. (F) qPCR of PDK4 mRNA in HCC cells treated with Aza or TSA. (G) Left: The methylated DNA levels of PDK4 promoter CpG island were quantified using the EpiTect methyl-profiler qPCR primer assay. Right: qPCR of PDK4 mRNA. Bar graphs: Data are shown as mean ± SEM (triplicate assays). *P<0.05 vs corresponding controls.

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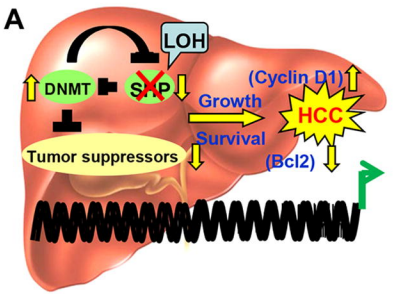
Fig. 3. *Pdk4*$^{-/-}$ livers showed increased hepatocytes proliferation which was diminished by arsenic treatment. (A) Left: Immunohistochemistry (IHC) staining for PCNA protein. Dark brown indicates positively stained nuclei. Right: Histogram showing quantification of positive PCNA staining results. *p<0.05 vs WT Con; $^{\#}$p<0.05 vs *Pdk4*$^{-/-}$ Con. (B-D) Western blot of cyclin & cyclin dependent kinase proteins, and cell cycle modulators in WT and *Pdk4*$^{-/-}$ mice treated with arsenic (50 ppm for 1 month). N=5/group, pooled samples run in duplicate lanes or single lane. Numbers indicate density relative to WT control (Con).

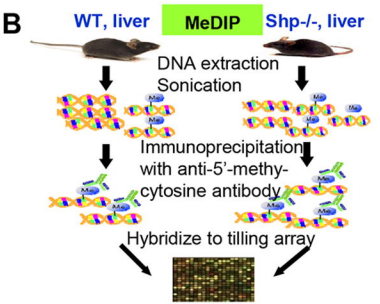
Fig. 4. FACS sorting showed rapid G2/M cell cycle progression in shPDK4 HCC cells. (A) shCTRL and shPDK4 cells synchronized in G1/S phase by Hydroxyurea (3 mM for 24h) treatment followed by FACS sorting. (B) shCTRL and shPDK4 cells synchronized in G2/M phase by Nocodazole (50 ng/ml for 16h) treatment followed by FACS sorting.

Fig. 5. Knockdown of PDK4 in HCC cells induced cell cycle proteins which was diminished by arsenic. (A) Western blot of PDK4 protein and qPCR of PDK4 mRNA in Huh7 cells showing lower (top) and higher (bottom) knockdown efficiency (shPDK4L). (B) Western blot of cell cycle proteins in control (shCTRL) or shPDK4L cells in the presence or absence of Arsenic (As) (15 μM for 24h). (C) Western blot of cell cycle proteins in shCTRL and shPDK4H cells. (D) Transient transfection assay to determine Cyclin E1 promoter reporter activity by E2F1. (E) Western blot of protein expression in WT and E2f1$^{-/-}$ mouse liver. (F) Western blot of protein expression in Huh7 cells with E2F1 knockdown using two different shRNAs against E2F1. (G) Western blot of protein expression in Huh7 cells treated with PDK4 inhibitor diisopropylamine dichloroacetate (DADA).

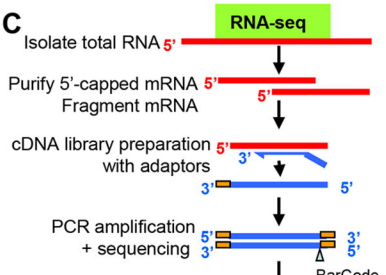
Figure 1



MeDIP (methylated DNA-IP-on-ChIP) and RNA-seq (RNA-sequencing) to identify novel tumor suppressors using Shp-/- mice



Identify hypermethylation and demethylation signals across the genome



Quantitative expression of Pol II gene RNAs

Figure 2

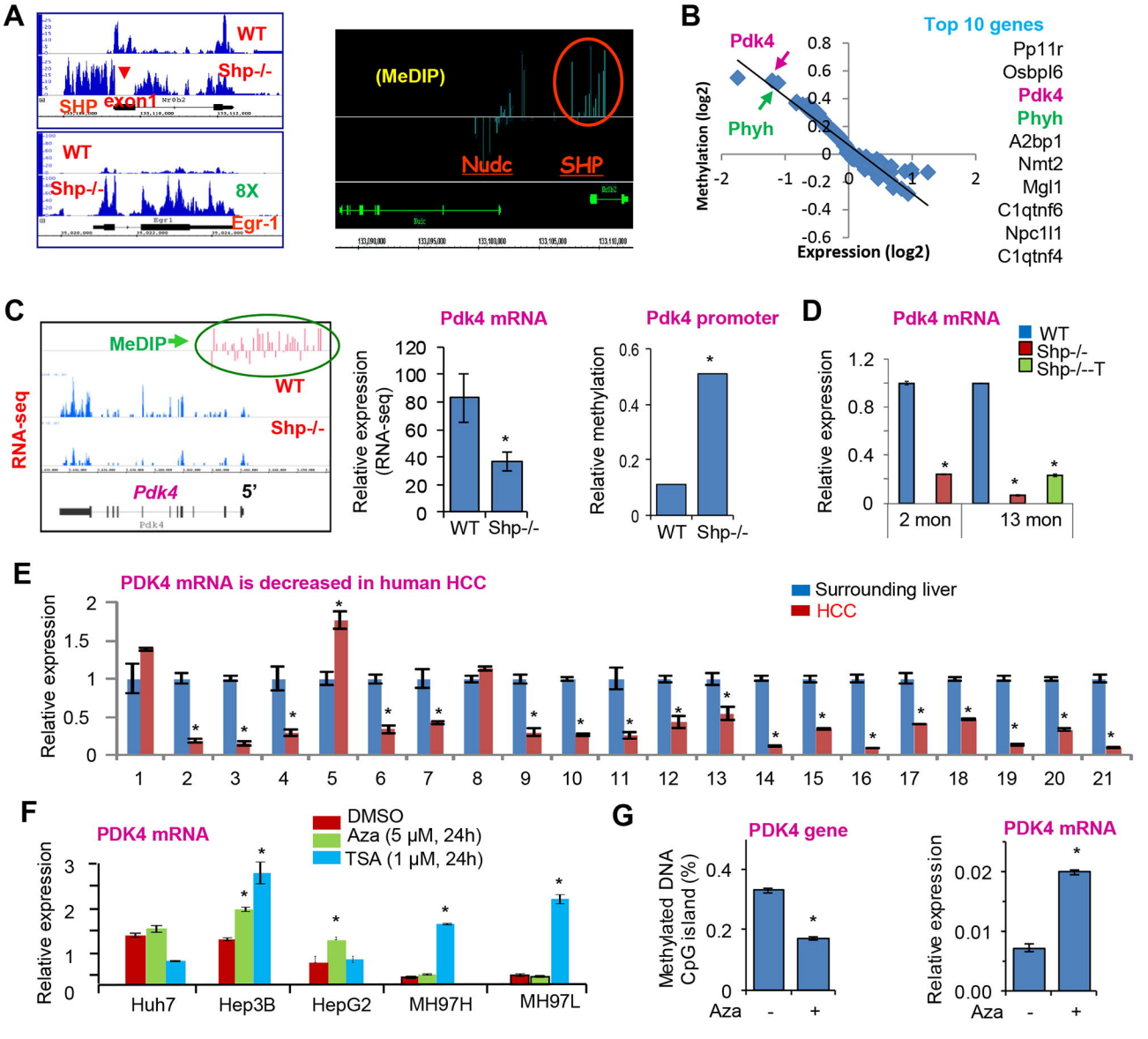

A
WT
Shp-/-
SHP exon1
WT
Shp-/-
8X
Egr-1
(MeDIP)
Nudc
SHP
B
Top 10 genes
Pdk4
Phyh
Methylation (log2)
Expression (log2)
Pp11r
Osbpl6
Pdk4
Phyh
A2bp1
Nmt2
Mgl1
C1qtnf6
Npc1l1
C1qtnf4
C
RNA-seq
MeDIP
WT
Shp-/-
Pdk4
5'
Pdk4 mRNA
Relative expression (RNA-seq)
WT
Shp-/-
Pdk4 promoter
Relative methylation
WT
Shp-/-
D
Pdk4 mRNA
Relative expression
WT
Shp-/-
Shp-/--T
2 mon
13 mon
E
PDK4 mRNA is decreased in human HCC
Relative expression
Surrounding liver
HCC
F
PDK4 mRNA
Relative expression
DMSO
Aza (5 μM, 24h)
TSA (1 μM, 24h)
Huh7
Hep3B
HepG2
MH97H
MH97L
G
PDK4 gene
Methylated DNA CpG island (%)
Aza
PDK4 mRNA
Relative expression
Aza

Figure 3

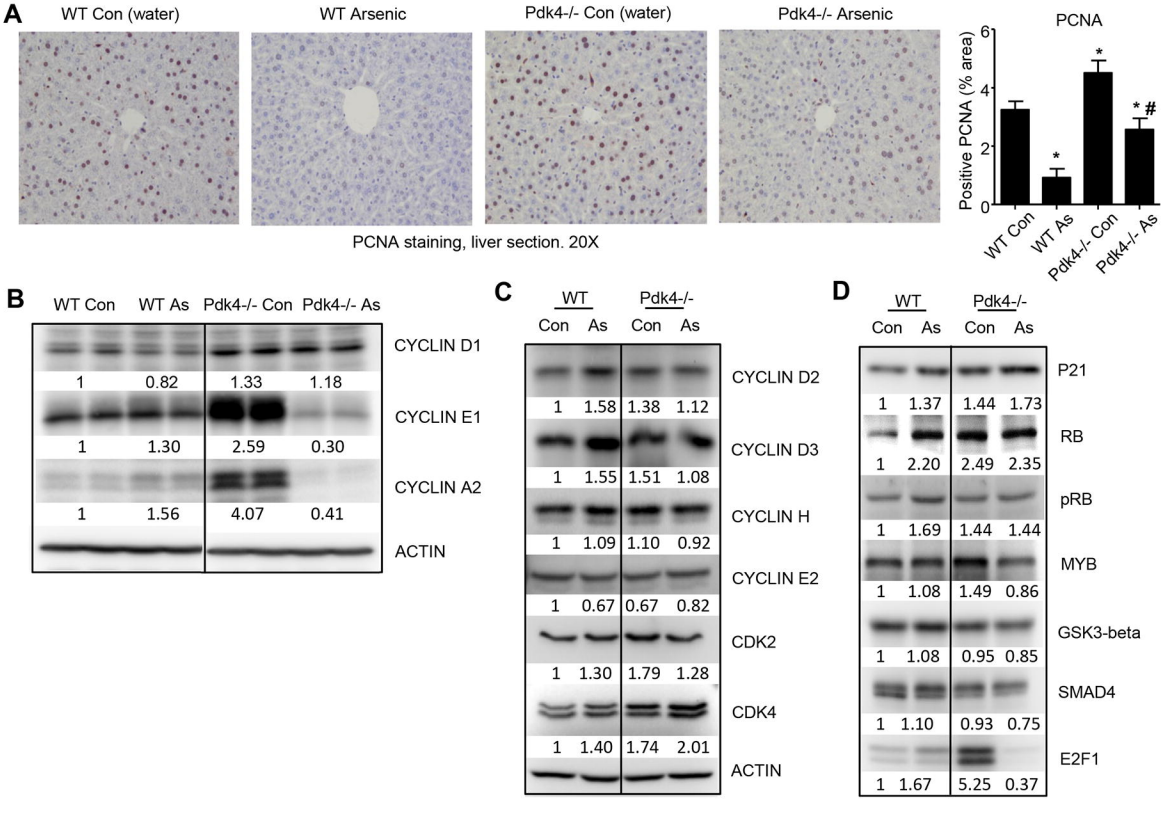

A
WT Con (water)
WT Arsenic
Pdk4-/- Con (water)
Pdk4-/- Arsenic
PCNA staining, liver section. 20X
PCNA
Positive PCNA (% area)
WT Con
WT As
Pdk4-/- Con
Pdk4-/- As
B
WT Con
WT As
Pdk4-/- Con
Pdk4-/- As
CYCLIN D1
1 0.82 1.33 1.18
CYCLIN E1
1 1.30 2.59 0.30
CYCLIN A2
1 1.56 4.07 0.41
ACTIN
C
WT
Pdk4-/-
Con As Con As
CYCLIN D2
1 1.58 1.38 1.12
CYCLIN D3
1 1.55 1.51 1.08
CYCLIN H
1 1.09 1.10 0.92
CYCLIN E2
1 0.67 0.67 0.82
CDK2
1 1.30 1.79 1.28
CDK4
1 1.40 1.74 2.01
ACTIN
D
WT
Pdk4-/-
Con As Con As
P21
1 1.37 1.44 1.73
RB
1 2.20 2.49 2.35
pRB
1 1.69 1.44 1.44
MYB
1 1.08 1.49 0.86
GSK3-beta
1 1.08 0.95 0.85
SMAD4
1 1.10 0.93 0.75
E2F1
1 1.67 5.25 0.37

Figure 4

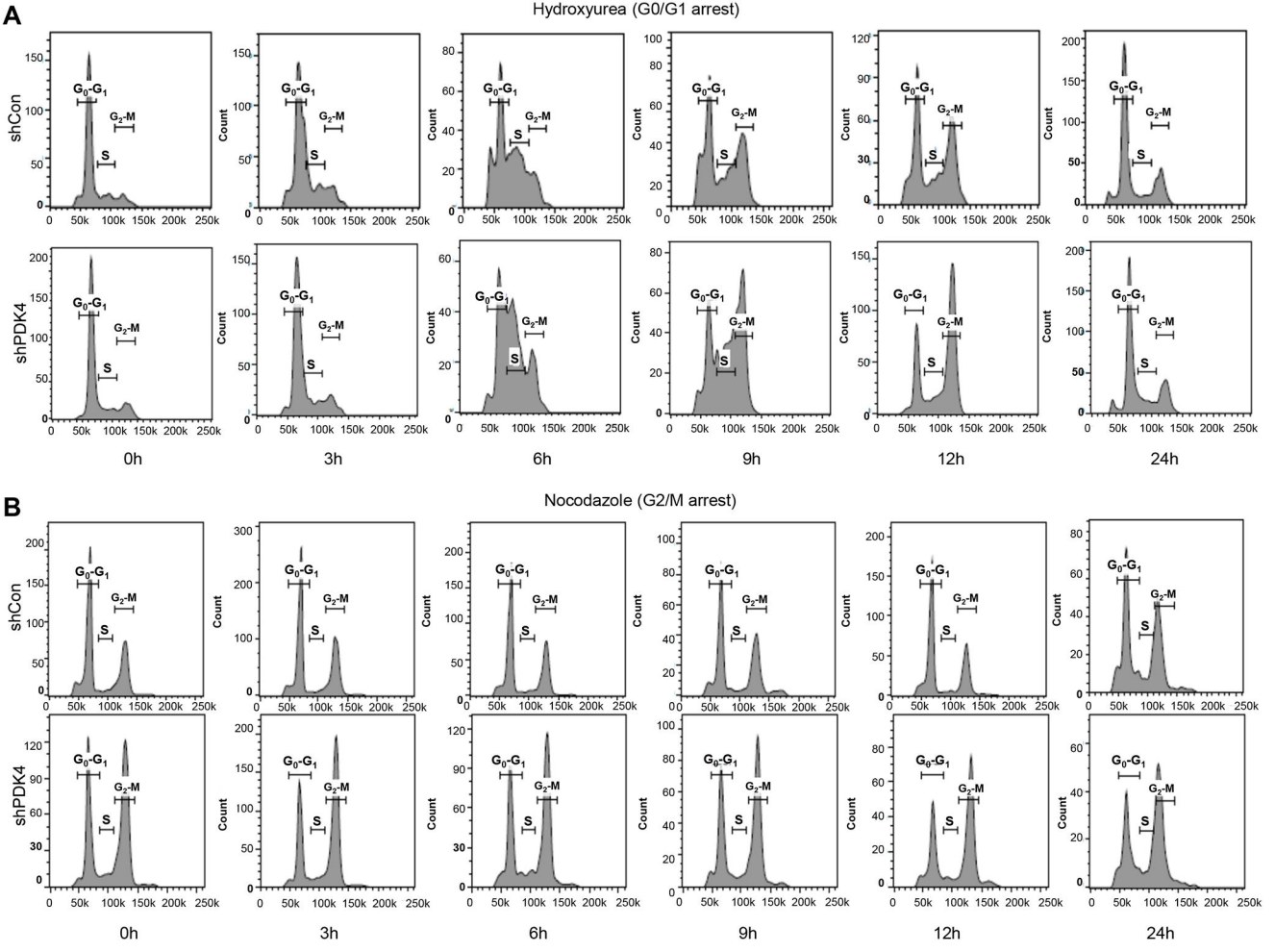
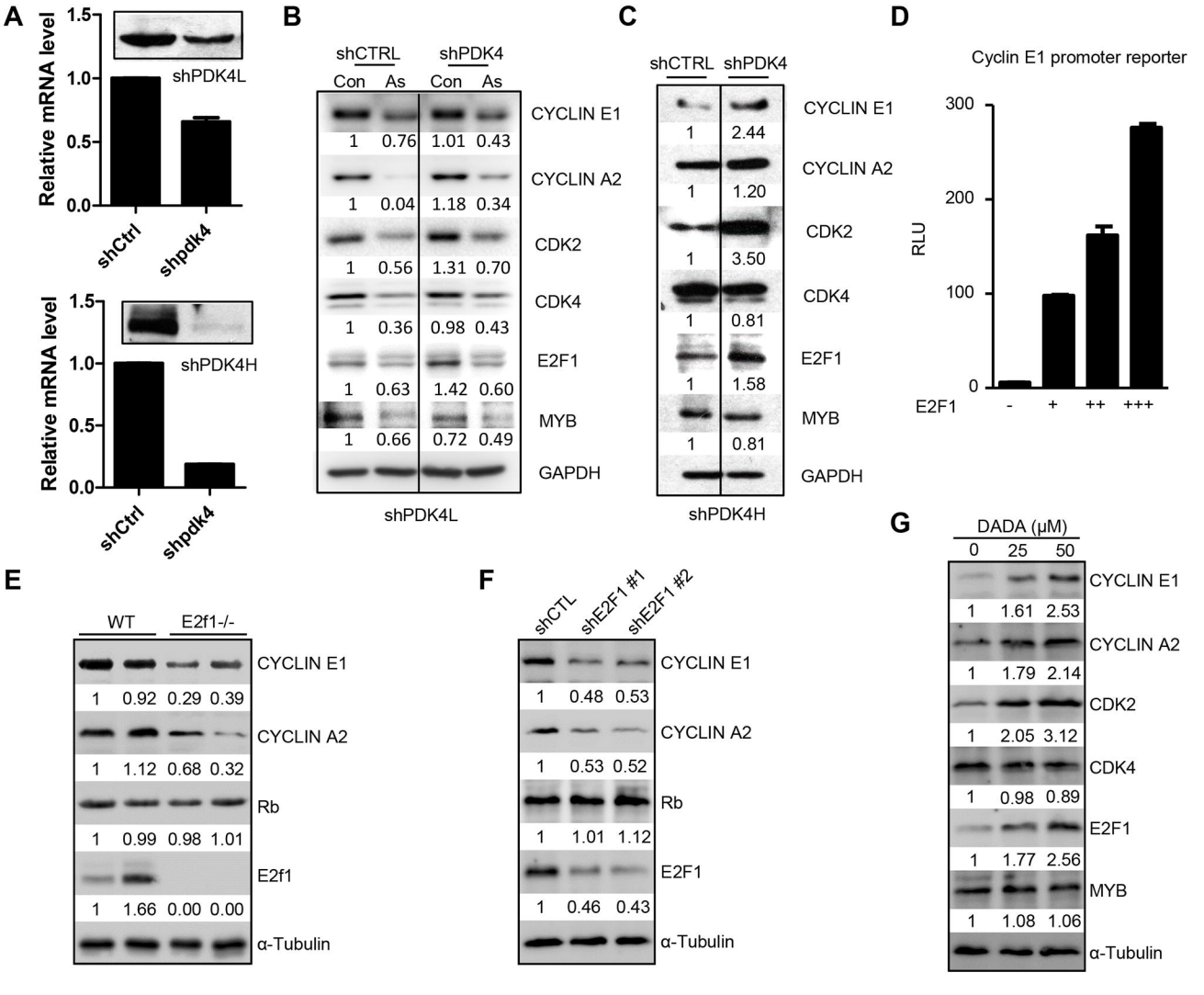

A
Hydroxyurea (G0/G1 arrest)
shCon
shPDK4
Count
G0-G1
G2-M
S
0h
3h
6h
9h
12h
24h
B
Nocodazole (G2/M arrest)
shCon
shPDK4
Count
G0-G1
G2-M
S
0h
3h
6h
9h
12h
24h

Figure 5


A
Relative mRNA level
1.5
1.0
0.5
0.0
shPDK4L
shCtrl
shpdk4
Relative mRNA level
shPDK4H
shCtrl
shpdk4
B
shCTRL
shPDK4
Con
As
Con
As
CYCLIN E1
1 0.76 1.01 0.43
CYCLIN A2
1 0.04 1.18 0.34
CDK2
1 0.56 1.31 0.70
CDK4
1 0.36 0.98 0.43
E2F1
1 0.63 1.42 0.60
MYB
1 0.66 0.72 0.49
GAPDH
shPDK4L
C
shCTRL
shPDK4
CYCLIN E1
1 2.44
CYCLIN A2
1 1.20
CDK2
1 3.50
CDK4
1 0.81
E2F1
1 1.58
MYB
1 0.81
GAPDH
shPDK4H
D
Cyclin E1 promoter reporter
RLU
300
200
100
0
E2F1
-
+
++
+++
E
WT
E2f1-/-
CYCLIN E1
1 0.92 0.29 0.39
CYCLIN A2
1 1.12 0.68 0.32
Rb
1 0.99 0.98 1.01
E2f1
1 1.66 0.00 0.00
α-Tubulin
F
shCTL
shE2F1 #1
shE2F1 #2
CYCLIN E1
1 0.48 0.53
CYCLIN A2
1 0.53 0.52
Rb
1 1.01 1.12
E2F1
1 0.46 0.43
α-Tubulin
G
DADA (μM)
0
25
50
CYCLIN E1
1 1.61 2.53
CYCLIN A2
1 1.79 2.14
CDK2
1 2.05 3.12
CDK4
1 0.98 0.89
E2F1
1 1.77 2.56
MYB
1 1.08 1.06
α-Tubulin

Supplementary Figure 1

PDK4-deficiency results in expedited cellular proliferation through increased cyclins

Jonathan Choiniere, Jianguo Wu, and Li Wang

Molecular Pharmacology

Analysis Type by Cancer	Cancer /Normal	
Bladder Cancer		1
Brain and CNS Cancer		
Breast Cancer		5
Cervical Cancer		
Colorectal Cancer		18
Esophageal Cancer	2	1
Gastric Cancer		2
Head and Neck Cancer		1
Kidney Cancer		
Leukemia		
Liver Cancer	1	3
Lung Cancer		7
Lymphoma		
Melanoma		
Myeloma		
Other Cancer		1
Ovarian Cancer		1
Pancreatic Cancer		2
Prostate Cancer		2
Sarcoma		4
Significant Unique Analyses	3	48
Total Unique Analyses	322	

Supp. Fig. 1 PDK4 gene is down-regulated in various human cancers. The data from 48 different studies in Oncomine are analyzed. Cell color is determined by the best gene rank percentile for the analyses within the cell. Blue: downregulation; red: upregulation. Threshold (*P* value): $1x10^{-4}$; Threshold (Gene rank): Top 10%; Threshold (Fold change): 2.