Pyridoxal kinase (PDXK) facilitates CRC progression by releasing beta-catenin from the GSK-3β destruction complex

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Abstract

Studies have suggested a close association between hyperactivation of the catenin beta 1 gene (CTNNB1) and the occurrence and progression of colorectal cancer (CRC). Here, we report that Pyridoxal kinase (PDXK) promotes the development of colorectal cancer through Wnt signaling pathway. The expression of PDXK is elevated in CRC patients and is associated with an unfavourable prognosis. Genetic depletion of PDXK significantly inhibited CRC cell viability, viability, and migration both in vitro and in vivo. Furthermore, we observed that overexpression of PDXK enhanced CRC cell viability, invasion, and migration, and these effects were dependent on its kinase activity. GSEA revealed a strong association between PDXK expression and the Wnt signaling pathway, which was validated through luciferase reporter assays and RT-qPCR. Mechanistically, PDXK was found to activate the Wnt signaling pathway by interacting with GSK-3β and releasing beta-catenin from the GSK-3β destruction complex, thereby promoting tumorigenesis. These findings provide direct insights into the molecular mechanisms underlying the functions of PDXK in CRC and suggest it as a potential therapeutic target for combating colorectal cancer.

Introduction

According to statistics, CRC is responsible for approximately 900,000 deaths worldwide annually, making it the fourth deadliest cancer[1]. In China, CRC has become the third most common cancer, following lung and stomach cancers[2]. Additionally, the incidence rate of early-onset CRC continues to rise, despite advancements in medical science[3]. Therefore, there is an urgent need to explore potential pathogenic factors and drug targets for colorectal cancer.

The Wnt signaling pathway is involved in various biological processes, including cell cycle regulation, embryonic development, inflammation, and cancer[4]. Currently, research on the Wnt pathway predominantly concentrates on the Wnt/β-catenin branch, with significant emphasis on the influence of a "destruction complex" comprising adenomatous polyposis coli (APC), axis formation inhibitor (AXIN2), casein kinase 1 (CK1), and glycogen synthase kinase 3β (GSK3β) on the cytoplasmic stability of beta-catenin [5–8]. The Wnt/β-catenin pathway is characterized by the binding of Wingless/integrase-1 (Wnt) to its core receptor complexes (LRP5 and LRP6), which interact with members of the FZD protein family[9, 10]. In the absence of Wnt ligand stimulation, cytoplasmic β-catenin forms complexes with GSK3β, CK-I, Axin2, and APC. Within this complex, cytoplasmic β-catenin is phosphorylated by GSK3β, and the phosphorylated β-catenin is degraded through a ubiquitin-mediated proteasome pathway in the cytoplasm. This prevents the accumulation of β-catenin in the cytoplasm, thereby inhibiting Wnt signaling[5, 11, 12].

An increasing number of studies have demonstrated the pivotal role of the Wnt/β-catenin signaling pathway in the generation and development of CRC(CRC)[13, 14]. Enhanced Wnt/β-catenin signaling has been observed in nearly all CRC patients, and the increased nuclear localization of β-catenin promotes the expression of Wnt target genes, thus contributing to CRC progression[15–17].
Vitamins are well-known essential nutrients for maintaining overall health. Recent studies have highlighted the role of vitamins in regulating cancer-related metabolic processes. For instance, vitamin C has been shown to impact leukemia-related processes and influence the phenotype of leukemia cells[19]. Another study demonstrated the dependency of acute myeloid leukemia (AML) cell viability on Vitamin B6[20]. Pyridoxal 5-phosphate (PLP) serves as the active form of Vitamin B6, and the phosphorylation of Pyridoxal (PL) by PDXK is a crucial step in Vitamin B6 synthesis[21, 22]. Upregulation of PDXK has been shown to promote the proliferation and metastasis of hepatocellular carcinoma cells (HCC) and serous ovarian cancer cells (SOC)[23, 24]. However, there is limited research on the role of PDXK in the occurrence and development of colorectal cancer.

In this study, we aimed to investigate the potential involvement of PDXK as a key mediator of β-catenin nuclear accumulation in CRC cells and to elucidate the biological consequences of PDXK-GSK3β interaction in colorectal tumor formation.

**Methods**

**Reagents and Antibodies**

Mouse monoclonal antibodies against Myc (Cat. #M562-5, MBL), HA (Cat. #M0291-3, MBL), GAPDH (Cat. #60004-1-Ig, Proteintech); β-actin (Cat. #81115-1-RR, Proteintech); rabbit monoclonal antibody against beta-catenin (Cat. #8480, CST), Phospho-beta-cateninSer33/37/Thr41 (Cat. #9561, CST), PDXK (Cat. #15309-1-AP, Proteintech), GSK-3β (Cat. #5676, CST); rabbit polyclonal antibodies against AXIN2 (Cat. #20540-1-AP, Proteintech), P21 (Cat. #10355-1-AP, Proteintech), β-tubulin (Cat. #10094-1-AP, Proteintech), LaminA/C (Cat. #10298-1-AP, Proteintech);

**Cell Culture**

The human CRC cell lines RKO, SW48, DLD1, HT29, SW480, LoVo, HCT116 and FHC were cultured in McCoy’s 5A medium (AppliChem, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA) and 100 U of penicillin-streptomycin (Gibco, Carlsbad, CA, USA). HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Monad) supplemented with 10% FBS and 100 U of penicillin-streptomycin. All cell lines were obtained from ATCC and incubated in a 5% CO2 incubator at 37°C. No mycoplasma contamination was detected. Wnt3α (Cat. #HY-P70453C, MCE; 40ng/ml) was used to activate Wnt signaling pathway.

**Transient Transfections and Lentivirus-Mediated Stable Overexpression**

To achieve overexpression of PDXK, the PDXK cDNA was amplified from 293T cells using PCR with the following primers: Forward 5’- ACGCGTCGACGCCACCATGGAGGAGTGCCGGGTG-3’, Reverse 5’-CTAGCTAGCCACCGTCGGCTGGCGAGTGGACGAC-3’. The amplified PDXK cDNA was then ligated into the pHAGE-puro-3×Myc vector. To eliminate the kinase activity of PDXK, we introduced mutations in the
active site residues Gly232/234 and Asp235, changing them to Ala. The resulting mutated cDNA, PDXK<sup>G232/234A D235A</sup>, was inserted into the pHAGE-puro-3×Myc vector. The recombinant vectors carrying PDXK<sup>G232/234A D235A</sup> or PDXK were transfected into 293T cells along with the packaging plasmids pVSVg (AddGene 8454) and psPAX2 (AddGene 12260). After 48 hours, the medium containing lentiviral particles was harvested through filtration and ultracentrifugation. RKO cells at 85% confluence were then infected with the prepared viruses at a multiplicity of infection (MOI) of 0.2. After 48 hours of infection, RKO cells were selected with puromycin (1 µg/mL) for 7 days, and the surviving cells were pooled for Western blot analysis.

**Genetic Knock-Out of PDXK in HCT116 Cells**

To deplete PDXK in HCT116 cells, sgRNA sequences targeting <i>PDXK</i> exons 1 and 2 (sgRNA1 and sgRNA2) were synthesized by Tsingke biotechnology company (Beijing, China). The prepared sgRNAs oligos were ligated into lentiCRISPRv2 (Addgene, USA). The recombinant lentiCRISPRv2-sgRNAs were packaged and amplified in 293T cells as mentioned before. The lentiviral production was harvested for 48 h infection to HCT116 cells. Finally, the infected cells were grown in 200 µl of 2 µg/ml puromycin. 10 days later, the survivals were amplified and verified by Western blot. The sgRNA1 (designed on http://crispor.tefor.net/) is Forward 5’-CACCGGAGCTCCAGGAGTTGTACGA-3’. Reverse 5’-AAACTCGTACAACTCCTGGAGCTCC-3’. The sgRNA2 (designed on http://crispor.tefor.net/) is Forward 5’-CACCGGAAGCAGCAGAACCCCAGGC-3’. Reverse 5’-AAACGCCTGGGGTTCTGCTGCTTCC-3’.

**CCK8 assay**

Cell proliferative ability was measured using a cell counting kit-8 (Beyotime, China). Each group of cells (1000 cells/well) were plated on 96-well plates. After 24 h, 48 h, 72 h, 96 h or 120 h maintaining, each well was subjected to 10 µL CCK8 at 37°C for 1 h posttreatment, the plates were read using a microplate reader at 450 nm.

**Colony formation**

To determine the long-term proliferation of cells, cells (400 cells/well) were grown on 6-well plates for 14 days. Afterwards, the cells were stained with 0.2% crystal violet for 30 min. After washed by water, images were captured and the formatted colonies were scored and plotted.

**Transwell migration assays**

To reflect the migration ability of cells under different conditions, 500 µL growth medium was supplemented on the 24-well plates, and the 20% FBS was added as the chemotactic factor. Cells (1×10<sup>4</sup>) were suspended in 100 µL serum-free media and applied onto the insert on the 24-well plates. After 24 h maintaining, the migrated cells on the lower side of the insert filter were fixed with 5% glutaraldehyde for 10 min before being counterstained 1% crystal violet in 2% ethanol for 20 min. The upper layer of cells was carefully wiped off, fixed, stained and photographed, and the number of tumor cells entering the lower layer was counted.
RT-PCR

Total RNA was extracted using TRIzol reagent (TaKaRa, #9109). RT Master Mix for qPCR kit (MCE, China) was employed to prepare cDNA. Real-time PCR was undertaken using SYBR Green PCR Mix Realtime kit (Beyotime, China) on the StepOnePlus Real-Time PCR System (Applied Biosystems, China). Comparative 2-ΔΔCt method was used for real-time quantitative PCR. The primers used for RT-qPCR were as follows: MYC: Forward 5'-GGCTCCTGGCAAAAGGTCA-3', Reverse 5'-CTGCAGTGGCTGATGT-3'; AXIN2: Forward 5'-CAACACCAGCGGAACGAA-3', Reverse 5'-GCCCAATAAGGATGTAAGGACT-3'; CTNNB1: Forward 5'-TCCCACCTGTCAGCCT-3', Reverse 5'-ATGGACATACTGCAGCCT-3'; GAPDH: Forward 5'-CATCACCATCTTCCAGGAGGAGA-3', Reverse 5'-TGCAGGAGGCATTGCTGATGATCT-3'. GAPDH was used as a control.

Western blot

RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 µg/mL aprotinin, 10 µg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride) was applied to prepare cell lysates. Bradford Protein Assay kit (Beyotime, China) was used to determine the protein concentration. Subsequently, the 20 µg proteins were subjected to 12% SDS-PAGE and electrotransferred onto polyvinylidene difluoride membranes (PVDF, Millipore, Cat# IPVH00010, Merck KgaA, Darmstadt, Germany). The membranes were blocked by 5% non-fat milk for 1 h at room temperature before exposure to primary antibodies over night at 4°C and subsequently secondary antibodies for 1 h at room temperature. Finally, the signals were visualized on a ChemiDocTM XRS + (Bio-Rad).

Co-immunoprecipitation (Co-IP)

Co-Immunoprecipitation was used to verify the physical interaction between Myc-PDXK and HA-GSK-3β. Briefly, the transfected 293T cells were lysed by ice-cold IP lysis buffer (30 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 10 µg/mL aprotinin, 10 µg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride) for 15 min. After centrifugation, the cell debris was discarded and the cell supernatant was collected. A/G-agarose beads (Smart-Lifesciences, Changzhou, China) pre-coated indicated antibodies were added and incubated in the cell lysates at 4°C. 6 h later, the Co-IP proteins were subjected to western blot analysis.

Luciferase reporter assay

200 ng TCF/LEF1-Luc vectors (Yeasen, China) were introduced into HCT116 cells together with the indicated dose of constructed PDXK-Myc vectors. 48 h later, using a dual-luciferase assay kit (Promega), the luciferase activity was recorded using the Promega luciferase system.

Immunofluorescence analysis

HEK293T cells were cultured on coverslips and transfected with the HA-GSK3β and Myc-PDXK expression plasmids. After 36 h, the cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. Then, the cells were washed with PBS and blocked with 5% BSA in PBS for 30 min. To visualize the expression of HA-GSK3β and Myc-PDXK, mouse anti-HA antibody and rabbit anti-Myc
antibody were used as primary antibodies, and mouse Alexa594- and rabbit Alexa488-conjugated antibodies (Invitrogen) were used as secondary antibodies. DAPI was used to stain the nuclei. Finally, the coverslips were observed and digitally photographed using a confocal microscope with a 60× oil objective.

**Animal study**

The BALB/c mice aged 4–6 weeks were purchased from the Experimental Animal Center of Wuhan University (Wuhan, China). All mice were grown under SPF conditions with 12: 12 dark/light. Food and water were provided sufficiently during the whole day. The Animal ethics approval was granted by the University of Wuhan University. After 21 days, the mice were euthanized by CO₂ asphyxiation, and the implanted tumors were resected for weight and volume. The tumor tissues were fixed with 4% paraformaldehyde (PFA). The 5-µm-thick sections were prepared for immunohistochemistry analysis and Hematoxylin and eosin (HE) staining.

**Immunohistochemical analysis**

Immunohistochemistry was performed as previously depicted. Briefly, slides were treated with 10% H₂O₂ and permeabilized with 0.1% TX-100. Next, primary antibodies against Cyclin D1, C-Myc and beta-catenin were added on slides to bind to target proteins at 37°C overnight and then immunoblotted by the secondary antibodies. Next, the slides were visualized with Avidin-Biotin Complex (ABC) staining systems (Vector Laboratories, Inc. USA) as instructed. The Immunohistochemical analysis was also conducted on the tissue microarray carrying tumoral (n = 15) and uncarcinomous tissues (n = 15) from individuals suffering from CRC (Shanghai Outdo biotech).

**Data and resources**

Gene expression data and associated clinical data for 20 cancer types were downloaded from the Cancer Genome Atlas data portal (TCGA, http://cancergenome.nih.gov/[25]. We downloaded microarray datasets including gene expression profiles and clinical information data from the Gene Expression Omnibus database (GEO, https://www.ncbi.nlm.nih.gov/geo/). We downloaded the CRC related datasets from the GEO database (GSE110224, GSE113513, GSE20916, GSE37364, GSE39582, GSE41258, GSE41328, GSE62932, GSE71187, GSE8671, GSE87211)[26].

**Statistical analysis**

Statistical significance was evaluated using GraphPad Prism software (GraphPad, USA). Data are expressed as the mean ± SEM. The student's t-test was employed to analyze the difference in the data between two groups. One-way analysis of variance (ANOVA) with Bonferroni post hoc analysis was used to verify the difference in the data from multiply groups. P < 0.05 was considered statistically significant.

**Results**

**PDXK is overexpressed in CRC**
In our investigation of PDXK expression in colorectal cancer, we employed the GEO database. Figure 1A-K clearly illustrates a significant elevation of PDXK expression in tumor tissue compared to normal tissue. Additionally, we examined PDXK expression levels across various human cancers using the TCGA database[27, 28]. Notably, tumor tissues of COAD (Colon adenocarcinoma) and READ (Rectum adenocarcinoma) exhibited higher PDXK expression when compared to normal tissues (Fig. 2A). Moreover, both sample-paired and unpaired expression difference analyses substantiated the marked and plausible disparity in PDXK expression between COAD and READ. Remarkably, the ROC curves employed for PDXK prediction demonstrated a high level of accuracy (Fig. 2B-G). These compelling findings strongly suggest the significant role of PDXK expression in the pathogenesis of colorectal cancer.

Subsequently, Western blot analysis was conducted to examine the protein levels of PDXK in colorectal tumors and adjacent normal tissues of 10 clinical colorectal samples from Wuhan Union Hospital, confirming the upregulation of PDXK in CRC (Fig. 3A, B). We further assessed PDXK expression in seven colorectal cancer-derived cell lines and one normal colorectal cell line (FHC). Figure 3C illustrates a significant increasing level of PDXK in most CRC cell lines compared to that in FHC. To validate these findings, immunohistochemistry (IHC) was performed on a tissue microarray consisting of tissues from 15 CRC patients and 15 adjacent colorectal normal tissues to evaluate PDXK expression. The IHC assay revealed a high expression of PDXK in CRC compared to normal colorectal tissue (Fig. 3D, E). Collectively, these results indicate a close association between PDXK and the progression of colorectal cancer.

Overexpressing PDXK boosts the viability, colony formation ability and migratory potential in CRC cells

To understand the role of PDXK in CRC development and progression, we introduced stable expression of PDXK in RKO colon cancer cell lines due to its relatively low expression of PDXK. Additionally, to investigate the impact of PDXK's kinase activity, we transfected a kinase-dead mutant (PDXK^{G232/234A/D235A}) into RKO cells, generating a stable cell line termed PDXK^{m} for simplicity. Previous studies have provided confirmation that the amino acid residues Gly232, Gly234, and Asp235 (collectively known as the GXGD motif) are crucial for maintaining the enzymatic activity of PDXK[29, 30]. The overexpression of PDXK and PDXK^{m} in RKO cells was confirmed by Western blot analysis (Fig. 4A). Subsequently, we conducted a series of functional assessments. Colony formation assay and cell growth assay demonstrated that ectopic expression of PDXK enhanced the colony formation rate of RKO cells and increased their cell viability compared to wild-type RKO cells (Fig. 4B, 4C, and 4D). Meanwhile, transfection of PDXK^{m} into RKO cells did not show any significant change in colony formation ability. Furthermore, PDXK overexpression promoted RKO cell migration, while mutation of the kinase catalytic active site of PDXK resulted in unnoticeable alteration in RKO cell migration (Fig. 4E, F). These findings suggest that PDXK promotes RKO cell viability, and migration, and these effects are dependent on its kinase activity.

Depletion of PDXK impairs the cell viability and migratory ability in CRC cells
To further investigate the physiological function of PDXK, we used the CRISPR-Cas9 gene-editing technique to deplete PDXK in HCT116 CRC cells. As a result, we generated a PDXK knockdown cell line (PDXK-KD) and a PDXK knockout cell line (PDXK\textsuperscript{−/−}). The PDXK-reduced and deficient HCT116 cell lines were successfully generated and confirmed by Western blot analysis based on the CRISPR/Cas9 strategy (Fig. 5A). As depicted in Fig. 5C and 5D, the PDXK reduction or deficiency in HCT116 cells led to a decrease in the number of colonies, indicating a weaker proliferative ability of HCT116 cells. Notably, complete knockout of PDXK resulted in significantly weakened cloning ability in HCT116 cells.

Furthermore, the CCK-8 assay demonstrated that PDXK-deficient HCT116 cells exhibited a lower cell viability compared to the parental HCT116 cells (Fig. 5B). In addition, we performed transwell assays to validate that PDXK deficiency significantly reduced the migratory activity of HCT116 cells. As shown in Fig. 2E and 2F, HCT116 cells with reduced or deficient PDXK displayed lower migratory activity compared to wild-type cells. These findings collectively demonstrate that the depletion of PDXK impairs cell growth and migration in HCT116 CRC cells.

**PDXK activates the Wnt signaling pathway**

To further investigate the potential mechanism underlying the malignant behaviors driven by PDXK in colorectal cancer, we performed RNA-seq analysis comparing PDXK-deficient HCT116 cells with wild-type HCT116 cells. We utilized gene set enrichment analysis (GSEA) to analyze the differentially expressed genes (Fig. 6B). The heatmap in Fig. 6C clearly demonstrates a significant difference in gene expression between the two cell types. The volcano plot analysis of the differentially expressed genes in PDXK knockout cells and wild-type cells (Fig. 6A) reveal that the absence of PDXK leads to substantial changes in mRNA expression levels in HCT116 cells, indicating that PDXK has broad regulatory effects on gene expression in these cells. We further enriched the differentially expressed genes in 50 disease-related signaling pathways. The results showed that the Wnt signaling pathway exhibited significant overall downregulation after the loss of PDXK expression (Fig. 6B), suggesting a positive correlation between PDXK expression and pathway activity. Based on these bioinformatics analyses, we hypothesized that PDXK regulates the Wnt signaling pathway. To verify this hypothesis, we investigated whether PDXK influences TCF/LEF1-mediated transcriptional activation through luciferase reporter assays. PDXK and luciferase reporters were transfected in HCT116 cells to analyze the effect of PDXK in the transcriptional activation of several common pathways. Among them, the most notable activation effect was observed in TCF/LEF signaling (Fig. 6D). As shown in Fig. 6E, PDXK enhanced Wnt-mediated luciferase activity in a dose-dependent manner. Similarly, the transcriptional activity of AXIN2, CTNNB1, and Myc, which are downstream targets of the Wnt pathway, were downregulated in PDXK-deficient HCT116 cells (Fig. 6F). Taken together, the data indicate that PDXK activates the Wnt signaling pathway.

**The interaction between PDXK and GSK-3β disrupts the binding between GSK-3β and beta-catenin to stabilize beta-catenin**

beta-catenin, as a transcription co-activator, plays a critical role in activating the WNT signaling pathway, and the alteration of its cellular content is pivotal for the functioning of this pathway[31]. In our study, we
observed a significant decrease in beta-catenin protein levels in PDXK knockout cell lines, which is consistent with previous findings (Fig. 7H). Considering the complex formation between beta-catenin and GSK3β, we conducted Co-IP between PDXK and GSK3B. Immunoblot analysis of cell lysates showed that PDXK co-immunoprecipitated with GSK3B when Myc-PDXK and HA-GSK3B were co-expressed in 293T cells (Fig. 7A, B). In order to further investigate the localization of these interactions within HCT116 cells, we conducted immunofluorescence analysis. The results indicated that Myc-PDXK primarily localized in the cell membrane and cytoplasm of HCT116 cells. Interestingly, Myc-PDXK exhibited a significant colocalization with HA-GSK3B specifically in the cytoplasmic region (Fig. 7C). Western blot analysis was performed to examine the effects of PDXK on the expression of GSK3B and the stability of beta-catenin. The results indicated that PDXK did not significantly influence the expression level of GSK3B. However, it was observed that PDXK had a notable impact on the stability of beta-catenin (Fig. 7D and 7E). Based on these findings, we proposed a hypothesis that PDXK competes with beta-catenin to bind GSK3B, preventing GSK3B from phosphorylating CTNNB1 and thus inhibiting its degradation. To investigate the competitive binding between PDXK and beta-catenin to GSK3B, we conducted a competitive inhibition experiment. Figure 7F shows that the presence of PDXK significantly inhibited the binding of GSK3B and beta-catenin. Wnt3a stimulation is known to act as a potent activator of the Wnt signaling pathway and can effectively release beta-catenin from the "destructive complex" [32]. To investigate whether the Wnt pathway is inhibited in cancer cells following the knockdown or knockout of PDXK under Wnt3a stimulation, we conducted a stimulation experiment using Wnt3a on cells. Interestingly, we consistently observed that the presence of PDXK resulted in enhanced stability of beta-catenin (Fig. 7G). Previous studies have indicated that the phosphorylation of GSK3B at sites Ser33/37Thr41 of beta-catenin leads to its ubiquitination and subsequent degradation [25]. Subsequently, we performed nucleocytoplasmic fractionation on HCT116 wild-type (WT), PDXK knockdown (KD), and PDXK −/− cells. We found that in the presence of PDXK, the cytoplasmic content of beta-catenin significantly increased, which was consistent with lower phosphorylation levels at its Ser33/37Thr41 sites compared to PDXK KD and PDXK −/− cells (Fig. 7I, J). The findings suggested that the interaction between PDXK and GSK3B hinders the binding of GSK3B to beta-catenin, subsequently decreasing the phosphorylation level of beta-catenin at the Ser33/37Thr41 sites. As a result, beta-catenin is stabilized in the cytoplasm, thereby activating the Wnt/β-catenin signaling pathway.

**PDXK boosts CRC tumorigenesis in vivo**

To assess the impact of PDXK in CRC in vivo, we established a xenograft mouse model by subcutaneously injecting PDXK-deficient HCT116 cells and wild-type HCT116 cells into nude mice. We observed that the size and weight of tumors were significantly reduced in mice injected with PDXK-deficient HCT116 cells compared to those injected with wild-type cells (Fig. 8A-B). Throughout the experimental period, the tumor volumes were consistently smaller in the PDXK-deficient group (Fig. 8C). Histological analysis using H&E staining and immunohistochemistry against C-Myc, beta-catenin, and Cyclin D1 was performed to evaluate the expression levels in the tumor tissues (Fig. 8E). Western blot analysis confirmed that the protein levels of downstream genes in the Wnt/β-Catenin signaling pathway,
such as CCNB1 and CDK1, were downregulated in PDXK-deficient tumor tissues (Fig. 8D). These findings support our previous experiments and indicate that the depletion of PDXK inhibits colorectal tumorigenesis in a xenograft model, at least partially through the stabilization of beta-catenin.

**Discussion**

Nowadays, the generation of early-onset CRC (eoCRC) has a significant surge in individuals under the age of 50[33]. Notably, there has been a consistent observation of heightened Wnt/β-catenin signaling in nearly all CRC(CRC) patients, highlighting the critical role of this pathway in potential therapeutic interventions[15]. Moreover, a strong correlation has been found between high beta-catenin expression and improved overall survival in CRC patients[34]. The Wnt/β-catenin signal pathway activation, which is strictly controlled by a “destruction complex” (APC/AXIN2/CK1/GSK3 complex), is crucial to cell viability, survival, differentiation and migration[35]. In particular, β-catenin is captured and phosphorylated by GSK3β, activating the process of β-catenin degradation which inhibits the transcription of target genes[7, 36].

As an important kinase involved in intermediate metabolism, PDXK is aberrantly expressed in various types of cancer, where it regulates cancer cell viability, migration, invasion and survival[37].

We have observed that PDXK is aberrantly expressed in CRC tissues and cell lines, which is consistent with previous reports [38]. Our phenotype experiments with CRC cell lines further support the notion that PDXK acts as a key driver in the generation and progression of colorectal cancer. Interestingly, we found that PDXK exerts a stabilizing effect on β-catenin, a key component of the Wnt signaling pathway. Based on the presence of the "destruction complex" in cells, we hypothesize that PDXK competes with beta-catenin for binding to GSK3B. Our experiments have shown that the binding of PDXK to GSK3B hinders its interaction with beta-catenin, leading to a decreasing phosphorylation level of beta-catenin. This prevents beta-catenin from undergoing degradation in the cytoplasm through the ubiquitination-proteasome pathway, thereby activating the Wnt pathway. Our findings share similarities with previous studies, where researchers have found that Sec62 competitively disrupts the interaction between β-catenin and APC, leading to the inhibition of β-catenin destruction complex assembly [39].

Our study did not investigate the impact of PDXK on the other components of the "destruction complex" such as CK1 and APC and we did not explore the potential of PDXK to phosphorylate GSK3B or any specific phosphorylation site in our investigation. However, recent literature reports the existence of a novel site, alanine 228, which can impact PDXK enzyme activity. This site is situated in close proximity to the ATP-binding pocket of PDXK[40]. Further research is required to determine the phosphorylation targets of PDXK and elucidate the functional implications of the alanine 228 site on PDXK activity. Such investigations would provide valuable insights into the intricate underlying mechanisms that how is PDXK involved in modulating the Wnt/β-catenin signaling pathway.

In our study, we have identified PDXK as a novel positive regulator of the Wnt/β-catenin signaling pathway. We propose that PDXK exerts its regulatory role by dissociating β-catenin from the destruction
complex, which is composed of APC, AXIN2, CK1, and GSK3. In the absence of PDXK, the destruction complex captures and phosphorylates beta-catenin, leading to its degradation and inhibition of target gene transcription. However, when PDXK is present, it interacts with GSK3B and competes with beta-catenin for binding, preventing beta-catenin phosphorylation and degradation (Fig. 9). This results in the stabilization and nuclear translocation of beta-catenin, leading to the activation of the Wnt/β-catenin signaling pathway and subsequent transcriptional activation of target genes involved in cell viability, survival, differentiation, and migration. Our findings highlight the importance of PDXK in regulating the Wnt/β-catenin signaling pathway and provide new insights into the molecular mechanisms underlying CRC development and progression.

Declarations

Ethical Approval

The tissue microarray carried tumoral (n = 15) and noncancerous tissues (n = 15) from individuals suffering from CRC(Xian Alenabio).

Authors’ Contributions

M.Q. Fan: Investigation, formal analysis and writing editing; Meng. Gao: Investigation, editing; Jie. Gao: Investigation and editing; X.X. Xie: Investigation; Y.Y. Liu: Investigation; L.Z. Qi: editing; X.D. Zhang: supervision; R.L. Du: Resources, conceptualization, funding acquisition and supervision; S.Z. Li: Conceptualization, supervision, funding acquisition and editing.

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Competing interests

The authors declare that they have no conflicts of interest.

Availability of data and materials

The datasets generated/analyzed during the current study are available.

Ethics Statement

- Approval of the research protocol by an Institutional Reviewer Board: With the approval of the research protocol by the Ethics Committee of Wuhan University.

- Informed Consent: All patients had signed informed consent forms.
Registry and the Registration No. of the study/trial. N/A.

Animal Studies: Ethics approval for the animal experiments was granted by the University of Wuhan University, and all animal care was provided according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health.

References


Figures
Figure 1

The expression levels of PDXK in normal and tumor tissues. The expression levels of PDXK in normal and tumor tissues were compared in the GEO database (GSE110224, GSE113513, GSE20916, GSE37364, GSE39582, GSE41258, GSE41328, GSE62932, GSE71187, GSE8671, GSE87211).
Figure 2

**Differential analysis of PDXK expression in human cancers.** (A) Expression level of PDXK in tumors and corresponding normal tissues in the TCGA database. (B-D) PDXK expression profile in COAD (colon adenocarcinoma) based on data from the TCGA database. The area under the ROC curves showed the predictive accuracy of PDXK. (E-G) PDXK expression profile in READ (rectum adenocarcinoma) based on data from the TCGA database. The area under the ROC curves showed the predictive accuracy of PDXK. (*p < 0.05, **p < 0.01, ***p < 0.001).
**Figure 3**

**PDXK is overexpressed in colorectal cancer.** (A, B) The protein expression of PDXK in 10 representative pairs of primary CRC(T) and adjacent non-tumor tissues (N). (C) The protein expression of PDXK in the normal human colon cell line FHC and seven colorectal cell lines (HCT116, SW48, SW480, RKO, DLD1, LOVO, and HT29). (D) IHC staining for PDXK in CRC tissue microarrays containing adjacent tissues and normal tissues. (E) Analysis of PDXK expression in CRC tissue microarrays from 15 tumor and 15 adjacent tissues. COAD, colon adenocarcinoma; READ, rectal adenocarcinoma. The data are presented as the means ± SEM. Statistical significance was analyzed by Student’s *t* test. **p < 0.01.
Overexpressing PDXK boosts the viability, colony formation ability and migratory potential in CRC cells.

(A) The expression of Myc-PDXK and Myc-PDXK^{G232/234A D235A} (Abbreviated as Myc-PDXK^m) was assessed by Western blotting. (B) A CCK-8 assay was conducted to examine the growth and proliferation ability of Myc-PDXK-overexpressing, Myc-PDXK^m-overexpressing and control cells. (C, D) Colony formation assays demonstrated the proliferation ability of Myc-PDXK-overexpressing, Myc-PDXK^m-overexpressing and control cells. The number of clones was counted and plotted. (E, F) Transwell migration assay. The migration ability of Myc-PDXK-overexpressing, Myc-PDXK^m-overexpressing and control cells was measured. **P < 0.01 and ***P < 0.001 compared with controls.
Depletion of PDXK impairs the cell viability and migratory ability in CRC cells. (A) HCT116 PDXK-KD and PDXK<sup>−/−</sup> cell lines were constructed, and protein expression was detected by Western blotting. (B) CCK8 assays were performed to assess the growth and proliferation ability of the HCT116 PDXK-KD, PDXK<sup>−/−</sup> and control cell lines. (C, D) Colony formation assays were conducted to examine the proliferation capacity of the HCT116 PDXK-KD, PDXK<sup>−/−</sup> and control cell lines. Cell numbers were counted using ImageJ. (E, F) Transwell migration assay. The migration ability of the HCT116 PDXK-KD, PDXK<sup>−/−</sup> and control cell lines was measured. **<i>p</i> < 0.01, ***<i>p</i> < 0.001 and ****<i>p</i> < 0.0001 compared with controls.
Figure 6

**PDXK activates the Wnt signaling pathway.** (A, C) Volcanic map and heat map of differentially expressed genes in PDXK knockout cells and wild-type cells. (B) gene set enrichment analysis (GSEA). (D) PDXK enhanced TCF/LEF activation as shown by luciferase reporter pathway identification in HCT116 cells (n = 3). (E) Luciferase assays showing TCF/LEF activation as indicated by increased amounts of the PDXK plasmid expressed in HCT116 cells (n = 3). (F) The mRNA levels of genes with TCF/LEF-triggered transcription in PDXK-depleted HCT116 cells were determined by RT-qPCR analysis (n = 3).
Figure 7

**PDXK interacts with GSK-3β and disrupts the binding between GSK-3β and beta-catenin, thereby stabilizes beta-catenin.** (A, B) Co-IP was performed to examine the interaction between Myc-PDXK and HA-GSK3β in 293T cells. (C) The colocalization of Myc-PDXK (green) and HA-GSK3β (red) in HEK293T cells was determined by immunofluorescence. DAPI was used to stain nuclei (blue). (D) The protein level is not correlated with HA-GSK3β and increasing Myc-PDXK protein (0, 200 ng, 400 ng, 600 ng and 800 ng) in HCT116 cells by immunoblot assay. (E) The protein level is positively correlated with HA-beta-catenin and increasing Myc-PDXK protein (0, 200 ng, 400 ng, 600 ng and 800 ng) in HCT116 cells by immunoblot assay. (F) PDXK blocks binding between beta-catenin and GSK3B. Immunoprecipitation was performed to show the interaction between beta-catenin and GSK3B in the presence or absence of PDXK. (G) Immunoblotting of the beta-catenin in HCT116 PDXK-KD, PDXK−/− and WT cell lines after Wnt3a treatment for 0, 15, 30, 60, 120, 240 min. (H) The protein levels of the indicated Wnt-targeted genes in HCT116 PDXK-KD, PDXK−/− and WT cell lines were determined by immunoblot analysis. (I) HCT116 PDXK-KD, PDXK−/− and WT cells were fractionated into cytoplasmic and nuclear fractions. Cytoplasmic and nuclear fractions were detected using the beta-catenin antibody. β-Tublin and Lamin A/C were used as the cytoplasmic and nuclear fraction loading control separately. (J) The protein level of total beta-catenin and phosphorylated beta-catenin (Ser33/37/Thr41) in HCT116 PDXK-KD, PDXK−/− and WT cell lines was measured by immunoblot assay.
Figure 8

PDXXK boosts CRC tumorigenesis in vivo. (A) The sizes of subcutaneous tumors from nude mice were observed and compared. (B) Tumor tissues from the HCT116 PDXXK-KD, PDXXK<sup>−/−</sup> and control groups were collected and weighed. (C) The tumor sizes in the HCT116 PDXXK-KD, PDXXK<sup>−/−</sup> and control groups were measured using a vernier caliper and recorded regularly. (D) PDXXK, CDK1 and CCNB1 protein levels in tumor tissues were assessed by Western blotting. (E) Image of immunohistochemical staining for beta-catenin, C-Myc, Cyclin D1 and H&E staining of tumor tissue from the xenograft model are shown. Data are shown as the mean (± SEM) of technical replicates from one representative experiment out of three; **p < 0.01, ***p < 0.001 and ****P < 0.0001
Figure 9

Schematic of the PDXK/Wnt signaling pathway.