Alcohol-induced C/EBP β-driven KIAA1429 decreases oxidative stress and promotes pancreatic cancer growth and metastasis via the m6A/YTHDF2/SLC43A2 pathway

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Abstract

N6-methyladenosine (m6A) is implicated in tumorigenesis. To data, the role of KIAA1429, an RNA methyltransferase in pancreatic cancer (PC) has not been clearly defined. The present study demonstrated that KIAA1429 was upregulated in PC. Overexpression of KIAA1429 accelerated PC proliferation and metastasis, whereas knockdown of KIAA1429 had an opposite effect. Mechanistically, KIAA1429 promoted the degradation of SLC43A2 mRNA via an m6A-YTHDF2 mechanism, and downregulation of SLC43A2 reduced phenylalanine absorption and oxidative stress, thus enhancing the progression of PC. In addition, alcohol induced the expression of C/EBP β, which bound to the promoter of KIAA1429 and boosted its transcription. The findings of the present study revealed a potential link between alcohol consumption, m6A and phenylalanine absorption in PC progression, providing a novel means to combat this disease.

Introduction

According to the estimates, 62,210 new cases of pancreatic cancer (PC) will occur in 2022[1]. PC is characterized by late diagnosis and poor prognosis. Current therapies, including surgery and chemotherapy, have limited benefits for PC patients with metastasis. Therefore, further studies on the mechanism of PC progression are urgently needed to explore novel strategies to combat this disease.

N6-methyladenosine (m6A) refers to adenosine methylated at the sixth N atom. m6A is the most common epigenetic modification that acts a regulator in the processes of RNA splicing, localization, translation and degradation[2]. m6A is a reversible modification involving the methyltransferase complexes (writers) and demethylases (erasers), and is recognized by certain proteins (readers), which are implicated in PC growth[3], stemness[4], metabolism[5], and metastasis[6]. Depletion of KIAA1429, which is an important adaptor of the methyltransferase complex, has been reported to cause a significant decrease in m6A levels[7, 8]. Lan *et al.* reported that KIAA1429 promotes liver cancer proliferation and metastasis through posttranscriptional suppression of GATA3 in an m6A-dependent manner[9]. A study by Xu *et al.* demonstrated that KIAA1429 induces DAPK3 degradation mediated by m6A modification, thereby promoting lung cancer growth[10]. Jie *et al.* reported that KIAA1429 promotes PC cell proliferation *in vitro*[11]. However, the mechanism of action of KIAA1429 in PC is unknown.

It is a well-known fact that alcohol consumption contributes to PC risk, and alcohol-induced pancreatitis acts as a factor predisposing for PC[12]. A study demonstrated that pancreatitis patients who had continual alcohol consumption harbored a higher number of K-RAS mutations, leading to oncogenesis in pancreas[13]. Recently, Yu *et al.* reported that alcohol consumption generated cancer stem cells by inducing STAB2 binding to the promoters of certain genes, such as KLF4, OCT4 and cMyc[14], and the pancreas isolated from KC mice fed ethanol manifested more pronounces features characteristic for stem cells and epithelial-mesenchymal transformation[15]. To data, the mechanisms linking alcohol and m6A regulation have not been reported.

The present study demonstrated that the expression of m6A and KIAA1429 was increased in PC the tissues. KIAA1429 accelerated SLC43A2 degradation via an m6A-YTHDF2 mechanism. Downregulation of SLC43A2 reduced phenylalanine absorption and oxidative stress, and promoted PC progression. In addition, alcohol-induced C/EBP β increased KIAA1429 expression by binding to its promoter.

Results

1. Upregulated KIAA1429 in PC predicated poor prognosis

To determine whether RNA m6A modifications occurs in PC, we initially measured the m6A level in 16 pairs of the PC tissues. The data of dot blotting showed that the PC tissues had a higher m6A level than the normal pancreas (Fig. 1A). Then, mRNA was purified from the total RNA, and the results of m6A quantification revealed that the m6A level was increased in PC (Fig. 1B). The same results were observed in the PC (BxPC-3, PANC-1, AsPC-1, SW1990 and CFPAC-1) and normal (HPDE6-C7) cell lines (Fig. 1C). The m6A modification is catalyzed by a methyltransferase and removed by a demethylase. To determine the mechanisms of the changes in the m6A levels, we detected the mRNA levels of these enzymes in pancreatic cells, The data obtained by qRT–PCR showed that KIAA1429 and METTL14 were stably upregulated in all PC cells (Fig. 1D and Supplementary Fig. 1A-E). Previous studies have demonstrated that METTL14 promotes PC progression[16, 17] and chemoresistance[18, 19]. Several studies have implicated KIAA1429 in the regulation of multiple tumors[9, 10, 20–22]; however, the role of KIAA1429 in PC is unclear. Therefore, we focused our studies on this gene. Consistent with the results obtained by the RNA assays, the protein expression of KIAA1429 was significantly elevated in PC cells (Fig. 1E). Based on GEPIA (http://gepia.cancer-pku.cn/index.html), we determined that KIAA1429 was expressed at a higher level in PC (Supplementary Fig. 1F). In addition, the levels of KIAA1429 RNA (Fig. 1F) and protein (Fig. 1G) were significantly elevated in PC compared with those in the normal pancreas. The comparison of the Kaplan–Meier survival curves demonstrated (http://kmplot.com/analysis/index.php?p=background) that KIAA1429 was negatively associated with overall survival (OS) and recurrence-free survival (RFS). These results suggested that KIAA1429 was an oncogene and predicted poor prognosis in PC.

2. Alcohol-induced C/EBP β promoted KIAA1429 transcription

Based on the clinical data, we demonstrated a significant correlation between alcohol consumption and KIAA1429 mRNA expression (Supplementary Table 1). The m6A level was significantly higher in patients with a drinking history than that in patients who never consumed alcohol (Supplementary Fig. 2A). Lifestyles, such as drinking, are of great importance for PC progression [12, 23]. We suggested that alcohol consumption led to KIAA1429 dysregulation. Initially, we established the cell models by culturing the cells in the presence of alcohol for three months. The data of qRT–PCR (Fig. 2A) and western blotting

(Fig. 2B) showed that alcohol clearly induced KIAA1429 expression, indicated that alcohol influenced RNA methylation in PC by regulating the RNA methyltransferase KIAA1429.

To reveal the mechanism of KIAA1429 upregulation, we obtained the promoter sequence of KIAA1429 as a 2,000 bp sequence upstream of the transcription start site (http://genome.ucsc.edu/). The 2,000 bp sequence (H2000) and five gradually truncated sequences (H1012, H512, H262, H137 and H94) were cloned into the PGL3 plasmid to detect the promoter activities (Fig. 2C). No significant reduction in luciferase activity was observed by comparison of H2000 with truncated sequences, including H1012, H512, H262 and H137. However, the luciferase activity was clearly decreased when the sequence was truncated to 94 bp, indicating that the basal promoter region was located between 137 and 94 bp (Fig. 2C). Based on the sequence, we obtained a list of four potential transcription factors from the PROMO database (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3). Then, we overexpressed these four transcription factors in HEK293T cells transfected with H137-PGL3. Only C/EBP β promoted the transcriptional activity of H137-PGL3 (Fig. 2D). A significant correlation between KIAA1429 and C/EBP β was identified using the GEPIA database (Fig. 2E). The expression of C/EBP β mRNA was significantly upregulated in PC patients (Supplementary Fig. 2B). Survival curves demonstrated that high C/EBP β expression predicted poor OS (Fig. 2F). C/EBP β overexpression upregulated KIAA1429 at both the RNA (Fig. 2G) and protein levels (Fig. 2H). Furthermore, the results of a ChIP assay revealed that C/EBP β was bound to the promoter of KIAA1429 (Fig. 2I). Alcohol treatment upregulated C/EBP β at both the RNA (Fig. 2J-L) and protein levels (Fig. 2B). These findings demonstrated that alcohol upregulated C/EBP β, which was able to bind to the KIAA1429 promoter to activate its transcription.

3. Overexpression of KIAA1429 promoted PC growth and metastasis

The data of the present study indicated that the expression of KIAA1429 was associated with the pT, pN and pTNM stages (Supplementary Table 1). The results of gene set enrichment analysis implied that KIAA1429 was involved in tumor stemness and metastasis[11]. To elucidate the function of KIAA1429 during the progression of PC, a stable KIAA1429 overexpression model was established in CFPAC-1 cells using a lentivirus, and overexpression was confirmed at the RNA (Fig. 3A) and protein levels (Fig. 3B). The data of the dot blotting (Fig. 3C) and RNA methylation quantification assays (Fig. 3D) showed that the m6A levels in the total RNA and mRNA were significantly increased after KIAA1429 upregulation. Overexpression of KIAA1429 significantly promoted the proliferation of PC cells (Fig. 3E and 3F). In addition, overexpression of KIAA1429 accelerated the migration and invasion of PC cells (Fig. 3G).

4. KIAA1429 knockdown inhibited PC proliferation, migration and invasion

We established the stable knockdown models based on two independent shRNA sequences (shK-1 and shK-2). The knockdown efficiency was confirmed by qRT–PCR (Fig. 3H and Supplementary Fig. 3A) and

western blotting (Fig. 3I and Supplementary Fig. 3B). The data of the dot blotting (Fig. 3J and Supplementary Fig. 3C) and RNA methylation quantification assays (Fig. 3K and Supplementary Fig. 3D) showed that the m6A levels in the total RNA and mRNA were significantly decreased after KIAA1429 knockdown. The results of the CCK-8 (Fig. 3L and Supplementary Fig. 3E) and EdU (Fig. 3M and Supplementary Fig. 3F) assays demonstrated that knockdown of KIAA1429 attenuated the ability of the cells to proliferate. Furthermore, the migration and invasion were considerably inhibited after knockdown of KIAA1429, as indicated by the data of a transwell assay (Fig. 3N and Supplementary Fig. 3G). Furthermore, KPC1199 mouse PC cells were used to investigate the function of KIAA1429 *in vivo*. In an orthotopic transplantation model, KIAA1429 knockdown significantly inhibited the proliferation of PC cells (Fig. 3O). To reveal the function of KIAA1429 in metastasis, KIAA1429-knockdown and control cells were injected into the spleen of C57 mice. As expected, KIAA1429 knockdown diminished liver metastasis in PC (Fig. 3P). Overall, these findings demonstrated that KIAA1429 functioned as a pro-growth and pro-metastatic factor in *vitro and in vivo*.

5. SLC43A2 was identified as a target of KIAA1429 by RNA-seq and MeRIP-seq

To ascertain the mechanism of action of KIAA1429 in PC, we performed RNA-seq combined with MeRIP-seq to analyze the changes in the genes in BxPC-3 cells, control cells or cells with KIAA1429 knockdown (Fig. 4A). RNA-seq revealed 580 upregulated genes and 391 downregulated genes after KIAA1429 knockdown. These altered genes were mostly enriched in the metabolic pathways, such as synthesis and metabolism of amino acids (Fig. 4B). The MeRIP-seq data detected 19,407 peaks in control group and 20,110 peaks in the KIAA1429-deficient group, and the “RRACH” motif was highly enriched in the detected peaks (Fig. 4C). Most peaks were distributed in the promoter regions (≤ 1kb) and in 3'-UTRs (Fig. 4D). After selection, 12 genes with overlapping profiles according to the data of RNA-seq and MeRIP-seq were identified as potential targets of KIAA1429 (Fig. 4A). The SLC43A2 gene attracted our attention, and this gene encodes a member of the L-amino acid transporter family involved in the transport of leucine, phenylalanine, valine and methionine. The results of survival analysis (http://kmplot.com/analysis/index.php?p=background) demonstrated that the expression of SLC43A2 was significantly associated with OS (Fig. 4E) and RFS (Fig. 4F) in PC patients. MeRIP-seq showed a decrease in the peak (chr17:1575169–1575669) of SLC43A2 mRNA in KIAA1429-knockdown cells (Fig. 4G). The presence of a very high confidence m6A site was confirmed by the SRAMP database (http://www.cuilab.cn/sramp) (Fig. 4H). Furthermore, the data of MeRIP-qPCR confirmed that KIAA1429 knockdown significantly decreased the m6A level of SLC43A2 mRNA and overexpression of KIAA1429 increased this level (Fig. 4I).

6. KIAA1429 accelerated SLC43A2 mRNA degradation via the m6A-YTHDF2 pathway

The results of qRT–PCR showed that KIAA1429 knockdown upregulated the level of SLC43A2 mRNA and KIAA1429 overexpression downregulated this level (Fig. 5A). The same results were observed at the

protein level (Fig. 5B). Interestingly, the results of qRT–PCR indicated that SLC43A2 mRNA was downregulated in all PC cell lines (Fig. 5C). Then, the cells were treated with 3-deazaadenosine (DAA), a global methylation inhibitor, and the data demonstrated that SLC43A2 was upregulated at both the RNA (Fig. 5D) and protein levels (Fig. 5E). Next, we constructed the wild type (WT) pmirGLO plasmid with a segment of the SLC43A2 3'-UTR containing the m6A site. The mutant (MUT) plasmid contained a replacement of adenine (A) in the m6A site with thymine (T). The data of the dual-luciferase reporter assays showed that KIAA1429 knockdown promoted the expression of SLC43A2 in the presence of WT plasmid and overexpression of KIAA1429 inhibited the expression, while no changes were observed in the presence of the MUT plasmid (Fig. 5F). Furthermore, KIAA1429 knockdown significantly extended the half-life of SLC43A2 mRNA, and overexpression of KIAA1429 shorten the half-life (Fig. 5G). These results indicated that KIAA1429 accelerated SLC43A2 degradation in an m6A-dependent manner. Human YTH domain family 2 (YTHDF2) was reported to recognize the m6A site and promote mRNA degradation[24, 25]. We reduced the level of YTHDF2 in KIAA1429 overexpressing cells and overexpressed YTHDF2 in KIAA1429-knockdown cells. The results showed that YTHDF2 reversed the degradation of KIAA1429-modified SLC43A2 (Fig. 5H and 5I). The data of RIP-qPCR confirmed that YTHDF2 was directly bound to SLC43A2 mRNA (Fig. 5J). These results suggested that KIAA1429 accelerated the degradation of SLC43A2 mRNA degradation in the m6A-YTHDF2 pathway.

7. SLC43A2 was responsible for KIAA1429-modified PC proliferation and metastasis

Aiming to reveal the function of SLC43A2 in KIAA1429-mediated proliferation of PC, we inhibited SLC43A2 in stable KIAA1429-knockdown cells. The data of a CCK8 assay demonstrated that KIAA1429 knockdown significantly inhibited PC proliferation, and the downregulation of SLC43A2 rescued the inhibition caused by KIAA1429 knockdown (Fig. 6A and 6B). Then, we amplified SLC43A2 in stable KIAA1429-overexpressing cells. Overexpression of SLC43A2 counteracted the growth-promoting effect induced by KIAA1429 (Fig. 6C). The same results were observed in the EdU assay (Fig. 6D-G). Moreover, SLC43A2 knockdown rescued the changes in the migration and invasion caused by a decreased in KIAA1429 (Fig. 6H-J), and the overexpression of SLC43A2 abrogated this effect by inducing KIAA1429 amplification (Fig. 6H and 6K).

8. SLC43A2 induced phenylalanine absorption and ROS accumulation to abrogate PC proliferation and metastasis

SLC43A2 is a member of the solute carrier (SLC) family and functions in the reabsorption of amino acids (leucine, isoleucine, phenylalanine, valine and methionine)[26]. To test which amino acid restricts PC cells, increasing concentrations of amino acids were added in a separate experiment. The results of a CCK8 assay demonstrated that excessive concentration of phenylalanine significantly reduced cell viability (Fig. 7A). ELISA was used to detect the concentration of phenylalanine. Overexpression of SLC43A2 promoted phenylalanine absorption because the medium cultured with SLC43A2-overexpression cells

contained a lower concentration of phenylalanine (Fig. 7B). A study has reported that phenylalanine causes oxidative injury in rat astrocytes [27]. Therefore, we assumed that SLC43A2 upregulated phenylalanine absorption and increased oxidative stress to inhibit the progression of PC. We detected significantly elevated levels of reactive oxygen species (ROS) in SLC43A2-overexpressing cells, and this increase was not detected in the cells pretreated with N-acetyl-L-cysteine (NAC), an antioxidant (Fig. 7C). In addition, NAC rescued the inhibition of the proliferation (Fig. 7D and 7E) and metastasis (Fig. 7F) caused by SLC43A2 overexpression. Overall, the present study demonstrated that SLC43A2 modified by KIAA1429 is involved in PC progression via the regulation of phenylalanine metabolism and oxidation.

Discussion

Multiple studies have elucidated the prominent role of m6A in cancer progression[28]. Guo *et al.* demonstrated that ALKBH5 inhibits the malignant phenotypes of PC by stabilizing PER1 mRNA in an m6A-dependent manner, activating the ATM-CHK2-p53/CDC25C pathway[6]. Another study demonstrated that m6A-modified PIK3CB enhances PC growth and metastasis through AKT signaling[3]. All these findings provide solid evidence for a regulatory role of m6A in the progression of PC. Consistent with these findings, we detected a significant increase in the m6A level in the PC tissues and cells by the dot blotting and m6A quantification assays. Focusing on KIAA1429, an important adaptor of the methyltransferase complex, we proved the oncogenic role of KIAA1429 in the progression of PC based on the results of *in vitro and in vivo* assays, which were in accordance with the published data of other authors[9–11, 20–22]. KIAA1429 regulates cancer progression in an m6A-dependent manner in liver cancer[9] and non-small-cell lung cancer[10]; however, the regulation is m6A-independent in breast cancer[20], colorectal cancer[21] and gastric cancer[21]. To clarify this hypothesis, we treated the cells with DAA, a methylation inhibitor, and SLC43A2 was upregulated at both the RNA and protein levels. Therefore, we concluded that the expression of SLC43A2 was regulated by KIAA1429 in an m6A-dependent manner.

m6A, is a reversible epigenetic regulator that may be closely associated with lifestyle to participate in cancer progression. A study by Zhang [29] *et al.* demonstrated that cigarette smoking activates METTL3 and promotes the progression of PC. However, the role of alcohol and m6A in cancer is unclear. The data of the present study indicated that the expression of KIAA1429 mRNA was related to drinking and not to smoking. To explore the effect of drinking on KIAA1429, we initially established an *in vitro* model by treating the cells with alcohol. Consistent with the clinical data, alcohol treatment upregulated KIAA1429 both at the mRNA and protein levels. The results of the luciferase reporter and ChIP assays confirmed that C/EBP β served as transcription factor of KIAA1429. In addition, the results were supported by the TCGA data. Finally, we demonstrated that the transcription factor C/EBP β was upregulated, and this upregulation was responsible for an increase in KIAA1429.

A combination of the sequencing data and bioinformatics analysis with the results of qRT–PCR, western blotting, MeRIP-PCR, luciferase reporter and rescue experiments demonstrated that SLC43A2 was the downstream target of KIAA1429. SLC43A2 is an amino acid transporter located in the cell membrane and

is a member of the major facilitator superfamily; this protein is responsible for the transport of phenylalanine, leucine, isoleucine, methionine and valine[30, 31]. Bian et al. reported that SLC43A2 upregulated in the mouse melanoma cell line B16F10 is able to outcompete CD8 + T cells with regard to the transport of methionine, inducing apoptosis in CD8 + T cells [32]. In contrast, the results of the present study revealed that SLC43A2 was downregulated in PC cells, and this downregulation predicted poor prognosis. Indeed, the levels of multiple amino acids, such as phenylalanine, leucine, isoleucine and valine, were elevated in the circulation of PC patients[33]. Macropinocytosis and lysosomal degradation may represent an alternative approach to obtain essential amino acids for certain hypovascular cancers, such as PC[34]. These findings indicated the differences in metabolic reprogramming in various cancers.

ROS are generated by nicotinamide adenine dinucleotide phosphate oxidases and the mitochondrial respiratory chain[35]. Cancer cells produce large quantities of ROS during rapid proliferation, and the internal regulation of the antioxidant mechanisms is necessary for cancer survival. The role of ROS in tumor biology remains controversial. On the one hand, ROS cause the inactivation of the DNA repair enzymes, and DNA damage accumulation leads to precancerous lesions[36]. ROS promote tumor growth and epithelial-to-mesenchymal transition by acting as second messengers[35]. On the other hand, excessive ROS inhibit malignant phenotypes and thus may be used for cancer therapy[37, 38]. The present study demonstrated that the ROS level was increased after SLC43A2 overexpression, and administration of NAC rescued the inhibition caused by SLC42A2 overexpression. The downregulation of SLC43A2 and a decrease in the level of phenylalanine mitigated oxidative stress and were beneficial for cancer survival and metastasis, indicating that these events may be a novel target for PC therapy.

Overall, the present study provided a novel mechanism of the progression of PC involving alcohol consumption, m6A regulation and phenylalanine metabolism (Fig. 8). The oncogenic role and predictive characteristics were confirmed. Mechanistically, the transcription factor C/EBP β was able to bind to the promoter of KIAA1429 to activate its expression. Alcohol treatment induced the expression of C/EBP β to upregulate KIAA1429. KIAA1429 accelerated SLC43A2 degradation, thus reducing phenylalanine absorption and oxidative stress in an m6A-YTHDF2-dependent manner. These findings provide new ideas for conquering PC.

Materials And Methods

Cell culture

HEK293T, human PC (SW1990, BxPC-3, AsPC-1, CFPAC-1 and PANC-1) and normal pancreatic ductal epithelial cells (HPDE6-C7) were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The short tandem repeat profiling was used for cell authentication. HEK293T, SW1990, BxPC-3, CFPAC-1 PANC-1 and HPDE6-C7 cells were cultured in DMEM (Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco, USA). AsPC-1 cells were grown in RPMI-1640 (Gibco, USA) supplemented with 10% FBS. All cells were maintained at 37 °C in an atmosphere of 5% CO_2. In certain

experiments, the administration of specific amino acids (2 μM phenylalanine, 4 μM leucine, 4 μM isoleucine, 1 μM methionine and 4 μM valine) was used to detect an effect on PC.

Alcohol-induced cell model

HPDEC6-7, BxPC-3 and SW1990 cells were cultured as described above. Ethanol (100%; Sigma, USA) was added to the medium to a concentration of 20 mg/100 ml. The cells were cultured continuously for three months, and the medium was replaced every 3 days.

Human samples

The PC tissues and fragments of the normal pancreas were collected during surgery. The present study was approved by the Ethics Committee of the First Affiliated Hospital of Harbin Medical University. Informed consent was obtained from all patients.

Vector construction

The 2,000bp sequence upstream of the KIAA1429 TSS (-2000/0) was obtained from UCSC (http://genome.ucsc.edu/). The 2,000bp sequence and five deletion sequences (-1012/0, -512/0, -262/0, -137/0 and -94/0) were inserted into the PGL3-basic vector.

The full sequences of SLC43A2, YTHDF2, C/EBP β, GATA1, GR-α and TFII-I were cloned into the PCDNA3.1 vector.

The sequence adjacent to the m6A motif in the SLC43A2 3'-UTR was cloned into the pmirGLO vector (WT), and a mutant plasmid (MUT) wad produced by substituting A with T at the m6A site.

Gene knockdown or overexpression

The sequence for stable KIAA1429 knockdown was designed by Hanbio Tech (Shanghai, China) and inserted into the pHBLV-U6-MCS -PGK-PURO plasmid.

Stable KIAA1429 overexpression was produced by GeneChem Tech (Shanghai, China) using the Ubi-MCS-IRES-PURO plasmid.

siRNAs were used for transient knockdown of YTHDF2 or SLC43A2.

The detailed sequences are listed in Supplementary Table 3.

RNA preparation and qRT–PCR

An RNA extraction kit (Accurate Biology, China), a reverse transcription kit (Toyobo, Japan) and SYBR Green (Roche, Switzerland) were used for qRT–PCR. Gene expression was normalized to the level of GAPDH. Supplementary Table 2 contains a complete list of the primers.

Western blotting

The antibodies for western blotting were as follows: β-actin (Boster, China), GAPDH (Sangon Biotech, China), KIAA1429 (CST, USA), SLC43A2 (Sangon Biotech, China), C/EBP β (Santa Cruz, USA) and YTHDF2 (Proteintech, USA).

Cell proliferation and cell metastasis assays

CCK-8 (MedChemExpress) and EdU (Beyotime Biotechnology) experiments were performed to investigate cell growth as described elsewhere[39].

Transwell assays were used to evaluated the invasive and migratory capacities as described previously[16].

Dot blotting

A total of 800 ng of RNA was loaded onto a Hybond-N^+ membrane (Amersham, USA). After UV crosslinking, the membrane was blocked and immunoblotted with an anti-m6A antibody (CST, USA) and a secondary antibody. The signals on the membrane were visualized by enhanced chemiluminescence (Bio-Rad, USA). The samples were stained with methylene blue (MB), and this signal was used for normalization.

m6A quantification

An mRNA purification kit (ThermoFisher, USA) was used to separate mRNA from the total RNA. The percentage of methylated mRNA was detected by an EpiQuik™ m6A RNA methylation quantification kit (Epigentek, USA).

RNA-seq, MeRIP-seq and MeRIP-qPCR

Total RNA extraction and mRNA purification were conducted as described above. A NEBNext® Ultra™ RNA library prep kit (NEB, USA) was used to generate the sequencing libraries. The 150 bp paired-end reads were obtained using the Illumina HiSeq 4000 platform.

Me-RIP was performed as described previously [40]. Briefly, purified RNA was fragmented into ~100 nt oligonucleotides. A portion of the RNA served as the input, and the remaining RNA was immunoblotted with an anti- m6A antibody (Synaptic Systems). RNA captured by an anti-m6A antibody was collected on protein A beads (Thermo Fisher, USA). Finally, methylated RNA was used for standard RNA-seq or qRT–PCR. The SLC43A2 primers are listed in Supplementary Table 2.

RIP

The RIP experiment was carried out using a RIP kit (BersinBio, China). Briefly, the protein–RNA complexes were extracted using polysome lysis buffer. RNA bound to the target protein was immunoprecipitated

with an anti-YTHDF2 antibody (Protein, USA) and pulled down by magnetic beads.

ChIP

ChIP was performed using a chromatin IP kit (CST, USA). In brief, 1×10^7 cells were crosslinked with 1% formaldehyde; the reaction was stopped by glycine, and the adducts were collected. After nuclei isolation and chromatin fragmentation, 2% of the digested chromatin was used for the input, and remaining material was coincubated with an anti-C/EBP β antibody (Santa Cruz, USA) or IgG (CST, USA) for 4 h. Finally, the immunoprecipitated chromatin was collected using protein G magnetic beads, purified and quantified by PCR.

Luciferase reporter assay

A luciferase reporter assay was performed using a luciferase system (Promega, USA) following the manufacturer's protocol.

RNA stability

The cells were incubated with actinomycin D (ActD, 5 μg/ml) for 15, 30, 60, 120 and 180 min. The expression of SLC43A2 mRNA was detected by qRT–PCR.

Phenylalanine detection

The cells were transfected with SLC43A2-containing or empty PCDNA 3.1 plasmids. Fresh medium was replaced 48 h after the transfection, and the cells were continuously cultured for 48 h. The medium was collected, and the concentration of phenylalanine was detected by a phenylalanine enzyme linked immunosorbent assay (ELISA) kit (Spbio, China).

In vivo experiments

Six-week-old female C57BL/6 mice were purchased from Charles River (Beijing, China). The KPC1199 cell-derived KPC pancreatic ductal adenocarcinoma mouse model was provided by Dr. Jing Xue from Renji Hospital (Shanghai, China). To generate an orthotopic model, 5×10^5 KPC1199 cells with stable KIAA1429 knockdown and luciferase expression or the corresponding controls were surgically implanted in the pancreas. To generate a liver metastasis model, 5×10^5 KPC1199 cells were surgically injected into the spleen. Splenectomy was performed after 5 min. Four weeks later, mice were anaesthetized, injected with luciferin and imaged using a Night-OWL II LB983 imaging system (Berthold Technologies, Germany).

Statistics

The differences were evaluated using Student's t test. The correlations between KIAA1429 mRNA and the clinical data were analysed by chi-squared test. Kaplan–Meier plots used for survival analysis were compared using log-rank test. A P value < 0.05 was considered significant.

Declarations

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The KPC1199 cell-derived KPC pancreatic ductal adenocarcinoma mouse model was provided by Dr. Jing Xue from Renji Hospital (Shanghai, China).

Author contributions

LG: conceptualization, data curation, software, investigation, visualization, writing-original draft; GYL: data curation, validation, investigation; ZYL: data curation, validation, investigation; DST: software, investigation; CSY: formal analysis; JMM: data curation, validation; LC: investigation; LL: supervision, conceptualization; BS: supervision, conceptualization, methodology; GW: conceptualization, supervision, methodology, writing-review and editing; QST: supervision, conceptualization, methodology.

Conflict of Interest

All authors declare no conflicts of interest to disclose.

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Figures

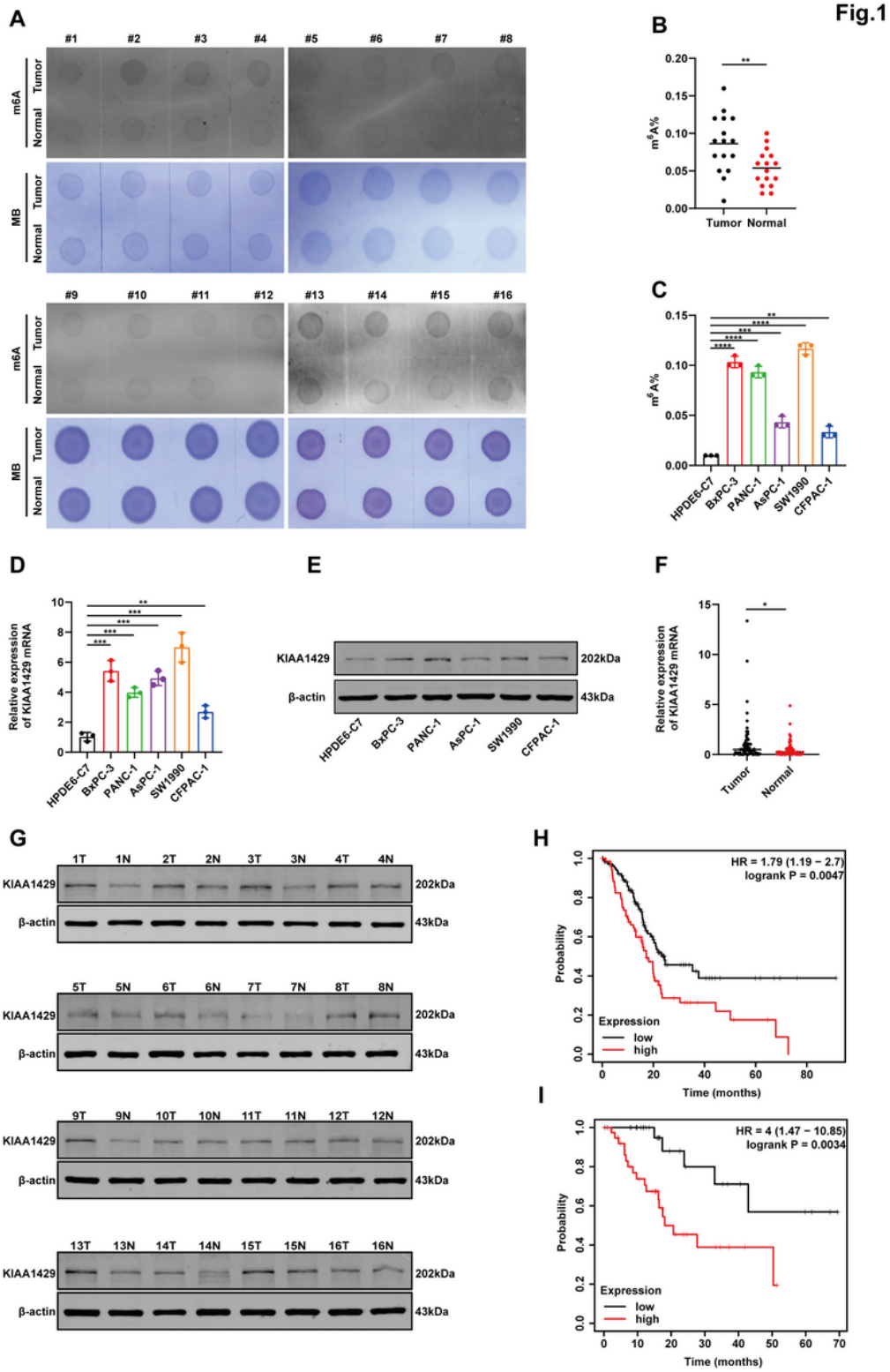

Figure 1

KIAA1429 was upregulated in PC and predicted poor prognosis.

(A) The m6A level in total RNA of the PC tissues was analyzed by dot blot.

(B) The m6A level in mRNA of the PC tissues was quantified by a colorimetric method.

(C) The m6A level in mRNA of the PC cells was analyzed by a colorimetric method.

(D) The expression of KIAA1429 mRNA in PC cells was quantified by qRT–PCR.

(E) The expression of KIAA1429 in PC cells was detected by western blotting.

(F) The expression of KIAA1429 mRNA in PC tissues was quantified by qRT–PCR.

(G) The expression of KIAA1429 in PC tissues was detected by western blotting.

(H) High expression of KIAA1429 predicted poorer OS.

(I) High expression of KIAA1429 predicted poorer RFS.

*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001

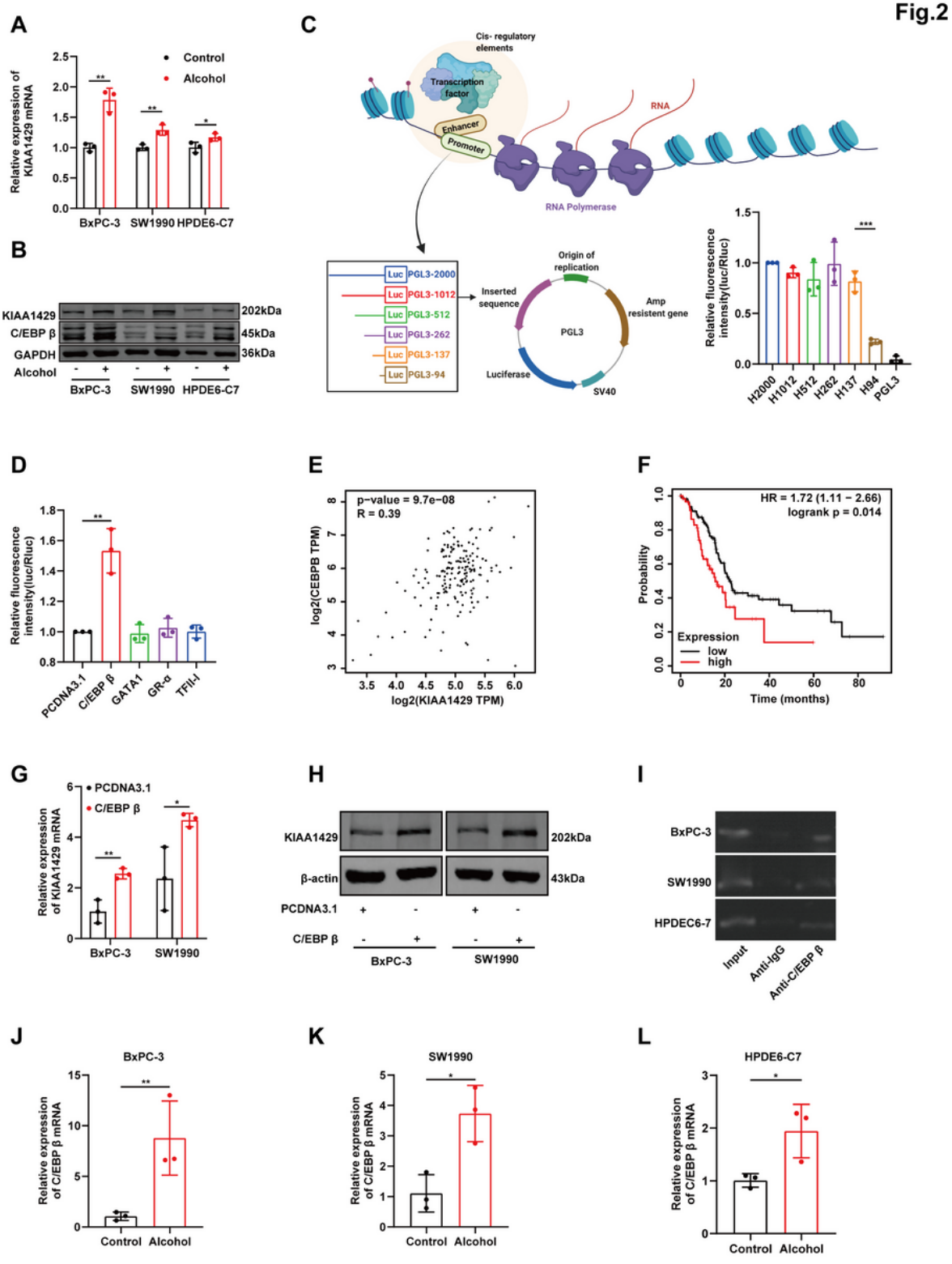

Figure 2

Alcohol-induced C/EBP β activated KIAA1429 transcription

(A) Alcohol treatment upregulated the expression of KIAA1429 mRNA.

(B) Alcohol treatment upregulated the expression of C/EBP β and KIAA1429.

(C) KIAA1429 promoters spanning from -2,000/-1,012/-512/-262/-137/-94 to 0 were generated, inserted into the PGL3 plasmid and transfected into HEK293T cells. The relative fluorescence signal was measured.

(D) C/EBP β overexpression significantly boosted the relative fluorescence signal of the cells transfected with the PGL3-137 plasmid.

(E) The expression of C/EBP β was positively correlated with KIAA1429.

(F) High expression of C/EBP β predicted poorer OS.

(G) The data of qRT–PCR indicated that C/EBP β promoted the expression of KIAA1429 mRNA.

(H) The data of Western blotting indicated that C/EBP β promoted the expression of KIAA1429.

(I) The ChIP assay proved that C/EBP β was bound to the promoter of KIAA1429.

(J-L) The data of qRT–PCR demonstrated that alcohol promoted the expression of C/EBP β mRNA.

*P<0.05, **P<0.01, ***P<0.001

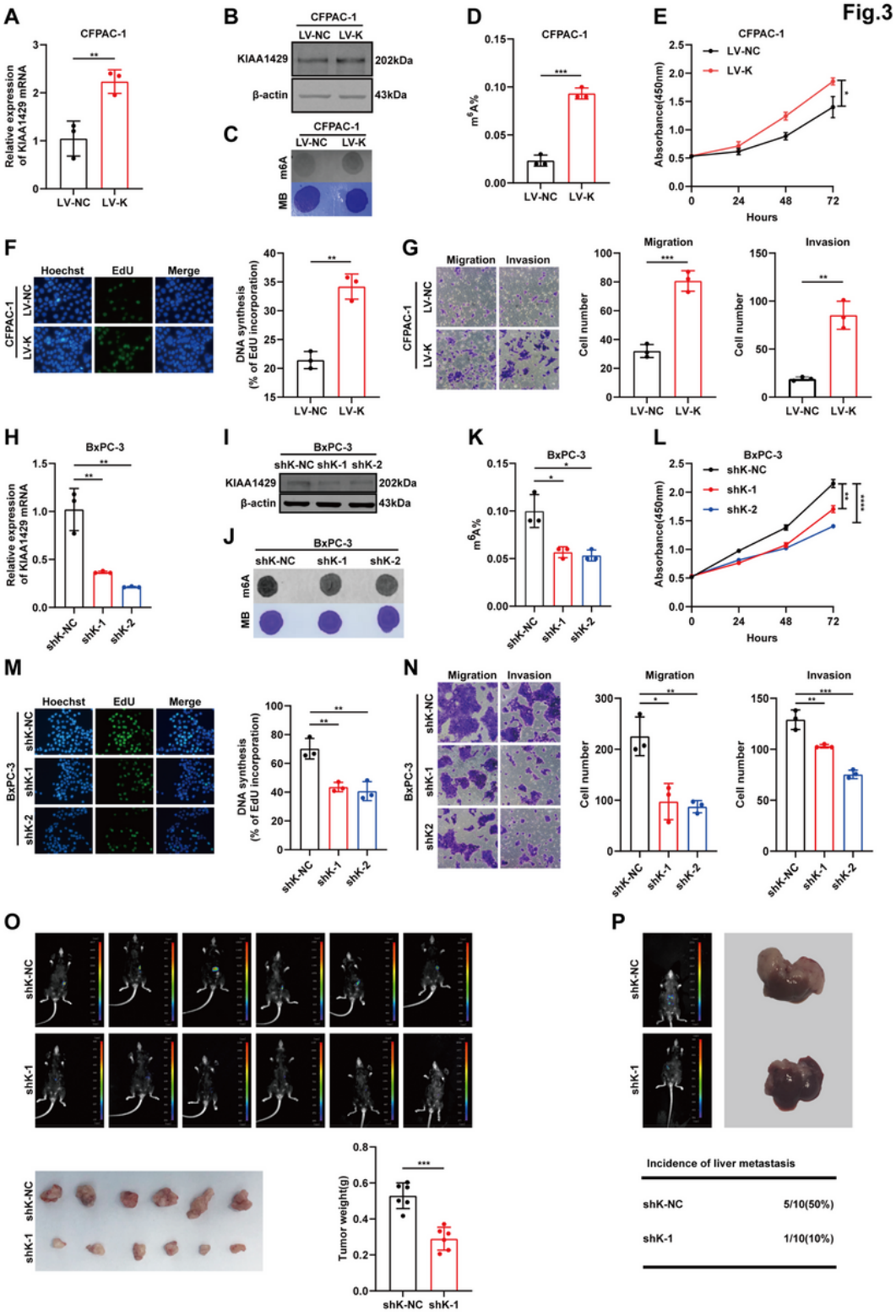

Figure 3

The function of KIAA1429 in PC progression *in vitro and in vivo.*

(A) The overexpression efficiency of KIAA1429 was quantified by qRT–PCR.

(B) The overexpression efficiency of KIAA1429 was measured by western blotting.

(C) The effect of KIAA1429 overexpression on the m6A level in total RNA was assessed by dot blotting.

(D) The effect of KIAA1429 overexpression on the m6A levels in mRNA was detected by a colorimetric assay.

(E) KIAA1429 overexpression improved cell viability.

(F) KIAA1429 overexpression promoted cell proliferation.

(G) KIAA1429 overexpression promoted cell metastasis.

(H) The knockdown efficiency of KIAA1429 was quantified by qRT–PCR.

(I) The knockdown efficiency of KIAA1429 was measured by western blotting.

(J) The effect of KIAA1429 knockdown on the m6A levels in total RNA was assessed by dot blotting.

(K) The effect of KIAA1429 knockdown on the m6A levels in mRNA was detected by a colorimetric assay.

(L) KIAA1429 knockdown attenuated cell viability.

(M) KIAA1429 knockdown inhibited cell proliferation.

(N) KIAA1429 knockdown inhibited cell metastasis.

(O) Knockdown of KIAA1429 inhibited PC growth *in vivo*.

(P) Knockdown of KIAA1429 inhibited PC metastasis *in vivo*.

*P<0.05, **P<0.01, ***P<0.001

Fig.4

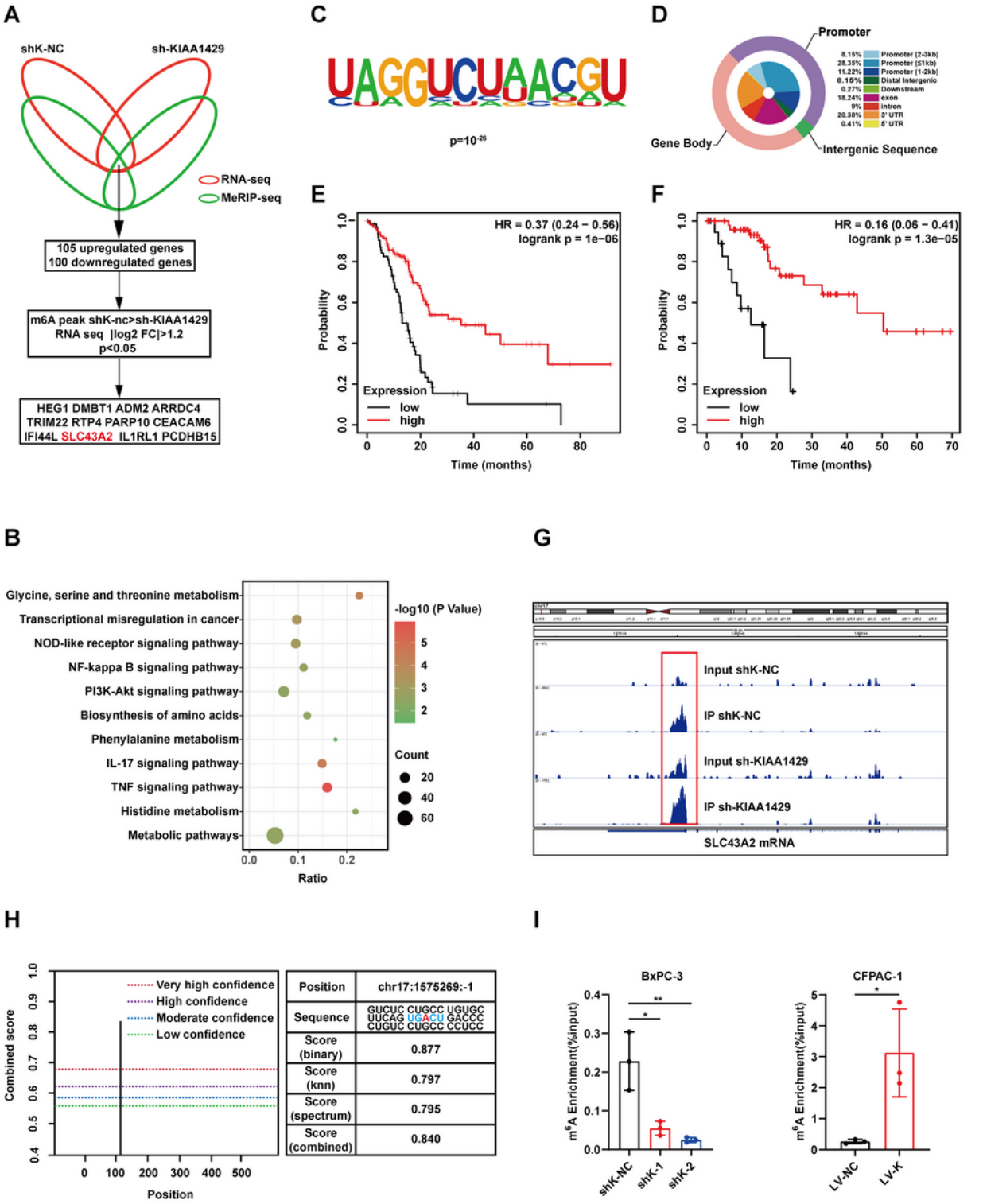

Figure 4

SLC43A2 was the target of KIAA1429.

(A) Schematic diagram of identification of the target of KIAA1429 by MeRIP-seq and RNA-seq.

(B) KEGG analysis shows the changes in the genes in the pathways influenced by KIAA1429 knockdown.

(C) The top m6A motif in BxPC-3 cells.

(D) The consensus distribution of the m6A peaks in BxPC-3 cells.

(E) Low expression of SLC43A2 predicted poorer OS.

(F) Low expression of SLC43A2 predicted poorer DFS.

(G) The m6A peak of SLC43A2 in KIAA1429 knockdown or control cells.

(H) The SRAMP database predicted the m6A site of SLC43A2 with very high confidence.

(I) The data of MeRIP-qPCR indicated that KIAA1429 elevated the m6A level of SLC43A2 mRNA.

*P<0.05, **P<0.01

Fig.5

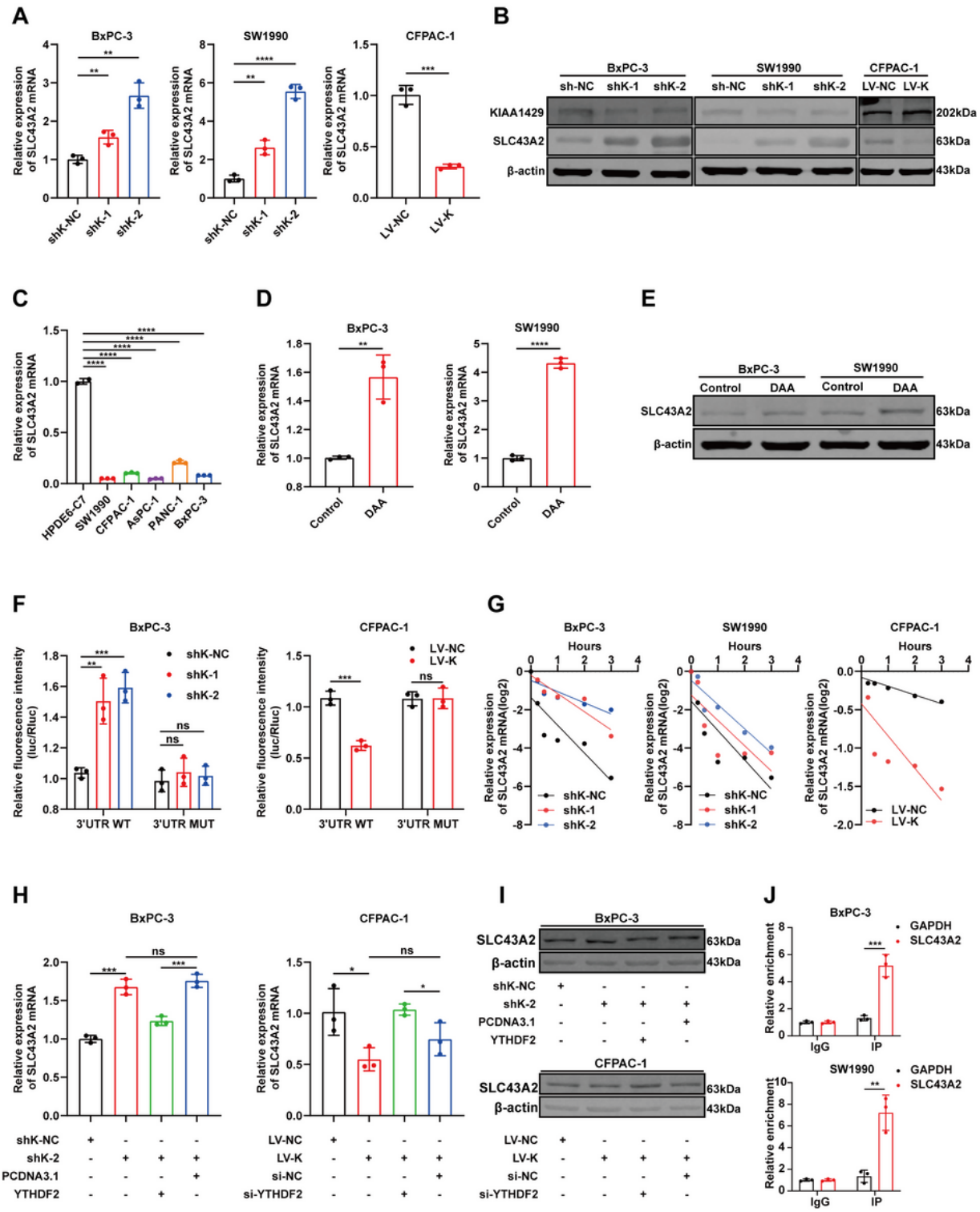

Figure 5

KIAA1429 promoted the degradation of SLC43A2 mRNA via the m6A-YTHDF2 pathway

(A) The data of qRT–PCR showed a negative correlation between SLC43A2 mRNA and KIAA1429.

(B) The data of western blotting indicated that SLC43A2 was negatively associated with KIAA1429.

(C) SLC43A2 mRNA was downregulated in PC cells.

(D) The data of qRT–PCR indicated that DAA treatment upregulated SLC43A2 mRNA.

(E) The data of western blotting indicated that DAA treatment upregulated SLC43A2.

(F) The cells were transfected with wild-type or mutant plasmids. The relative fluorescence signals were measured.

(G) KIAA1429 decreased the half-life of SLC43A2 mRNA.

(H-I) The data of qRT–PCR and western blotting indicated that YTHDF2 was involved in KIAA1429-modified degradation of SLC43A2.

j) The data of RIP-qPCR indicated that YTHDF2 was directly bound to SLC43A2 mRNA.

*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001

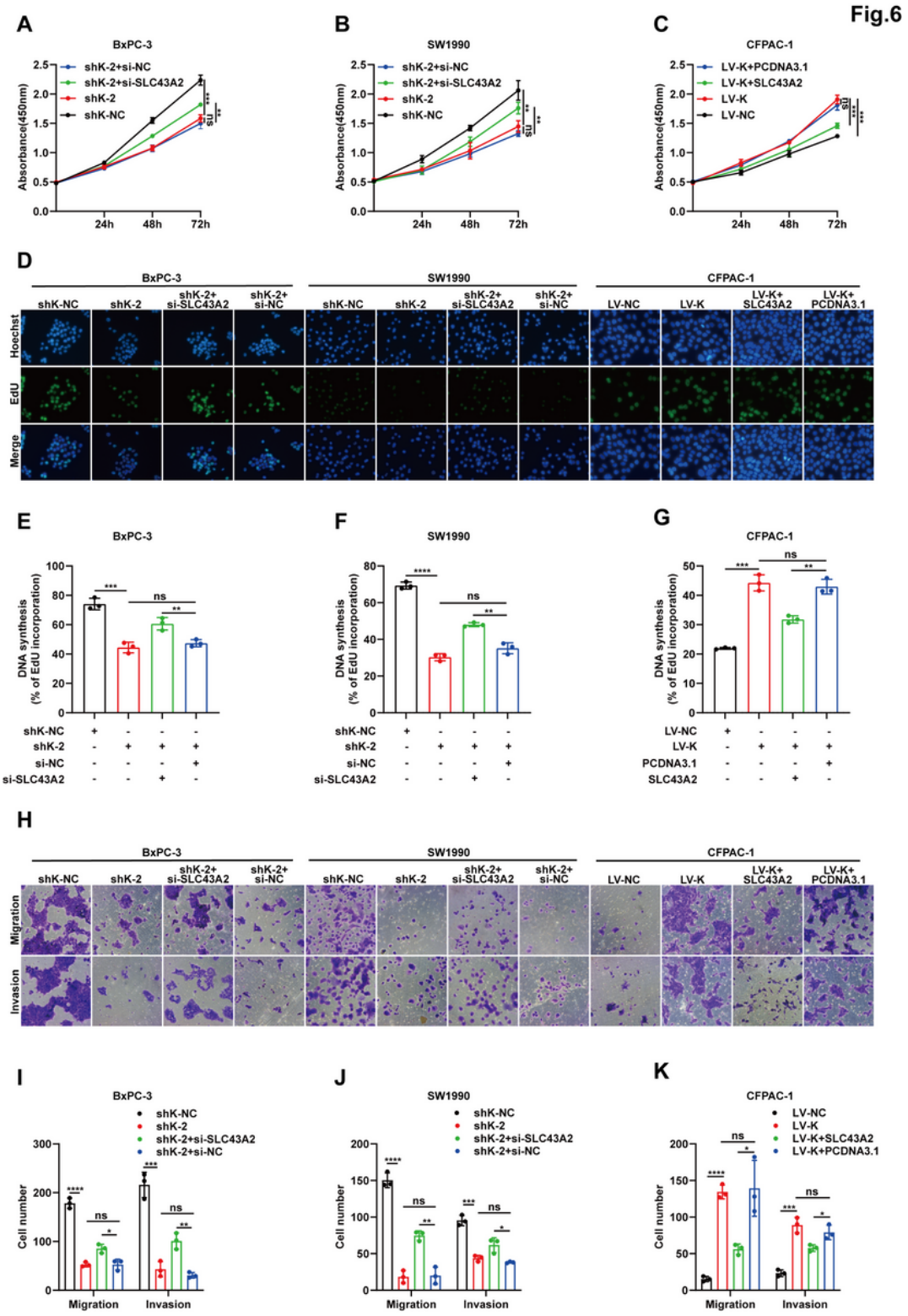

Figure 6

The role of SLC43A2 in PC progression

(A-D) SLC43A2 abrogated the proliferation of PC cells modified by KIAA1429.

(E-K) SLC43A2 abrogated metastasis of PC cells modified by KIAA1429.

*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001

Fig.7

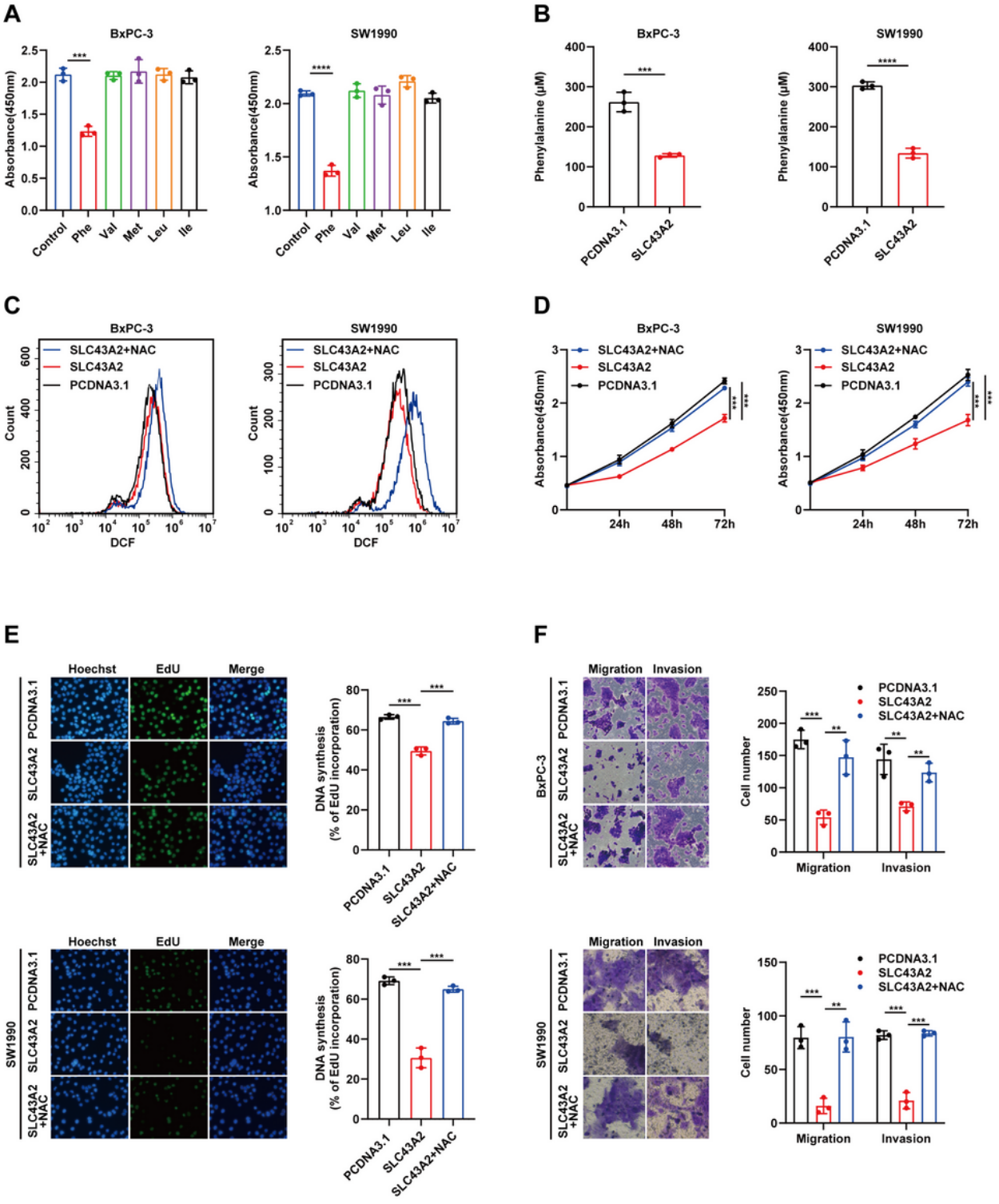

Figure 7

SLC43A2 induced phenylalanine absorption and ROS accumulation to abrogate PC proliferation and metastasis

(A) Cell viability after treatment with the indicated amino acids.

(B) SLC43A2 overexpression significantly decreased the concentration of phenylalanine in the medium.

(C) NAC pretreatment abrogated the elevated ROS level induced by SLC43A2 overexpression.

(D) NAC pretreatment rescued the adverse impact on cell viability induced by SLC43A2 overexpression.

(E) NAC pretreatment rescued the adverse impact on cell proliferation induced by SLC43A2 overexpression.

(F) NAC pretreatment rescued the adverse impact on cell metastasis induced by SLC43A2 overexpression.

**P<0.01, ***P<0.001, ****P<0.0001

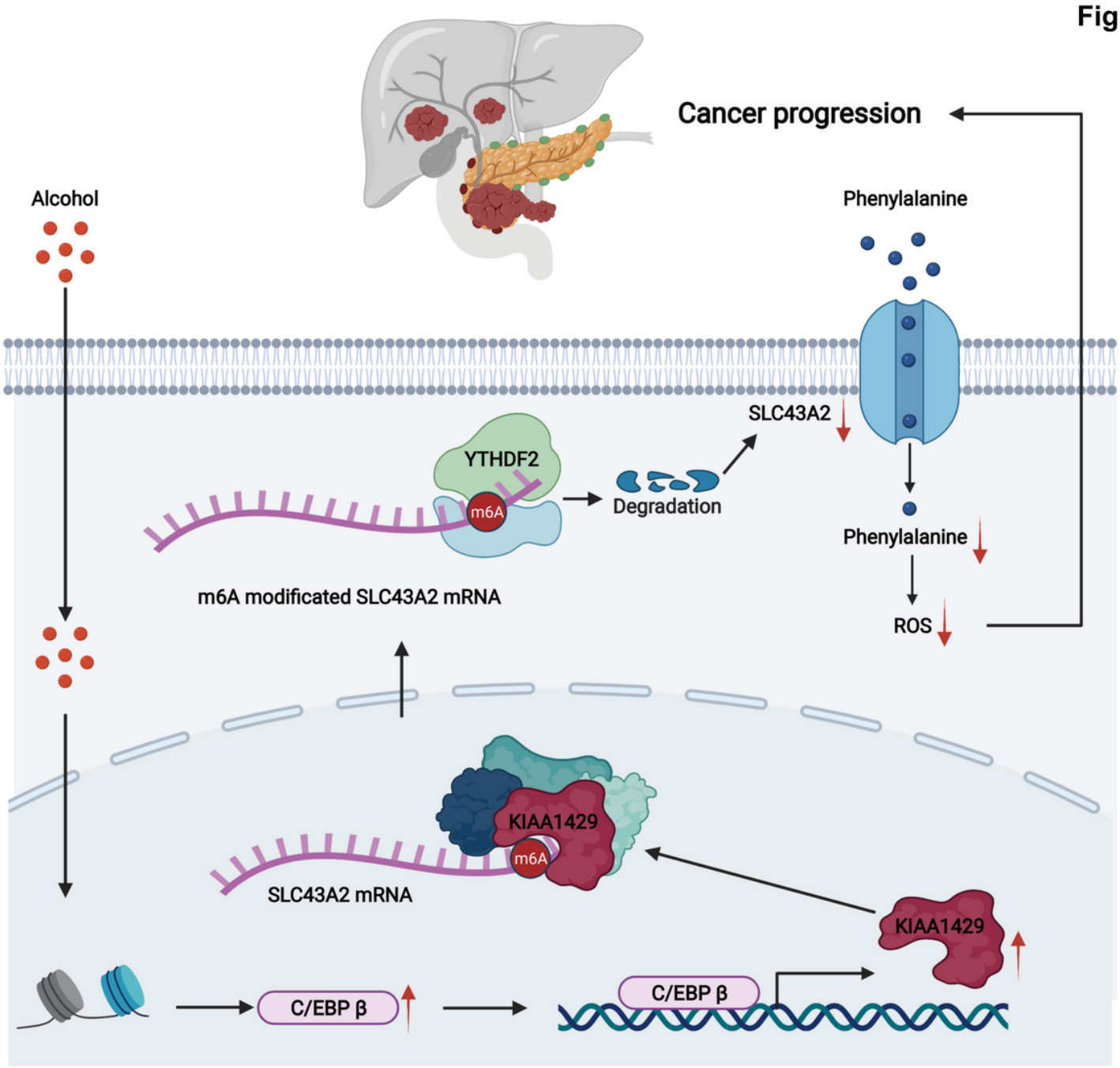

Figure 8

The proposed mechanism of the regulation of the progression of PC by KIAA1429.

Alcohol upregulated the expression of C/EBP β. C/EBP β was bound to the promoter of KIAA1429 and activated its transcription. KIAA1429 decreased phenylalanine absorption and ROS levels, thereby promoting PC growth and metastasis by destabilizing SLC43A2 in a m6A-YTHDF2-dependent manner.

Supplementary Files

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