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Original Research

Linc-UROD stabilizes ENO1 and PKM to strengthen glycolysis, proliferation and migration of pancreatic cancer cells

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ABSTRACT

Pancreatic cancer (PC) is a fatal malignancy, threatening human health in worldwide. Long non-coding RNAs (lncRNAs) have been acknowledged to be essential regulators in various biological processes of human cancers. However, the role of some novel lncRNAs in PC remain to be explored. In this study, we focused on the function and molecular mechanism of a novel lncRNA linc-UROD (also named TCONS_00002016 or XLOC_000166) in PC. The expression of linc-UROD was found to be upregulated in PC cells. The results of loss-of-function assays demonstrated that linc-UROD knockdown suppressed cell proliferation and migration, induced cell cycle G0/G1 arrest, and accelerated apoptosis of PC cells. Through mechanistic experiments, we found that IGF2BP3 stabilized linc-UROD through METTL3-mediated m6A modification. In addition, linc-UROD enhances the stability of ENO1 and PKM through interacting with them to inhibit ubiquitination. Detection on glucose consumption, pyruvate kinase activity and lactate production indicated that linc-UROD accelerated glycolysis of PC cells through PKM/ENO1-mediated pathway. To summarize, linc-UROD stabilized by IGF2BP3/METTL3 contributes to glycolysis and malignant phenotype of PC cells by stabilizing ENO1 and PKM. The findings suggest that linc-UROD may be a novel therapeutic target for PC patients.

Introduction

Pancreatic cancer (PC) is one of the malignant gastrointestinal tumors which mainly occurs in men aged between 40 and 85 years [1]. There are approximately 50,000 newly diagnosed PC patients each year [2]. According to the statistics of GLOBOCAN 2012, there are over 331,000 PC-associated deaths every year [3]. The risk factors of PC include obesity, diabetes, diet, and smoking [4,5]. So far, the combination of surgery, chemotherapy and radiotherapy is the optimal therapeutic method for PC patients [6]. However, most PC patients are diagnosed at advanced stages and has poor prognosis [7]. In addition, the overall 5-year survival rate of PC is unfavorable and the recurrence rate of PC remains high [8]. In recent years, the importance of molecular biology in PC has been expounded [9]. Hence, the identification of valuable biomarkers for PC is of great significance [10]. Glycolysis has been reported to be a hallmark of cancers [11]. Moreover, glucose metabolism plays an important role in the progression of PC [12]. Therefore, the regulatory mechanism underlying the glycolysis in PC is also worth investigating.

Long non-coding RNAs (lncRNAs) belong to non-protein coding transcripts family that are longer than 200 nucleotides. Emerging studies have implied that lncRNAs are involved in many biological processes of tumors [13]. For example, Shang et al. have demonstrated that lncRNA CCAT1 accelerates the progression of colorectal cancer via serving as a sponge for miR-181a-5p [14]. Chen et al. have proposed that lncRNA SNHG16 contributes to lung cancer development by sponging miR-520 to regulate VEGF expression [15]. Rui et al. have clarified that lncRNA DLG1-AS1 boosts cell proliferation in cervical cancer through miR-107/ZHX1 axis [16]. Peng et al. have illustrated that lncRNA TNRC6C-AS1 facilitates thyroid carcinoma through modulation of Hippo signaling pathway [17]. Furthermore, lncRNAs have been confirmed to participate in the malignant phenotypes of PC cells, including cell growth, invasion, and angiogenesis [18]. For instance, Gao et al. have confirmed that lncRNA ZEB2-AS1 influences cell growth and invasion in PC by functioning as a competing endogenous RNA

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(ceRNA) [19]. Deng et al. have determined that the SNHG14/miR-613/ANXA2 axis plays the promoting role in PC [20]. Wang et al. have elucidated that lncRNA CRNDE facilitates cell proliferation and metastasis in PC via enhancing IRS1 expression [21]. Nevertheless, most lncRNAs remain to be investigated in PC.

As a novel lncRNA, linc-UROD (also named TCONS_00002016 or XLOC_000166) has not been explored in any human diseases yet. Here, we focused on the function of linc-UROD in PC cells and its underlying mechanisms.

Materials and methods

Cell culture

Human normal pancreatic duct epithelial cell line (HPDE6-C7) was obtained from Huatuo Biological Technology Co., Ltd. (Shenzhen, China). PC cell lines (BxPC3, BxPC1, MIA, PANC-1 and AsPC-1) were all purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). AsPC-1 and BxPC3 cell lines were cultured in RPMI-1640 Medium supplemented with 10% fetal bovine serum (FBS). HPDE6-C7, MIA and PANC-1 cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) contained 10% FBS. All cell lines were cultured in a 10 cm dish at a density of 8×10^6/dish and incubated in air with 5% CO_2 at 37°C.

Cell transfection

For overexpression, the whole sequence of METTL3, ENO1 or PKM was separately sub-cloned into pcDNA3.1 vector to generate pcDNA3.1/METTL3, pcDNA3.1/ENO1 and pcDNA3.1/PKM, with pcDNA3.1 empty vector as the negative control (named as pcDNA3.1) Additionally, siRNAs targeting linc-UROD (si-linc-UROD#1, si-linc-UROD#2, si-linc-UROD#3), METTL3 (si-METTL3#1, si-METTL3#2, si-METTL3#3), METTL14 (si-METTL14#1, si-METTL14#2, si-METTL14#3), METTL16 (si-METTL16#1, si-METTL16#2, si-METTL16#3), IGF2BP3 (si-IGF2BP3#1, si-IGF2BP3#2, si-IGF2BP3#3) and negative control siRNA (siCtrl) were all synthesized by GenePharma (Shanghai, China). All transfections were used Lipofectamine 3000 (Invitrogen, USA) based on the manufacturer's instructions. For transfection, cells were planted onto 24-well plates with siRNA/medium or DNA/medium ratio at 1.0 μl/50 μl or 0.8 μg/50 μl.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA was extracted from PC cells using TRIzol reagent (Invitrogen, USA) according to the guidance. Then, total RNA was reversely transcribed to cDNA with RevertAid First Strand cDNA Synthesis Kit (Thermo fisher, IL, USA). SYBR Green PCR Master Mix (Applied Biosystems, USA) was used for RT-qPCR analysis. Thermocycling conditions were as follows: 95°C for 5min (40 cycles) for pre-denaturation, followed by denaturation at 95°C for 10 s (40 cycles), annealing and extension at 60°C for 30 s for fluorescence collection. The relative RNA expression was calculated using $2^{-\Delta\Delta Ct}$ method by normalizing to the internal control GAPDH or U6. The experiment was independently conducted in triplicate.

Colony formation assay

After 48 h transfection, cells (600 cells per well) were planted in 6-well plates and incubated for 14 days. Next, cells were fixed with 4% PFA for 30 min and stained with 0.5% crystal violet solution for 15 min. The number of colonies was counted eventually. The experiment was independently conducted in triplicate.

Transwell assay

A total of 2×10^4 PC cells were collected after transfection, and then seeded in the upper chamber of transwell chambers (24-well; Corning Incorporated, Corning, NY, USA) with serum-free medium for testing cell migration. The lower chamber was filled with the complete culture medium. Twenty-four hour later, the migrated cells were dyed by crystal violet and counted under an optical microscope (DMI1, Leica, Wetzlar, Germany). The assay was independently carried out in triplicate.

Flow cytometry analysis

Cell cycle distribution and apoptosis were monitored via Cell Cycle Detection Kit I (BD Biosciences, Franklin Lake, NJ, USA) and Annexin V-FITC/PI Apoptosis kit (Invitrogen, Carlsbad, CA, USA), respectively. PC cells were collected from pre-cooled PBS for staining. The samples were suspended and moved to culture tube with addition of Annexin V-FITC (0.2mg/ml) and PI (0.952mg/ml). The cell cycle distribution and apoptosis rate were detected by a flow cytometer (BD Biosciences, USA) and analyzed by FlowJo software version 10.5.3 (Tree Star, USA). The experiment was independently conducted in triplicate.

Extracellular acidification rate (ECAR) assay

Cells were plated onto 96-well plate with density of 1×10^4 cells/well. The extracellular acidification rate (ECAR) was measured using the Seahorse XF 24 Extracellular Flux Analyzer (Seahorse Bioscience, CA, USA). ECAR was measured using Seahorse XF Glycolysis Stress Test kit and Seahorse XF Cell Mito Stress Test kit (Agilent Technologies, CA, USA).

Detection of glucose consumption, pyruvate kinase activity and lactate production

PC cells were cultured in glucose-free DMEM for 16 h, and then incubated with high-glucose DMEM under normoxic conditions for an additional 24 h. Culture medium was then removed, and intracellular glucose levels were measured using a fluorescence-based glucose assay kit (BioVision, Milpitas, California, USA) according to the manufacturer's instructions. Lactate levels were measured using a lactate oxidase-based colorimetric assay read at 540 nm according to the manufacturer's instructions (Beyotime, Wuxi, China) and normalized to cell number. Pyruvate kinase activity was measured using the Pyruvate Kinase Activity Assay Kit (Jiancheng, China).

RNA pull down assay

Biotinylated linc-UROD and its antisense RNA probe were synthesized and incubated with cytoplasmic protein extract to form RNA-protein complexes. The complexes were separated from other components in the incubated solution through binding to streptavidin-labeled magnetic beads. After the complexes were eluted, proteins (IGF2BP1, IGF2BP2, IGF2BP3, PKM, ENO1) that bound to linc-UROD was detected by western blot. The assay was independently carried out in triplicate.

RNA immunoprecipitation (RIP) assay

RIP assay was conducted by using Z-Magna RIP™ RNA-binding Protein Immunoprecipitation kit (Millipore Corporation, USA). Cells lysates were obtained by treated cells with lysis buffer, Next, cell lysates were immunoprecipitated with anti-m6A (1mg/ml; Abcam, CA, USA), anti-METTL3 (1mg/ml; Abcam), anti-ENO1 (850μg/ml; Abcam), anti-PKM (550μg/ml; Abcam), anti-IGF2BP3 and negative control anti-IgG (1mg/ml; Abcam). Finally, the RNA complexes were extracted for RT-qPCR. The experiment was performed in triplicate.

Ubiquitination assay

HEK-293T cells were treated with 2 mM MG132 (proteasome inhibitor) for 16 h. Cells extracts were obtained using RIPA buffer and the cell extracts were incubated with 100 μl protein beads and 5 μg HA antibody overnight. After washing the beads and boiling the protein complex, ubiquitination of PKM or ENO1 was detected by western blot.

Western blot

Total protein was extracted from in cells with RIPA lysis buffer (Thermo Fisher, USA). The concentration of protein was measured using

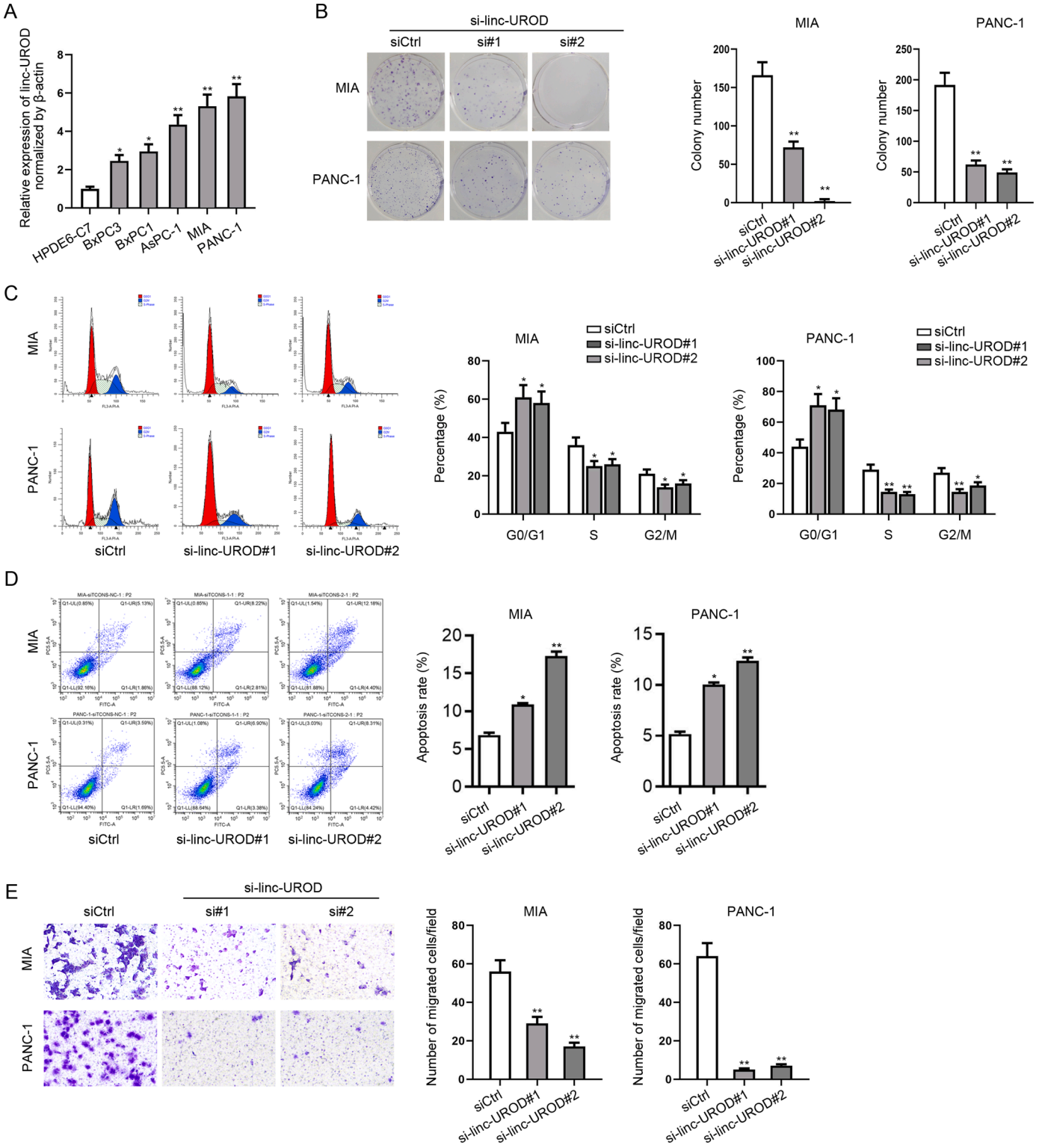

Fig. 1. Linc-UROD silencing suppressed PC cell growth and migration. (A) RT-qPCR tested linc-UROD expression in PC cell lines and normal cell line. (B) Cell proliferation was observed by colony formation assay. (C and D) Cell cycle and apoptosis were evaluated by flow cytometry analysis. (E) The migratory capacity of PC cells was measured by transwell assays. * $P < 0.05$, ** $P < 0.01$.

a BCA protein assay reagent (Beyotime, Shanghai, China). Protein was separated by 10% SDS-PAGE and transferred onto PVDF membranes (Millipore, USA). The membranes were blocked with 5% defatted milk in TBST and incubated with primary antibody, including Anti-PKM (1/10000), Anti-ENO1 (1/1000) and Anti-GAPDH (1/500-1/10000) at 4°C overnight. Next, the membranes were washed and then incubated with horseradish peroxidase-labeled secondary antibody (1/10000) at room temperature for 2 h. Finally, the blot was visualized by using the ECL western blotting substrate (Invitrogen). The experiment was independently conducted in triplicate. The original images were provided as Supplementary file.

Statistical analysis

All the experiments were performed in triplicate. Experimental data was analyzed by the statistical software SPSS 22.0 (International Business Machines Corporation, Armonk, NY, USA). All quantitative data were plotted by using Graphpad Prism 8 (San Diego, CA, USA) and exhibited as mean ± standard deviation (SD). Two groups comparison was conducted with student's t-test, while multiple groups comparison was carried out with one-way ANOVA. $P < 0.05$ indicated difference was statistical significant.

Results

Linc-UROD silencing suppresses PC cell growth and migration

First of all, RT-qPCR results unveiled that the expression of linc-UROD was much higher in PC cell lines (BxPC3, BxPC1, AsPC-1, MIA, and PANC-1) than in normal human pancreatic duct epithelial cell line HPDE6-C7 (Fig. 1A). To detect the functional role of linc-UROD in PC, loss-of-function assays were carried out in MIA and PANC-1 cells which exhibited the relative highest level of linc-UROD expression. Before that, linc-UROD was knocked down in two cells through transfecting with si-linc-UROD#1/2/3 (**Supplementary Fig. 1A**). Results of functions assays showed that knockdown of linc-UROD notably impeded the proliferation of PC cells (Fig. 1B), induced cell cycle arrested at G0/G1 phase (Fig. 1C) and accelerated cell apoptosis (Fig. 1D). In addition, we discovered that depletion of linc-UROD weakened the migratory capacities of PC cells (Fig. 1E). To summarize, linc-UROD contributes to the malignant processes of PC cells.

The up-regulation of linc-UROD is mediated by m6A methylation

m6A methylation plays a critical role in RNA metabolism [22]. Here, we wondered whether linc-UROD was influenced by such modification in PC cells. Interestingly, RIP assay results suggested that linc-UROD was abundant in the precipitate of anti-m6A was more evident in MIA and PANC-1 cells than that in HPDE6-C7 cells (Fig. 2A). Since m6A

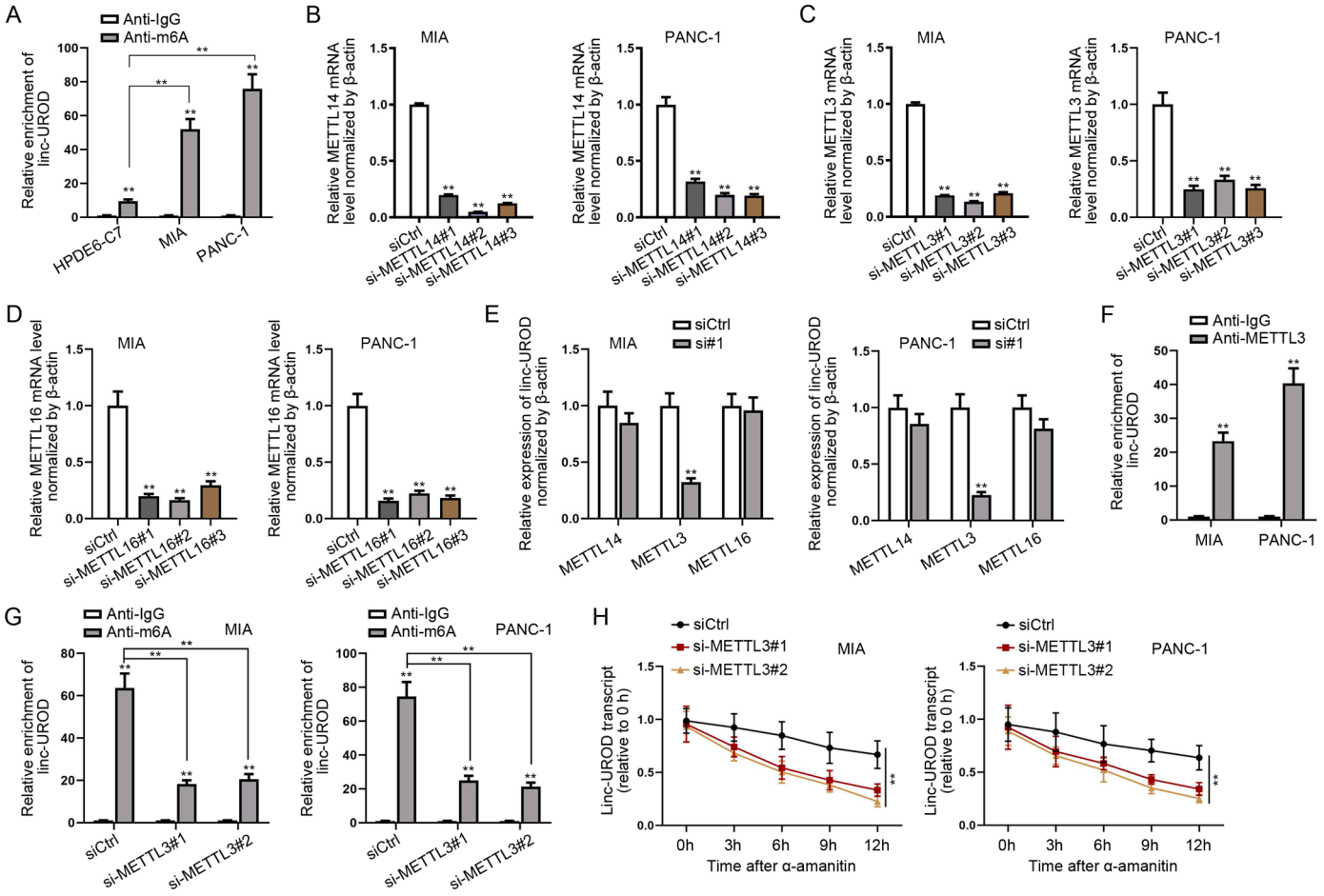

Fig. 2. The up-regulation of linc-UROD is mediated by m6A methylation. (A) The enrichment of linc-UROD in m6A antibody was assessed by m6A-RIP assays in PC cells. (B–D) RT-qPCR assay detected METTL14, METTL3 or METTL16 expression in MIA and PANC-1 cells upon METTL14/METTL3/METTL16 knockdown, respectively. (E) Linc-UROD expression was detected by RT-qPCR when METTL14, METTL3 or METTL16 was silenced. (F) The interaction between METTL3 and linc-UROD was verified by RIP assay. (G) The enrichment of linc-UROD in Anti-m6A group was examined by RIP assay in PC cells when METTL3 was inhibited. (H) The stability of linc-UROD was examined after METTL3 was depleted with treatment of α-amanitin. ** $P < 0.01$.

modification is mainly mediated by RNA methyltransferase such as METTL14, METTL3 or METTL16 [23], we respectively silenced these three genes in MIA and PANC-1 cells to identify the one affecting linc-UROD (Fig. 2B–D). The data revealed that the level of linc-UROD was only decreased after METTL3 interference (Fig. 2E). RT-qPCR verified that METTL3 expression in PC cell lines was aberrantly high (**Supplementary Fig. 1B**). In addition, the results of RIP assay confirmed the strong binding affinity between linc-UROD and METTL3 (Fig. 2F). Based on the subsequent m6A-RIP assay, we observed notably reduced m6A modification level in linc-UROD upon METTL3 silence (Fig. 2G). Given that m6A modification regulates RNA stability [24], we then tested the influence of METTL3 on linc-UROD degradation. The results demonstrated knockdown of METTL3 facilitated the degradation of linc-UROD in PC cells with α-amanitin treatment (Fig. 2H). Then, we validated the influence of METTL3/IGF2BP2/linc-UROD axis. The overexpression efficacy of pcDNA3.1/linc-UROD was proven (**Supplementary Fig. 1C**). From **Supplementary Fig. 1D–G**, we could conclude that IGF2BP2 knockdown could reverse the exacerbated PC cell malignant behaviors caused by METTL3 augment, while linc-UROD overexpression recovered the PC cell malignant behaviors. Overall, linc-UROD is stabilized by METTL3-induced m6A methylation in PC cells.

IGF2BP3 is responsible for the stabilizing effect of METTL3 on linc-UROD

Published reports have suggested that the stability of m6A-modified RNA is modulated by IGF2BPs [25]. Hence, we conducted RNA pull down assay to test the binding affinity of IGF2BPs and linc-UROD in PC cells. The results indicated that only IGF2BP3 could be substantially pulled down by Bio-linc-UROD (Fig. 3A and **Supplementary Fig. 3A**). Meanwhile, RIP assay data proved the high enrichment of linc-UROD in anti-IGF2BP3-precipitated complexes in PC cells (Fig. 3B). Importantly, we discovered that the level of linc-UROD was diminished in PC cells after interfering IGF2BP3 (Fig. 3C and D). Further, we noticed the degradation of linc-UROD was accelerated in IGF2BP3-inhibited PC cells (Fig. 3E). Next, we aimed to attest whether the impact of IGF2BP3 on linc-UROD relied on METTL3-mediated m6A modification. It was showed that the binding of IGF2BP3 to linc-UROD was inhibited due to METTL3 depletion (Fig. 3F). Then, we overexpressed METTL3 in MIA and PANC-1 cells (Fig. 3G). Interestingly, we found that METTL3 upregulation restrained linc-UROD degradation, while its effect was offset by IGF2BP3 knockdown (Fig. 3H). In short, IGF2BP3 recognizes

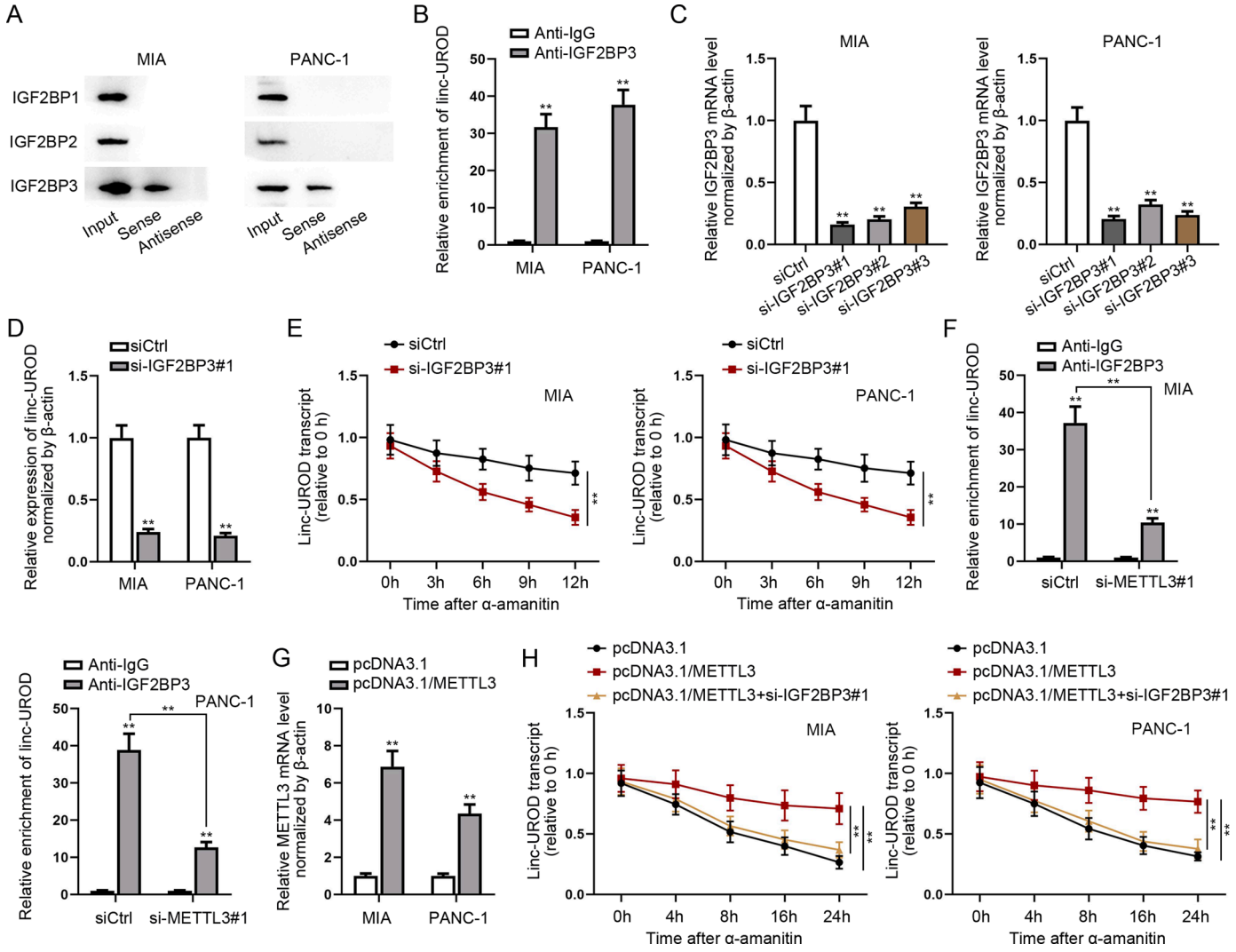

Fig. 3. IGF2BP3 stabilizes linc-UROD via METTL3-induced m6A modification. (A) RNA pull down assay detected the interaction of linc-UROD with IGF2BPs in PC cells. (B) RIP evaluated the binding of IGF2BP3 to linc-UROD in two PC cells. (C) RT-qPCR tested knockdown efficiency of IGF2BP3 in MIA and PANC-1 cells. (D) RT-qPCR measured the impact of IGF2BP3 on linc-UROD in PC cells. (E) RT-qPCR analyzed linc-UROD level in PC cells under α-amanitin treatment at different time points. (F) RIP tested the influence of METTL3 deficiency on IGF2BP3-linc-UROD interaction. (G) RT-qPCR verified the overexpression efficiency of METTL3 in PC cells. (H) RT-qPCR analyzed linc-UROD level in PC cells treated with α-amanitin with the indicated transfections. ** $P < 0.01$.

METTL3-mediated m6A modification on linc-UROD to stabilize linc-UROD.

Linc-UROD interacts with ENO1 and PKM protein to enhance their levels

After investigating the upstream mechanism of linc-UROD, we next explored its downstream mechanism in PC cells. Considering the functions of lncRNAs are often linked to their cellular distribution, we first detected the distribution of linc-UROD in PC cells. Results showed that most linc-UROD was present in the cytoplasm of PC cells (Fig. 4A). As known, cytoplasmic lncRNAs can interact with proteins to affect the expression or function of them, thereby modulating cancer cell processes [26,27]. Through RNA pull down analysis, we discovered that linc-UROD could interact with PKM and ENO1 (Fig. 4B and **Supplementary Fig. 3B**). Moreover, the two proteins were highly expressed in PC tissues and closely associated with overall survival of PC patients according to GEPIA data (**Supplementary Fig. 2A–D**). In addition, RIP assay also confirmed the interaction between linc-UROD and ENO1/PKM (Fig. 4C). On this basis, we speculated that ENO1 and PKM may be downstream effectors of linc-UROD in PC cells. Subsequently, data collected from RIP assay suggested that binding of linc-UROD to PKM or ENO1 was inhibited in linc-UROD-silenced MIA and PANC-1 cells (Fig. 4D and E). Next, western blot was conducted and we found PKM and ENO1 protein levels were decreased by linc-UROD knockdown (Fig. 4F and **Supplementary Fig. 3C**). In sum, linc-UROD interacts with PKM/ENO1 proteins to elevate their levels.

Linc-UROD inhibits ubiquitination and proteasome-mediated degradation of ENO1 and PKM proteins

To confirm the in-depth regulation of linc-UROD on PKM/ENO1 proteins, we treated PC cells with cycloheximide (CHX) to inhibit protein synthesis. As displayed in Fig. 5A and B and **Supplementary Fig. 3D and E**, the levels of both PKM and ENO1 proteins degraded much faster after linc-UROD silencing. The results indicated that the absence of linc-UROD expedited the degradation of both PKM and ENO1 proteins. Notably, it was found that linc-UROD depletion reduced PKM and ENO1 protein levels in PC cells, while having little impact on their protein levels in PC cells treated with MG132 (a protease inhibitor) (Fig. 5C and **Supplementary Fig. 3F**), implying that linc-UROD affected

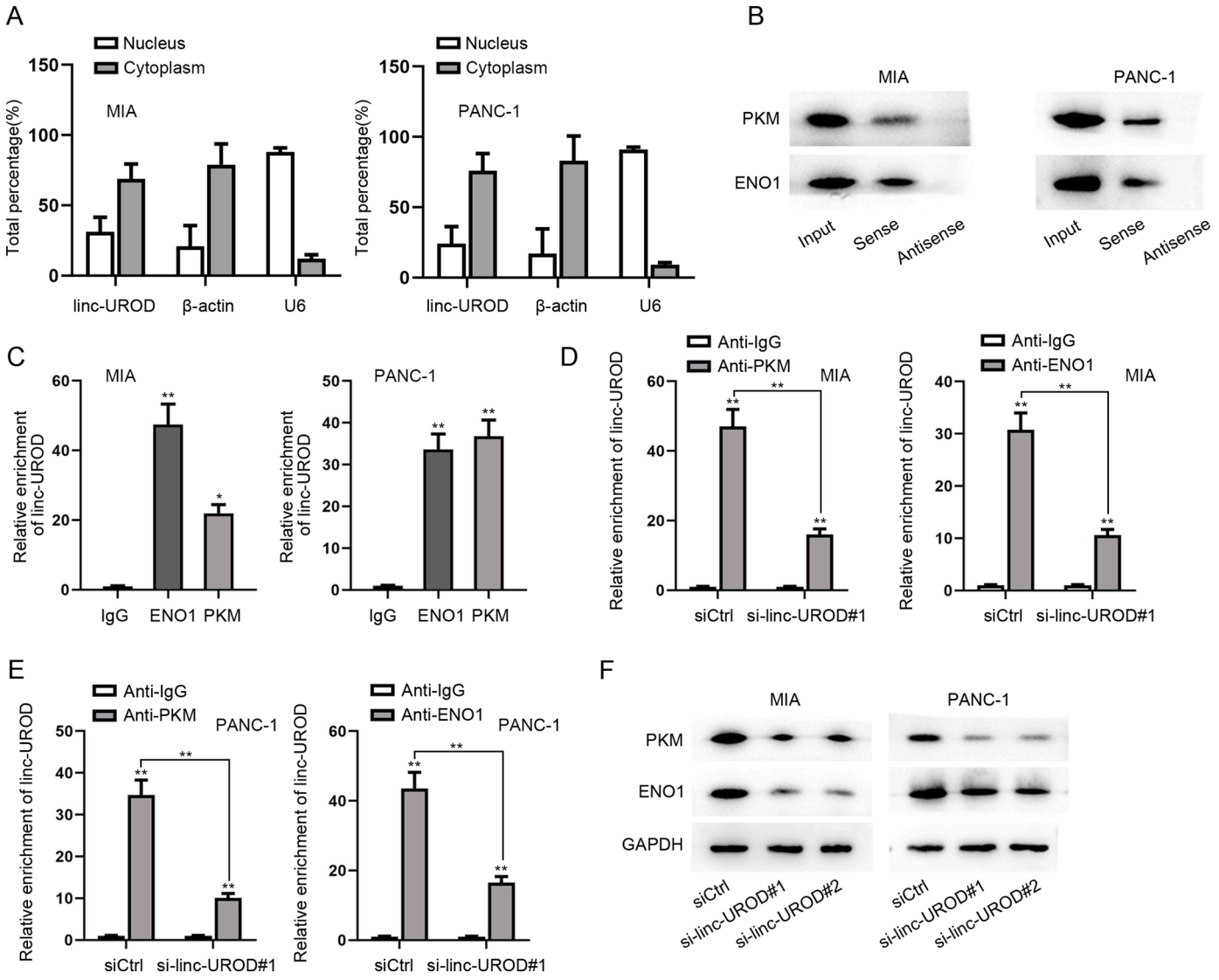

Fig. 4. Linc-UROD interacts with ENO1 and PKM to enhance their levels in PC cells. (A) Subcellular fractionation experiment analyzed the distribution of linc-UROD in MIA and PANC-1 cells. (B and C) RNA pull down and RIP assays exhibited the binding affinity of linc-UROD with ENO1 and PKM. (D and E) RIP examined the effect of linc-UROD interference on its binding to ENO1 and PKM. (F) Western blot analyzed the protein levels of ENO1 and PKM when linc-UROD was down-regulated. * $P < 0.05$, ** $P < 0.01$.

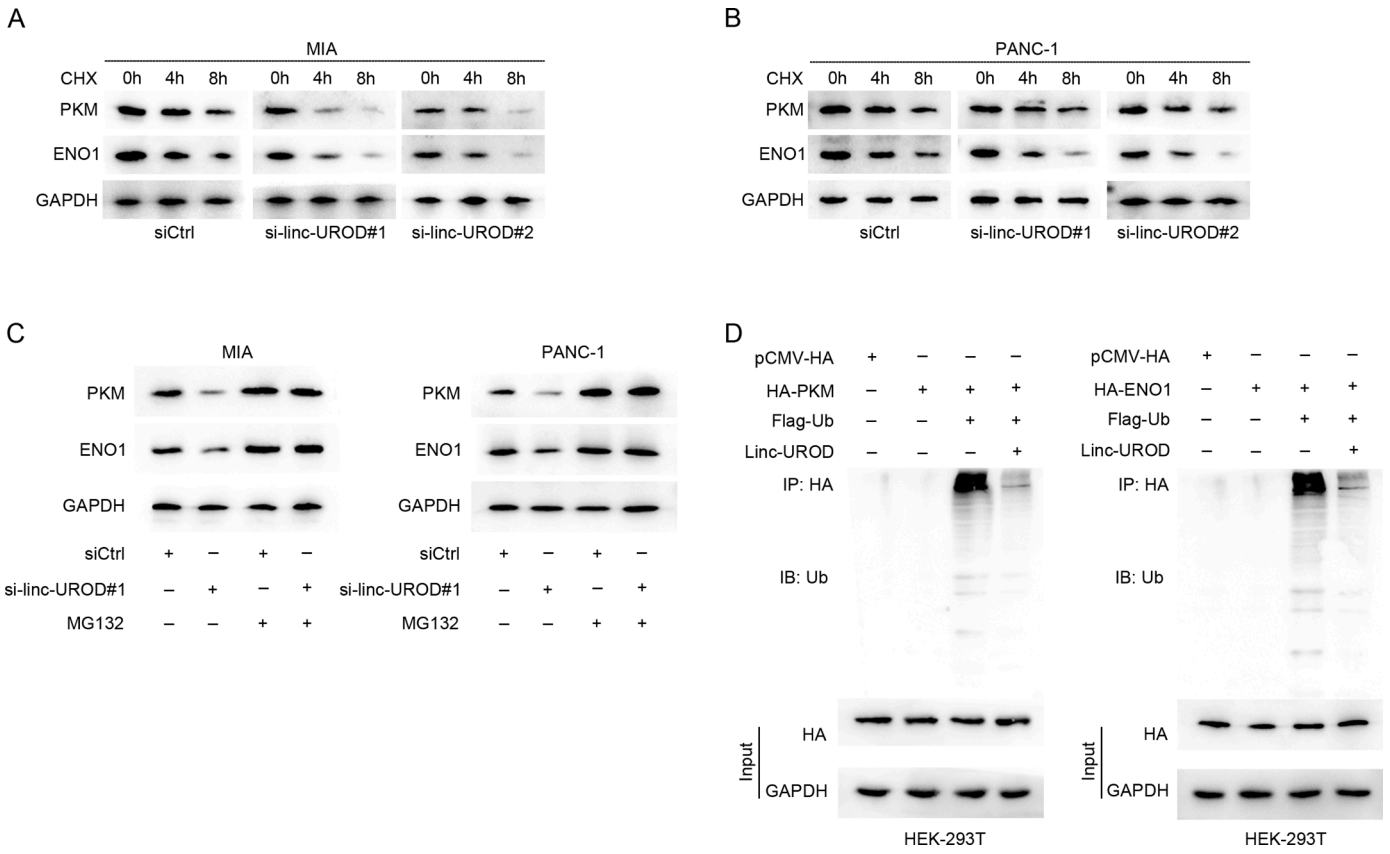

Fig. 5. Linc-UROD stabilizes ENO1 and PKM proteins by restraining their ubiquitination and degradation. (A and B) Western blot assayed the degradation of PKM and ENO1 in PC cells after CHX treatment. (C) The protein levels of ENO1 and PKM in different groups were analyzed via western blot. (D) Ubiquitination assay detected the ubiquitination of PKM and ENO1 proteins in PC cells with or without the presence of linc-UROD.

proteasome-mediated degradation of ENO1 and PKM proteins. The following IP-WB revealed that the existence of linc-UROD diminished the ubiquitination levels of both PKM and ENO1 proteins (Fig. 5D), evidencing that linc-UROD hindered ubiquitination of both proteins. Taken together, linc-UROD stabilizes ENO1 and PKM proteins through repressing their ubiquitination levels.

Linc-UROD depends on PKM and ENO1 to promote glycolysis in PC cells

Since both PKM and ENO1 proteins are glycolytic enzymes, we deduced that linc-UROD might affect glycolysis of PC cells. Experimental results unveiled that inhibition of linc-UROD lessened overall glycolytic flux in both PC cells, with the glycolytic capacity and reserve dramatically reduced (Fig. 6A). In addition, it turned out that loss of linc-UROD hindered glucose consumption (Fig. 6B) and pyruvate kinase activity (Fig. 6C). Meanwhile, linc-UROD deficiency led to decrease in lactate production (Fig. 6D). All these data revealed that linc-UROD worked as a contributor to PC cell glycolysis. After pcDNA3.1/ENO1 and pcDNA3.1/PKM plasmids were respectively transfected into PC cells to elevate ENO1 and PKM expression (**Supplementary Fig. 2E and F**), rescue assays were done. It was shown that ENO1 or PKM overexpression partly counteracted the influence of linc-UROD interference on ECAR rate, glucose consumption, pyruvate kinase activity, and lactate production (Fig. 6E–H). In a word, linc-UROD facilitates PC cell glycolysis via regulating PKM and ENO1.

Linc-UROD aggravates PC cell malignant behaviors through regulating PKM and ENO1

As glycolysis is related to PC progression [28], we speculated that linc-UROD regulated ENO1 and PKM to promote glycolysis, consequently facilitating PC cell malignant behaviors. To verify the speculation, rescue assays were conducted. Colony formation assays indicated that linc-UROD knockdown-induced suppression on PC cell proliferation was partly offset by overexpression of PKM or ENO1 (Fig. 7A). Moreover, G0/G1 arrest and the cell apoptosis induced by linc-UROD depletion were partially recovered after PKM or ENO1 upregulation (Fig. 7B and C). Furthermore, PKM or ENO1 overexpression led to partial recovery of the PC cell migration restrained by linc-UROD depletion (Fig. 7D). To conclude, linc-UROD exacerbates PC cell malignant behaviors via regulating PKM and ENO1.

Discussion

Molecular targets have attracted our attention for their critical roles in the development of various diseases. For instance, malignant downstream signaling pathways of mutated granulocyte-colony stimulating factor receptor (G-CSFR) have been reported to engage in controlling myeloid progenitor proliferation and differentiation to neutrophils, which also associated with a unique spectrum of myeloid disorders and related malignancies [29,30]. Recent studies have also pointed out that lncRNAs are important participators in biological processes of PC [18, 31]. A large number of lncRNAs have been determined as potential biomarkers for PC, including HOATIR, PVT1, and CCDC26 [32,33]. Presently, we probed into the function of linc-UROD in PC cells and confirmed that linc-UROD expression was high in PC cells. Moreover, we proved that linc-UROD knockdown hindered PC cell proliferation and migration while stimulating cell cycle G0/G1 arrest and apoptosis of PC cells. The above findings suggested linc-UROD might be used as a potential biomarker for PC diagnosis.

RNA m6A methylation is an important modification affecting RNA processing and RNA translation [34]. Methyltransferase including

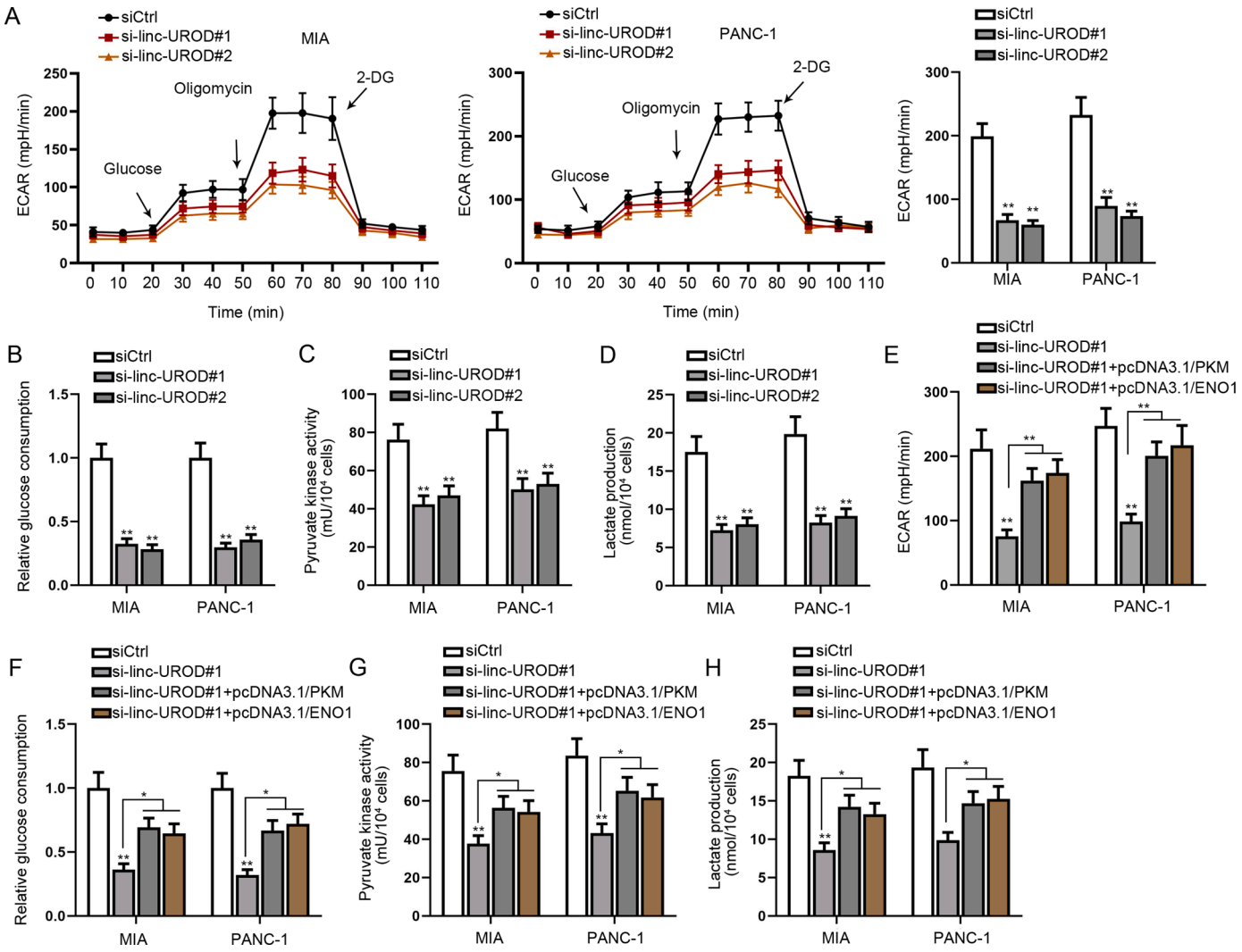

Fig. 6. Depletion of linc-UROD influences the glycolysis of PC cells via ENO1 and PKM. (A) ECAR assay analyzed the glycolytic flux of MIA and PANC-1 cells after linc-UROD was silenced. (B) The glucose consumption was detected when linc-UROD was down-regulated. (C and D) Pyruvate kinase activity and lactate production were analyzed when linc-UROD was down-regulated. (E–H) The changes in glycolysis under different conditions were measured via detecting ECAR rate, glucose consumption, pyruvate kinase activity and lactate production. * $P < 0.05$, ** $P < 0.01$.

METTL14, METTL3 and METTL16 have been identified to induce m6A modification of target RNAs [35]. Herein, we firstly confirmed that up-regulation of linc-UROD in PC cells was related to its high m6A modification mediated by METTL3. A previous report has uncovered that IGF2BPs can recognize RNA m6A modification to enhance the stability and translation of target RNAs [36]. In this work, we further validated that m6A-modified linc-UROD was stabilized by IGF2BP3. It has been reported that METTL3 promotes m6A modification on ABHD11-AS1 to enhance its stability [36], and m6A-modified KCNMB2-AS1 could be stabilized by IGF2BP3 [37]. Consistent with the former literature, our study uncovered that IGF2BP3 recognized METTL3-mediated m6A modification on linc-UROD to stabilize linc-UROD in PC cells.

ENO1 and PKM are both glycolytic enzymes that affect glycometabolism and therefore play a vital part in human cancer. As reported, ENO1 has been regarded as a potential marker in cancer immunotherapy [38]. Additionally, ENO1 has been mentioned to be involved in the adhesion, invasion and metastasis of PC cells [39]. In the meantime, PKM has been validated to accelerate tumorigenesis [40], including the progression of PC [41]. Consistent with these findings, we observed that ENO1 and PKM both exhibited high expression in PC tissues and were positively linked to prognosis of PC patients. We also found that linc-UROD interacted with ENO1 and PKM proteins in the cytoplasm of PC cells. As reported, the interactions between lncRNAs and proteins may affect the expression or function of the proteins. For instance, lncRNA CF129 interacts with p53 to facilitate its ubiquitination and degradation in PC cells [42]. Linc01232 binds to HNRNPA2B1 to inhibit its ubiquitination and increase its level in PC cells [43]. In this study, we unveiled that the binding of linc-UROD to ENO1 and PKM proteins reduced their ubiquitination level and enhanced their stability.

A large number of studies have implied that glycolysis occupies an important position in the progression and therapy of cancers [11]. Dai et al. have clarified that glycolysis facilitates PC development and influences drug resistance of PC cells [44]. Herein, we validated that linc-UROD strengthened glycolysis via elevating PKM and ENO1 expression, therefore accelerating the malignant phenotypes of PC cells.

Conclusion

This work demonstrates that linc-UROD interacts with ENO1 and PKM protein to hinder their ubiquitination and stabilize them, consequently promoting glycolysis and malignant processes of PC cells. Our study first unveils the importance of linc-UROD in PC and offers evidences for linc-UROD to be a potential biomarker for PC diagnosis.

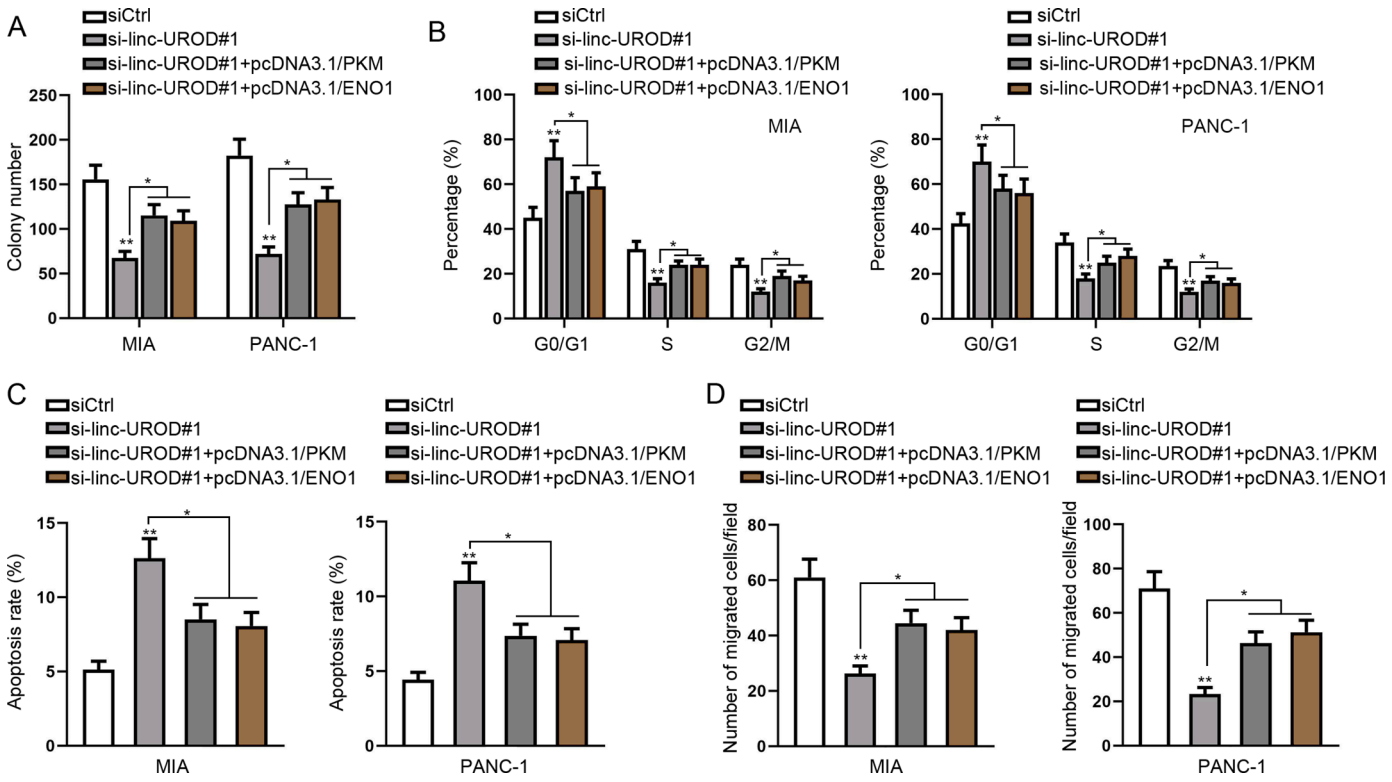

Fig. 7. Linc-UROD depends on ENO1 and PKM to facilitate PC cell progression. (A) Colony formation assay tested the proliferation capacity of PC cells under indicated conditions. (B and C) Changes in cell cycle and apoptosis upon different conditions were analyzed through flow cytometry. (D) Transwell assay examined PC cell migration under indicated conditions. * $P < 0.05$, ** $P < 0.01$.

However, there are also some limitations in this study. For instance, *in vivo* assays and clinical data were not involved. In addition, whether there are other possible regulatory mechanisms behind linc-UROD in PC remains to be explored.

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CRediT authorship contribution statement

Yuan He: Investigation. **Yaxing Liu:** Investigation. **Dongkai Wu:** Methodology. **Luyao Chen:** Investigation. **Zhonglin Luo:** Methodology. **Xingsong Shi:** Methodology. **Keyan Li:** Writing – review & editing. **Hao Hu:** Writing – review & editing. **Gexi Qu:** Writing – review & editing. **Qiang Zhao:** Writing – original draft. **Changhong Lian:** Writing – original draft.

Declaration of Competing Interest

No conflicts of interest exist.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2022.101583.

References

[1] V. Goral, Pancreatic cancer: pathogenesis and diagnosis, Asian Pac. J. Cancer Prev. 16 (14) (2015) 5619–5624.
[2] A. Gharibi, Y. Adamian, J.A. Kelber, Cellular and molecular aspects of pancreatic cancer, Acta Histochem. 118 (3) (2016) 305–316.
[3] M. Ilic, I. Ilic, Epidemiology of pancreatic cancer, World J. Gastroenterol. 22 (44) (2016) 9694–9705.
[4] P. Maisonneuve, A.B. Lowenfels, Epidemiology of pancreatic cancer: an update, Dig. Dis. 28 (4-5) (2010) 645–656.
[5] A.B. Lowenfels, P. Maisonneuve, Risk factors for pancreatic cancer, J. Cell. Biochem. 95 (4) (2005) 649–656.
[6] M.C. Safioleas, K.G. Moulakakis, Pancreatic cancer today, Hepatogastroenterology 51 (57) (2004) 862–868.
[7] D. Ansari, et al., Pancreatic cancer: yesterday, today and tomorrow, Futur. Oncol. 12 (16) (2016) 1929–1946.
[8] R. Gupta, I. Amanam, V. Chung, Current and future therapies for advanced pancreatic cancer, J. Surg. Oncol. 116 (1) (2017) 25–34.
[9] G.H. Sakorafas, V. Smyrniotis, Molecular biology of pancreatic cancer: how useful is it in clinical practice? JOP 13 (4) (2012) 332–337.
[10] M. Verma, Pancreatic cancer epidemiology, Technol. Cancer Res. Treat. 4 (3) (2005) 295–301.
[11] S. Ganapathy-Kanniappan, J.F. Geschwind, Tumor glycolysis as a target for cancer therapy: progress and prospects, Mol. Cancer 12 (2013) 152.
[12] L. Yan, et al., Glucose metabolism in pancreatic cancer, Cancers 11 (10) (2019) 1460 (Basel).
[13] Y. Fang, M.J. Fullwood, Roles, functions, and mechanisms of long non-coding RNAs in cancer, Genom. Proteom. Bioinform. 14 (1) (2016) 42–54.
[14] A. Shang, et al., Long non-coding RNA CCAT1 promotes colorectal cancer progression by regulating miR-181a-5p expression, Aging 12 (9) (2020) 8301–8320 (Albany NY).
[15] L. Chen, et al., LncRNA SNHG16 drives proliferation, migration, and invasion of lung cancer cell through modulation of miR-520/VEGF axis, Eur. Rev. Med. Pharmacol. Sci. 24 (18) (2020) 9522–9531.
[16] X. Rui, et al., lncRNA DLG1-AS1 promotes cell proliferation by competitively binding with miR-107 and Up-regulating ZHX1 expression in cervical cancer, Cell. Physiol. Biochem. 49 (5) (2018) 1792–1803.

[17] X. Peng, et al., Long non-coding RNA TNRC6C-AS1 promotes methylation of STK4 to inhibit thyroid carcinoma cell apoptosis and autophagy via Hippo signalling pathway, J. Cell. Mol. Med. 24 (1) (2020) 304–316.
[18] X. Huang, et al., LncRNAs in pancreatic cancer, Oncotarget 7 (35) (2016) 57379–57390.
[19] H. Gao, et al., LncRNA ZEB2-AS1 promotes pancreatic cancer cell growth and invasion through regulating the miR-204/HMGB1 axis, Int. J. Biol. Macromol. 116 (2018) 545–551.
[20] P.C. Deng, et al., LncRNA SNHG14 potentiates pancreatic cancer progression via modulation of annexin A2 expression by acting as a competing endogenous RNA for miR-613, J. Cell. Mol. Med. 23 (11) (2019) 7222–7232.
[21] G. Wang, et al., Long non-coding RNA CRNDE sponges miR-384 to promote proliferation and metastasis of pancreatic cancer cells through upregulating IRS1, Cell Prolif. 50 (6) (2017) e12389.
[22] X. Zhou, et al., The RNA-binding protein SRSF1 is a key cell cycle regulator via stabilizing NEAT1 in glioma, Int. J. Biochem. Cell Biol. 113 (2019) 75–86.
[23] Q. Lan, et al., The critical role of RNA m(6)A methylation in cancer, Cancer Res. 79 (7) (2019) 1285–1292.
[24] X. Wang, et al., N6-methyladenosine-dependent regulation of messenger RNA stability, Nature 505 (7481) (2014) 117–120.
[25] H. Huang, et al., Recognition of RNA N(6)-methyladenosine by IGF2BP proteins enhances mRNA stability and translation, Nat. Cell Biol. 20 (3) (2018) 285–295.
[26] W. Ni, et al., Long noncoding RNA GAS5 inhibits progression of colorectal cancer by interacting with and triggering YAP phosphorylation and degradation and is negatively regulated by the m(6)A reader YTHDF3, Mol. Cancer 18 (1) (2019) 143.
[27] N. Gu, et al., Silencing lncRNA FOXD2-AS1 inhibits proliferation, migration, invasion and drug resistance of drug-resistant glioma cells and promotes their apoptosis via microRNA-98-5p/CPEB4 axis, Aging 11 (22) (2019) 10266–10283 (Albany NY).
[28] F. Wang, et al., p38γ MAPK is essential for aerobic glycolysis and pancreatic tumorigenesis, Cancer Res. 80 (16) (2020) 3251–3264.
[29] P. Dwivedi, K.D. Greis, Granulocyte colony-stimulating factor receptor signaling in severe congenital neutropenia, chronic neutrophilic leukemia, and related malignancies, Exp. Hematol. 46 (2017) 9–20.
[30] P. Dwivedi, et al., Time resolved quantitative phospho-tyrosine analysis reveals Bruton's Tyrosine kinase mediated signaling downstream of the mutated granulocyte-colony stimulating factor receptors, Leukemia 33 (1) (2019) 75–87.
[31] P. McDonel, M. Guttman, Approaches for understanding the mechanisms of long noncoding RNA regulation of gene expression, Cold Spring Harb. Perspect. Biol. 11 (12) (2019).
[32] Z. Xie, et al., Salivary HOTAIR and PVT1 as novel biomarkers for early pancreatic cancer, Oncotarget 7 (18) (2016) 25408–25419.
[33] W. Peng, A. Jiang, Long noncoding RNA CCDC26 as a potential predictor biomarker contributes to tumorigenesis in pancreatic cancer, Biomed. Pharmacother. 83 (2016) 712–717.
[34] T. Sun, R. Wu, L. Ming, The role of m6A RNA methylation in cancer, Biomed. Pharmacother. 112 (2019), 108613.
[35] Y. Geng, et al., Identification of m6A-related genes and m6A RNA methylation regulators in pancreatic cancer and their association with survival, Ann. Transl. Med. 8 (6) (2020) 387.
[36] L. Xue, et al., m(6) A transferase METTL3-induced lncRNA ABHD11-AS1 promotes the Warburg effect of non-small-cell lung cancer, J. Cell. Physiol. 236 (4) (2021) 2649–2658.
[37] Y. Zhang, et al., Long noncoding RNA KCNMB2-AS1 stabilized by N(6)-methyladenosine modification promotes cervical cancer growth through acting as a competing endogenous RNA, Cell Transplant. 29 (2020), 963689720964382.
[38] P. Cappello, et al., Alpha-Enolase (ENO1), a potential target in novel immunotherapies, Front Biosci 22 (2017) 944–959 (Landmark Ed).
[39] M. Principe, et al., Alpha-enolase (ENO1) controls alpha v/beta 3 integrin expression and regulates pancreatic cancer adhesion, invasion, and metastasis, J. Hematol. Oncol. 10 (1) (2017) 16.
[40] K. Zahra, et al., Pyruvate kinase M2 and cancer: the role of PKM2 in promoting tumorigenesis, Front. Oncol. 10 (2020) 159.
[41] T.Y. Cheng, et al., Pyruvate kinase M2 promotes pancreatic ductal adenocarcinoma invasion and metastasis through phosphorylation and stabilization of PAK2 protein, Oncogene 37 (13) (2018) 1730–1742.
[42] M. Liu, et al., Hypoxia-induced feedback of HIF-1α and lncRNA-CF129 contributes to pancreatic cancer progression through stabilization of p53 protein, Theranostics 9 (16) (2019) 4795–4810.
[43] L.D. Meng, et al., Linc01232 promotes the metastasis of pancreatic cancer by suppressing the ubiquitin-mediated degradation of HNRNPA2B1 and activating the A-Raf-induced MAPK/ERK signaling pathway, Cancer Lett. 494 (2020) 107–120.
[44] S. Dai, et al., Glycolysis promotes the progression of pancreatic cancer and reduces cancer cell sensitivity to gemcitabine, Biomed. Pharmacother. 121 (2020), 109521.