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Original Articles

# UHRF1 promotes aerobic glycolysis and proliferation via suppression of SIRT4 in pancreatic cancer

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ABSTRACT

UHRF1 (ubiquitin like with plant homeodomain and ring finger domains 1) is an epigenetic modifier that is overexpressed in some cancers, including pancreatic cancer, and mediates silencing of tumor suppressor genes. However, the role of UHRF1 in regulating pancreatic cancer metabolism and metastasis is not clear. In the present study, we demonstrated that silencing UHRF1 significantly inhibited aerobic glycolysis in pancreatic cancer cells. Furthermore, we demonstrated that UHRF1 knockdown decreased hypoxia inducible factor (HIF)1α levels and HIF1α targeted glycolytic genes. The Cancer Genome Atlas dataset analysis supported this observation. The Sirtuin (SIRT) family members regulate aerobic glycolysis in many cancers. We analyzed the correlation between UHRF1 and SIRT3–5 expression and found a significant negative correlation between UHRF1 and SIRT4. Further transcriptional and functional analysis demonstrates that SIRT4 is a downstream target of UHRF1 and negatively regulated aerobic glycolysis, cell proliferation and tumor growth. Our study identified a novel UHRF1/SIRT4 axis in regulation of pancreatic cancer cell proliferation, metabolism, and metastasis.

## 1. Introduction

Pancreatic cancer is an aggressive malignant disease with poor prognosis, and its death rate is almost equal to its incidence. Despite significant progress, the 5-year overall survival of the disease remains at about 6% [1,2]. The poor prognosis of pancreatic cancer is due to its early metastasis, with aggressive invasion to nearby and distant organs. Moreover, resistance to traditional radiotherapy and chemotherapy also accounts for the unsatisfactory prognosis [3,4]. Therefore, uncovering the molecular mechanism of oncogenesis, proliferation and metastasis will provide novel insights into pancreatic cancer development and improve overall survival.

The oncogenesis, development and metastasis of pancreatic cancer is a multistep process that is associated with accumulation of somatic mutations, including *Kras*, *p53*, *SMAD4* and *CDKN2A* [5]. The application of these genetic aberrations into the development of engineered genetic mouse models and association with the establishment and progression of pancreatic cancer has revolutionized our understanding of the disease [6,7]. Furthermore, epigenetics research has provided a more comprehensive framework for the understanding of the pathological and biological mechanisms underlying pancreatic cancer [8]. The term epigenetics refers to the inheritance of genetic information that is not based on DNA sequence but can pass to the next generation via DNA and histone modifications as well as miRNA-dependent mechanisms [9]. Some well-established tumour suppressors in pancreatic cancer may be epigenetically altered through DNA methylation and chromatin modifications [10]. For example, besides mutations in CDKN2A, this tumour suppressor can also be silenced via DNA methylation and chromatin modifications in the promoter region [11]. Given the vital role of epigenetic modification in cancer development and progression, it becomes imperative for us to understand the epigenetic-based events that promote and maintain malignancy of pancreatic cancer [12].

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UHRF1 [ubiquitin like with plant homeodomain (PHD) and ring finger domains 1] is a chromatin modifier with multistructural domains and functions and it is overexpressed in many cancers [13]. UHRF1 contains a SET and RING-associated (SRA) domain that specifically recognizes hemimethylated CpG and a tandem tudor domain (TTD) and a PHD that bind cooperatively the H3 tails with H3K9me2/3. Based on the unique structures of UHRF1, this chromatin modifier is reported to participate in DNA methylation and heterochromatin formation that lead to gene silencing [14,15]. UHRF1 plays important roles in the oncogenesis and progression of cancer. For example, UHRF1 mediates silencing of tumour suppressors like genes like *CDKN2A* and Kelch like ECH associated protein (*KEAP*)1 and it is believed to play important roles in oncogenesis of pancreatic cancer [16]. However, the role of UHRF1 in cancer cell metabolism reprogramming, which is one of the new hallmarks of cancer described by Robert A. Weinberg, has seldom been discussed [17].

Over the first half of the 20th century, Otto Warburg published a body of work demonstrating that cancer cells exhibit atypical metabolism characteristics that distinguish them from normal tissues, also known as the Warburg effect or aerobic glycolysis [18]. Aerobic glycolysis links the high rate of glucose fermentation to uncontrolled proliferation and progression of cancer cells. Together with glutamine metabolism, glycolysis provides the carbon skeletons, NADPH and ATP as building blocks for macromolecule synthesis of proliferated cancer cells, which persist in hypoxia, which in turn rewires the metabolic pathway for cell growth and survival [19]. Central to the aerobic glycolysis is the hypoxia inducible factor (HIF)1α-mediated signalling pathway, which enables cancer cells to survive under hypoxic stress conditions by changing glucose metabolism toward a glycolytic phenotype. Moreover, HIF1α also induces angiogenesis and regulates the pH balance with proliferation rate [20]. Mounting evidence has demonstrated that oncogenic mutations in pancreatic cancer reprogram cancer cell metabolism. For example, the oncogenic Kras mutation has been reported to reprogram glucose and glutamine metabolism in pancreatic cancer [21]. Another well-studied example of genetic aberration leading to aerobic glycolysis is the p53-mediated glucose metabolism transformation. The TP53-inducible glycolysis and apoptosis regulator (TIGAR), also known as fructose-2,6-bisphosphatase, is an enzyme encoded by the C12orf5 gene. TP53 regulates aerobic glycolysis by inducing TIGAR expression [22]. However, the role of epigenetic modifiers in pancreatic cancer aerobic glycolysis control has seldom been studied.

In the present study, we demonstrated that overexpression of UHRF1, a chromatin modifier, predicted worse prognosis of pancreatic cancer patients. UHRF1 contributed to pancreatic cancer cell proliferation via inducing aerobic glycolysis. UHRF1 caused aerobic glycolysis and suppressed HIF1α protein levels. UHRF1 silenced sirtuin (SIRT)4 expression, a mitochondrial negative regulator of aerobic glycolysis and tumor suppressor. Taken together, our present study uncovered a novel axis of UHRF1/SIRT4 in the regulation of proliferation and aerobic glycolysis of pancreatic cancer.

## 2. Materials and methods

### 2.1. The Cancer Genome Atlas (TCGA) dataset analysis

TCGA-PAAD(Pancreatic Adenocarcinoma) on RNA expression (Level 3) of pancreatic cancer patients in terms of RNA-seq by Expectation–Maximization was downloaded from the Cancer Genomics Brower of the University of California, Santa Cruz (https://genome-cancer.ucsc.edu/). In total, 178 primary pancreatic cancer samples from patients with detailed expression data were chosen from the updated TCGA database according to the parameters mentioned.

### 2.2. Cell culture

The human pancreatic cancer cell lines PANC-1 and MIA PaCa-2 were obtained from American Type Culture Collection and cultured according to the standard protocols. PANC-1 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), containing foetal bovine serum (FBS) at a final concentration of 10%. MIA PaCa-2 cells were cultured in DMEM, with 10% FBS and 2.5% horse serum.

### 2.3. RNA isolation and quantitative real-time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and PrimeScript RT reagent (TaKaRa, Dalian, China) was used for reverse transcription to obtain cDNA samples. The expression status of specific genes and β-actin were determined by quantitative real-time RT-PCR using an ABI 7900HT Real-Time PCR System (Applied Biosystems, Frederick, MD, USA). All reactions were run in triplicate. Primer sequences are listed in Supplementary Table 1.

### 2.4. Protein extraction and western blotting

Cells were harvested and lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 50 mM Tris/HCl, pH 8.0 and 10% glycerol) supplemented with protease and phosphatase inhibitors for 10 min. Cell debris was removed by centrifugation at 12 000 rpm for 20 min at 4 °C. Protein concentrations were determined by using Thermo Pierce BCA Protein Assay Kit(Rockford, IL, USA). Equal amounts of total protein lysates were subjected to denaturing 10% SDS-PAGE, and then transferred to a membrane for subsequent blotting with specific antibodies. Rabbit monoclonal antibody against UHRF1 was purchased from Abcam (Cambridge, MA, USA). Antibodies to β-actin, HIF1α, HK2(hexokinase 2), Glut1(glucose transporter 1), LDHA (lactate dehydrogenase A) were purchased from Proteintech(Rosemont, IL, USA).

### 2.5. Lentivirus production and stable cell line selection

To silence UHRF1 expression, we used lentivirus-mediated transfection. pLKO.1 TRC cloning vector (Addgene plasmid: 10 878, Watertown, MA, USA) was used to generate shRNA constructs against UHRF1. Targets (21 bp) against UHRF1 were 5′-ATGTGGGATGAGAC GGAATTG-3′ and 5′-GCCTTTGATTCGTTCCTTCTT-3’. To obtain SIRT4 overexpression constructs, pCDH-CMV-MCS-EF1-Puro plasmid (Systembio, SBI, Palo Alto, CA, USA) was used to express SIRT4. Targets against SIRT4 were 5′-CCAGCGGTACTGGGCGAGAAA-3’. Lentiviral particles were produced by cotransfection of pLKO.1-shUHRF1, pLKO.1-shSIRT4 or SIRT4-expressing constructs with psPAX2 and pMD2. G into HEK-293T cells in a ratio of 4: 3: 1. Cell lines were obtained by infection of PANC-1 and MIA PaCa-2 cells with lentiviral particles followed by puromycin selection.

### 2.6. CCK-8 proliferation assay

Cell proliferation was determined by CCK-8 assay using CCK-8 reagents (Dojindo, Kumamoto, Japan).

### 2.7. Colony-formation assay

PANC-1 and MIA PaCa-2 cells (n = 500) stably expressing shRNA against UHRF1 or SIRT4 and the relative control cells were seeded. After cultivating for 10 days, 4% paraformaldehyde was used to fix the cells followed by staining with 1% crystal violet. The colonies were counted subsequently.

### 2.8. Measurement of extracellular acidification rate (ECAR) and oxygen consumption rate (OCR)

Cellular mitochondrial function and glycolytic capacity were measured using the Seahorse Bioscience XF96 Extracellular Flux Analyzer, according to the manufacturer's instructions of Seahorse XF Cell Mito Stress Test Kit or Glycolysis Stress Test Kit (Seahorse Bioscience, Billerica, MA, USA). Cells were plated in XF96 Cell Culture Microplates (Seahorse Bioscience) at an initial cellular density of $4 \times 10^4$ cells/well the day before determination. Seahorse buffer consists of DMEM, phenol red, 25 mM glucose, 2 mM sodium pyruvate, and 2 mM glutamine. For ECAR measurement, 10 mM glucose, 1 μM oligomycin, and 100 mM 2-deoxy-glucose were automatically added to measure ECAR value. After monitoring baseline respiration, 1 μM oligomycin, 1 μM FCCP(Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone), and 1 μM rotenone were automatically injected into XF96 Cell Culture Microplates to measure OCR. ECAR and OCR values were calculated after normalization to cell number.

### 2.9. Cell apoptosis measurement

To detect apoptotic rate, cells were stained by using a FITC Annexin V Apoptosis Detection Kit (BD, La Jolla, CA, USA) complied with the manufacturer's instructions and counted using a FACSCalibur flow cytometer.

### 2.10. Mitochondrial membrane potential measurement

JC-1 probe was employed to measure mitochondrial depolarization in pancreatic cancer cells. Briefly, Cells cultured in six-well plates after indicated treatments were incubated with an equal volume of JC-1 staining solution (5 g/ml) at 37°Cfor 20 min and rinsed twice with PBS. Mitochondrial membrane potentials were monitored by determining the relative amounts of dual emissions from mitochondrial JC-1 monomers or aggregates using an Olympus fluorescent microscope under Argon-ion 488 nm laser excitation. Mitochondrial depolarization is indicated by an increase in the green/red fluorescence intensity ratio.

### 2.11. Promoter activity with dual luciferase assay

SIRT4 promoter region covering from −2500 to +200 was amplified and cloned into pGL3-Basic vector to generate the pGL3-SIRT4 construct. Hypoxia response element (HRE)-luciferase plasmid was obtained from Addgene (Addgene plasmid 26 731). Renilla luciferase expressing vector pRL-TK was purchased from Promega (Madison, WI, USA). To assess the impact of luciferase activity, Dual-Luciferase Reporter Assay System (Promega) was used.

### 2.12. Chromatin immunoprecipitation (ChIP) assay

ChIP assay to assess the binding status of UHRF1 on SIRT4 promoter was performed according to the standard manuals provided by Magna ChIP A/G Chromatin Immunoprecipitation Kit (Merck Millipore Corporation, Darmstadt, Germany). The nuclear DNA extracts were amplified using two pairs of primers that spanned the SIRT4 promoter region. The primer sequences are listed in Supplementary Table 1 [23].

### 2.13. Tissue specimens and immunohistochemical(IHC) staining

The clinical tissue samples used in this study were obtained from patients diagnosed with pancreatic cancer at Fudan University Shanghai Cancer Center. Prior patient consent and approval from the Institutional Research Ethics Committee were obtained. Immunohistochemical staining of paraffin-embedded tissues with antibodies against UHRF1 and SIRT4 were performed to detect their expression according to standard IHC procedures. Anti-UHRF1 antibody (ab 57 083; Abcam) was used in a dilution factor of 1: 100. SIRT4 antibody (21 440-1-AP; Proteintech) was used at a dilution factor of 1: 50. Three different fields under the microscope for each slide were randomly chosen for scoring. Positive proportion and intensity were semiquantitatively scored. Protein expression levels were calculated by multiplying the positivity (0, < 5% of the total cells; 1, 5–25%; 2, 25–50%; 3, 50–75; and 4, > 75%) and intensity scores (0, no coloration; 1, pale yellow; 2, clay bank; and 3, brown) and were classified as follows: negative (0); weakly positive (1–4); moderately positive (6–8); and strongly positive (8 − 12).

### 2.14. Statistical analysis

Statistical analyses were performed by SPSS version 17.0 (IBM Corp., Armonk, NY, USA) using independent t tests (for continuous variables) and Pearson's $\chi^2$ tests (for categorical variables). Logistic regression was used to determine the correlation between UHRF1, Glut1, HK2, LDHA, SIRT3, SIRT4 and SIRT5 expression level and clinicopathological characteristics in the TCGA cohorts. Statistical significance was based on two-sided p values of $< 0.05$.

## 3. Results

### 3.1. UHRF1 overexpression predicts worse prognosis and promotes proliferation of pancreatic cancer cells

Previous studies have demonstrated that UHRF1 is overexpressed in pancreatic cancer patients; however, its expression in the prognosis of pancreatic cancer has seldom been reported. Here, by using TCGA dataset analysis, we demonstrated that higher expression of UHRF1 predicted worse prognosis of pancreatic cancer (Fig. 1A). The Clinical information regarding the samples is presented in Supplementary Table 2. For the selection of pancreatic cancer cell lines for further investigations, we analyzed the expression status of UHRF1 in pancreatic cancer cell lines, and our results demonstrated that UHRF1 expression was higher in PANC-1 and MIA PaCa-2 cells (Fig. S1). Next, we generated stable shRNA expression in pancreatic cancer cell lines of PANC-1 and MIA PaCa-2. The efficacy of knockdown was validated by quantitative RT-PCR and western blotting with UHRF1 antibodies (Fig. 1B and C). To confirm the role of UHRF1 in proliferation, we performed a CCK-8 proliferation assay. Decreased UHRF1 expression inhibited viability of PANC-1 and MIA PaCa-2 cells (Fig. 1D). The clone formation capacity of UHRF1 was assessed, which indicated that UHRF1 silencing attenuated clone formation capacity of PANC-1 and MIA PaCa-2 cells (Fig. 1E and F). Silencing UHRF1 increased the apoptosis of PANC-1 and MIA PaCa-2 cells (Fig. 1G and H). Overall survival analysis and *in vitro* proliferation assays further validated the role of UHRF1 in prognosis prediction and proliferation of pancreatic cancer cells.

### 3.2. UHRF1 promoted aerobic glycolysis in pancreatic cancer cells

It is generally perceived that proliferated solid cancer cells shift their glucose metabolism pattern to hypoxic glycolysis. Due to the positive role of UHRF1 in prediction of prognosis and proliferation of cancer cells, we supposed that UHRF1 participated in regulation of aerobic glycolysis. By using Seahorse XF Extracellular Flux Analyzers, we first examined the impact of UHRF1 silencing on glycolysis. ECAR decreased significantly in UHRF1-silenced PANC-1 and MIA PaCa-2 cells, reflecting the positive role of UHRF1 glycolysis in pancreatic cancer cells (Fig. 2A and B). Oxygen consumption by cells reflects mitochondrial respiration. In the process of aerobic glycolysis, cells decreased OCR. Consistent with the ECAR results, we observed a significant increase in OCR in UHRF1 knockdown PANC-1 and MIA PaCa-2 cells, which reinforced the positive role of aerobic glycolysis (Fig. 2C and D). By measuring mitochondrial membrane potential with JC-1 dye, we observed that silencing UHRF1 expression decreased

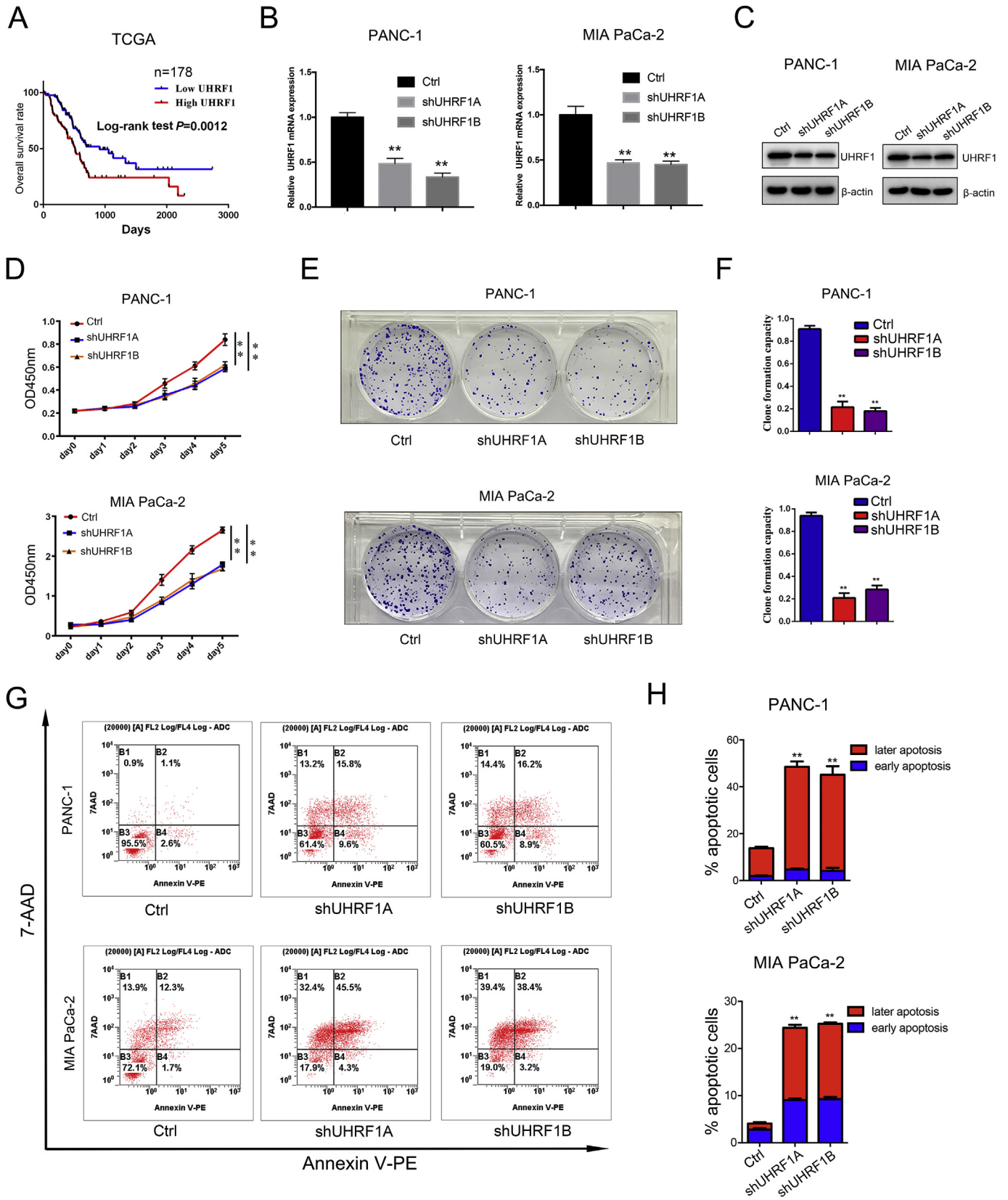


**Fig. 1. UHRF1 overexpression predicted worse prognosis and promoted proliferation of pancreatic cancer cells.** (A) In the TCGA cohort, patients with higher levels of UHRF1 expression displayed shorter overall survival. (B and C) Quantitative real-time RT-PCR and western blotting confirmed the silencing efficacy of UHRF1 in PANC-1 and MIA PaCa-2 cells. (D) Knockdown of UHRF1 decreased cell viability as reflected by CCK-8 proliferation assay. (E and F) Silencing UHRF1 expression in PANC-1 and MIA PaCa-2 cells decreased colony formation capacity of these cells. (G and H) Decreased UHRF1 expression increased apoptosis of PANC-1 and MIA PaCa-2 cells.

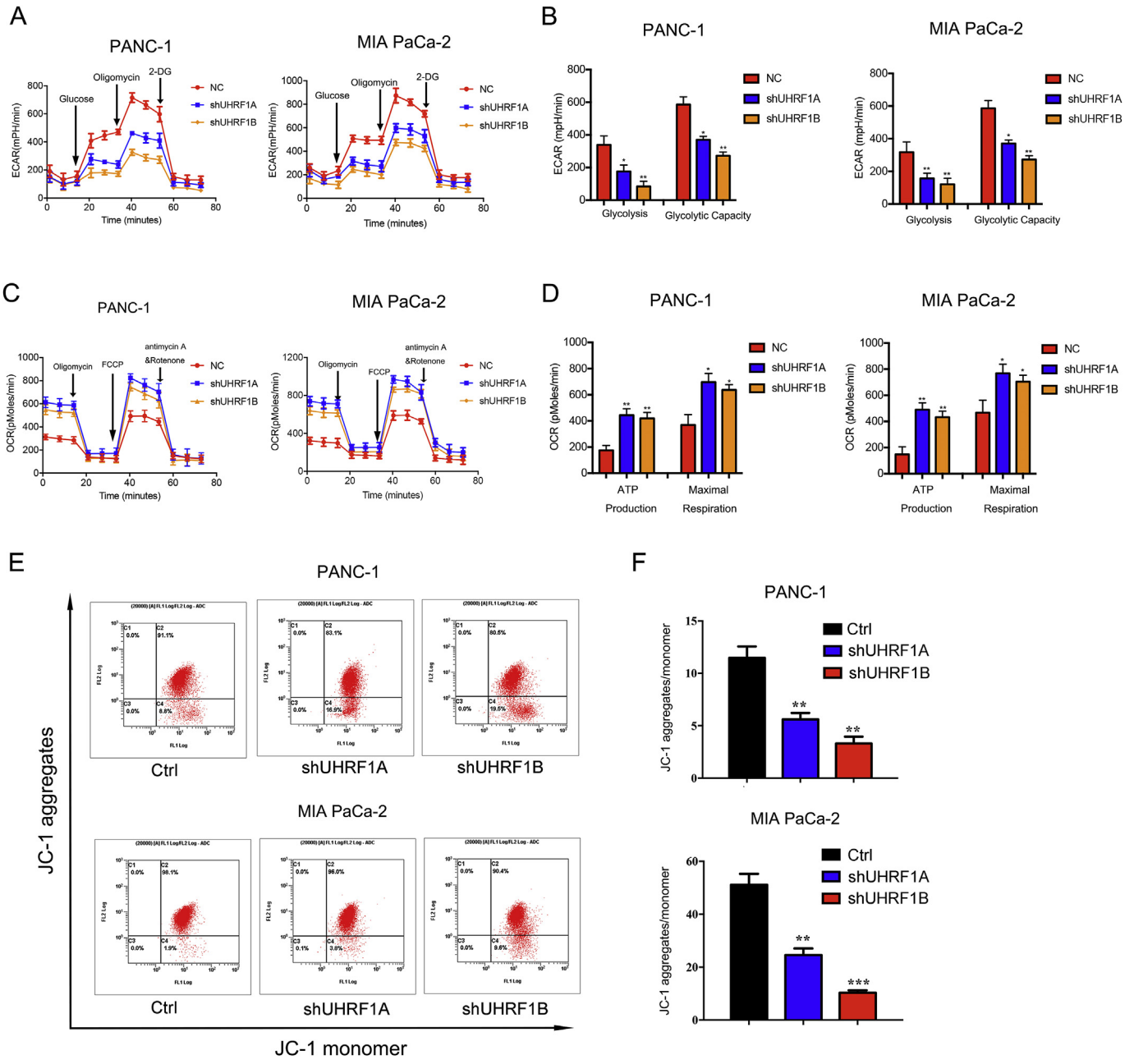


**Fig. 2. UHRF1 promoted aerobic glycolysis in pancreatic cancer cells.** (A) Representative image of ECAR measurement in UHRF1 silencing and control PANC-1 and MIA PaCa-2 cells. (B) Knockdown of UHRF1 decreased glycolysis and glycolytic capacity of PANC-1 and MIA PaCa-2 cells. (C) Chart of OCR measurement in UHRF1-silenced PANC-1 and MIA PaCa-2 cells. (D) Knockdown of UHRF1 increased mitochondrial respiration of PANC-1 and MIA PaCa-2 cells. (E and F) UHRF1 knockdown decreased mitochondrial membrane potential in PANC-1 and MIA PaCa-2 cells.

membrane potential of PANC-1 and MIA PaCa-2 cells (Fig. 2E and F). Therefore, UHRF1 positively regulated aerobic glycolysis in pancreatic cancer cells.

### *3.3. UHRF1 positively regulated HIF1α protein level and HIF1α targeted glycolytic genes*

HIF1α is a master regulator of aerobic glycolysis and hypoxia adaptation for solid tumours. To assess whether UHRF1 regulated aerobic glycolysis via HIF1α, we measured the protein level of HIF1α in UHRF1-silenced PANC-1 and MIA PaCa-2 cells. HIF1α protein level decreased significantly when UHRF1 expression was silenced (Fig. 3A). We assessed the impact of UHRF1 on HIF1α transcriptional activity as reflected by HRE-luciferase activity. We observed that UHRF1 positively regulated HIF1α transcriptional activity in a dose-dependent manner, reflecting a positive role of UHRF1 in HIF1α pathway regulation (Fig. 3B). HIF1α regulated aerobic glycolysis via transcriptional regulation of a series of glycolytic genes, including GLUT1, HK2 and LDHA. Thus, we examined the expression status of these glycolytic genes in UHRF1-silenced PANC-1 and MIA PaCa-2 cells. Consistent with the glycolysis analysis, expression of GLUT1, HK2 and LDHA decreased in UHRF1-silenced pancreatic cancer cells (Fig. 3C). Western blotting validated the role of UHRF1 in regulation of these glycolytic genes (Fig. 3D). To confirm the role of UHRF1 in regulation of these glycolytic genes, we analysed the expressional correlation between UHRF1 and GLUT1, HK2 or LHDA in a TCGA cohort of pancreatic cancer patients. Our analysis revealed that UHRF1 positively correlated with GLUT1, HK2 and LDHA expression in pancreatic cancer patients (Figs. 3E–4G).

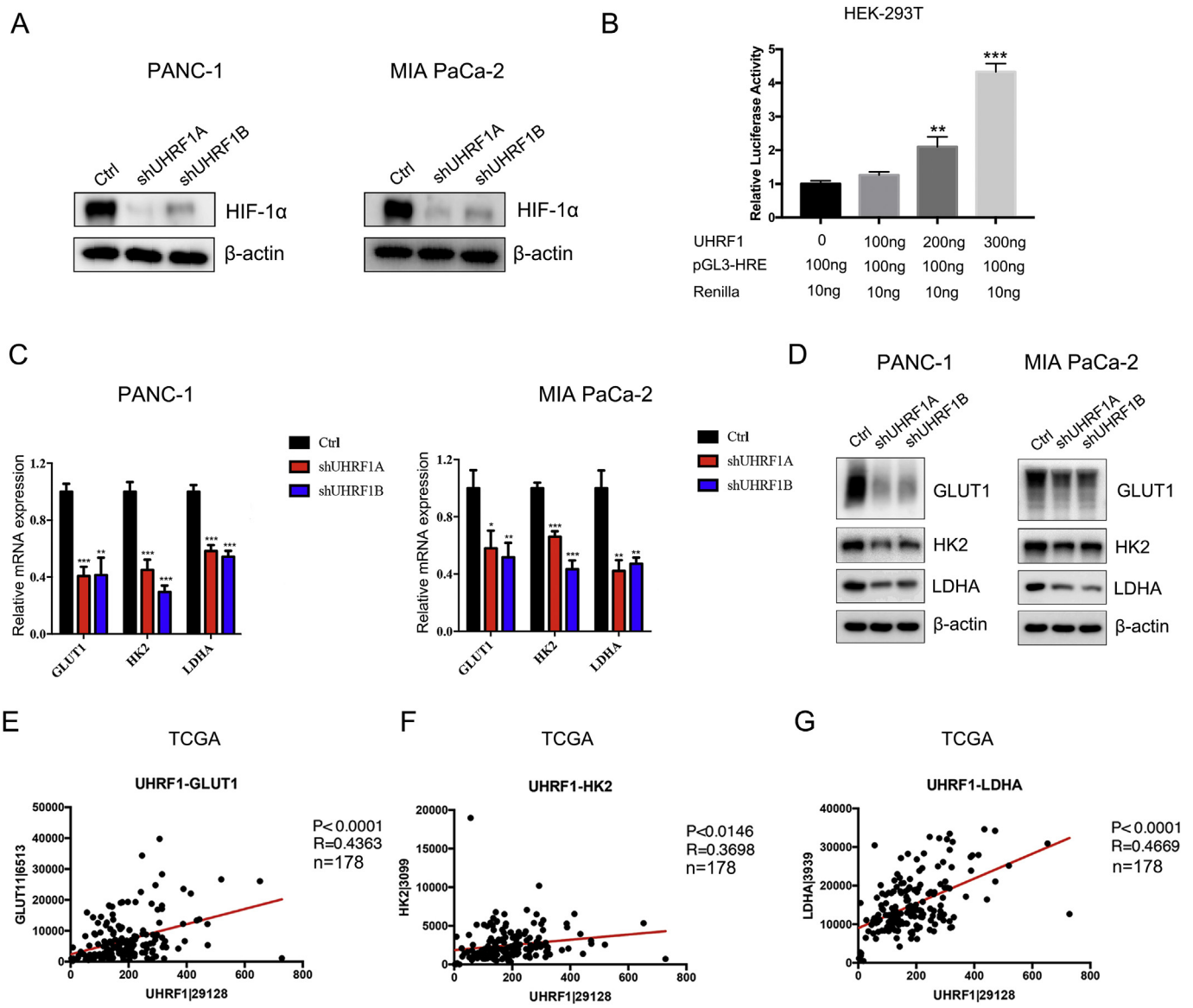


**Fig. 3. UHRF1 positively regulated HIF1α protein level and HIF1α targeted glycolytic genes.** (A) Knockdown of UHRF1 in PANC-1 and MIA PaCa-2 cells resulted in a reduction in HIF1α protein levels. (B) In HEK293T cells, UHRF1 positively increased HRE-luciferase activity in a dose-dependent manner. (C) Silencing UHRF1 expression in PANC-1 and MIA PaCa-2 cells decreased mRNA levels of GLUT1, HK2 and LDHA. (D) Knockdown of UHRF1 decreased protein levels of Glut1, HK2 and LDHA in PANC-1 and MIA PaCa-2 cells. (E–G) UHRF1 positively correlated with GLUT1, HK2 and LDHA expression in the TCGA cohort of pancreatic cancer patients.

### *3.4. UHRF1 negatively correlated with SIRT4 expression in pancreatic cancer patients and regulated SIRT4 expression in pancreatic cancer cells*

Mitochondrial SIRT3, SIRT4 and SIRT5 play negative roles in mediating glucose metabolism and tumour progression. We analyzed the expressional correlation between UHRF1 and SIRT3, SIRT4 or SIRT5 in TGGA pancreatic cancer patients. Expression of UHRF1 negatively and significantly correlated with SIRT4 expression in the TCGA cohort of pancreatic cancer patients, whereas, we observed no significant correlation between UHRF1 and SIRT3 or SIRT5 (Fig. 4A–C). To validate the TCGA results, we examined the expression status between UHRF1 and SIRT4 by immunohistochemical staining in patients from our center. The scoring parameters are listed in Supplementary Fig. 2. As demonstrated, patients with higher UHRF1 expression exhibited lower SIRT4 expression (Fig. 4D). The correlation is of significance and was confirmed by the negative correlation between UHRF1 and SIRT4 in pancreatic cancer patients (Fig. 4E). In UHRF1-silenced PANC-1 and MIA PaCa-2 cells, we observed an increase in SIRT4 mRNA and protein levels, validating the hypothesis that UHRF1 negatively regulates SIRT4 expression (Fig. 4F and G). Dual-luciferase assay demonstrated that UHRF1 suppressed SIRT4 promoter activity in HEK293T cells (Fig. 4H). ChIP assay demonstrated that UHRF1 specifically bound to SIRT4 promoter (Fig. 4I). Based on these observations, we propose that UHRF1 suppresses SIRT4 expression in pancreatic cancer cells.

### *3.5. SIRT4 is a tumour suppressor in pancreatic cancer*

The physiological role of SIRT4 has seldom been discussed in pancreatic cancer. Therefore, we analyzed the impact of SIRT4 on cell proliferation. We generated SIRT4-overexpressing PANC-1 and MIA PaCa-2 cells and the overexpression effect was confirmed by Western blotting with SIRT4 antibody (Fig. 5A). The CCK-8 proliferation assay demonstrated that overexpression of SIRT4 inhibited viability of PANC-1 and MIA PaCa-2 cells (Fig. 5B). The clone formation assay confirmed that overexpression of SIRT4 strongly inhibited clone formation

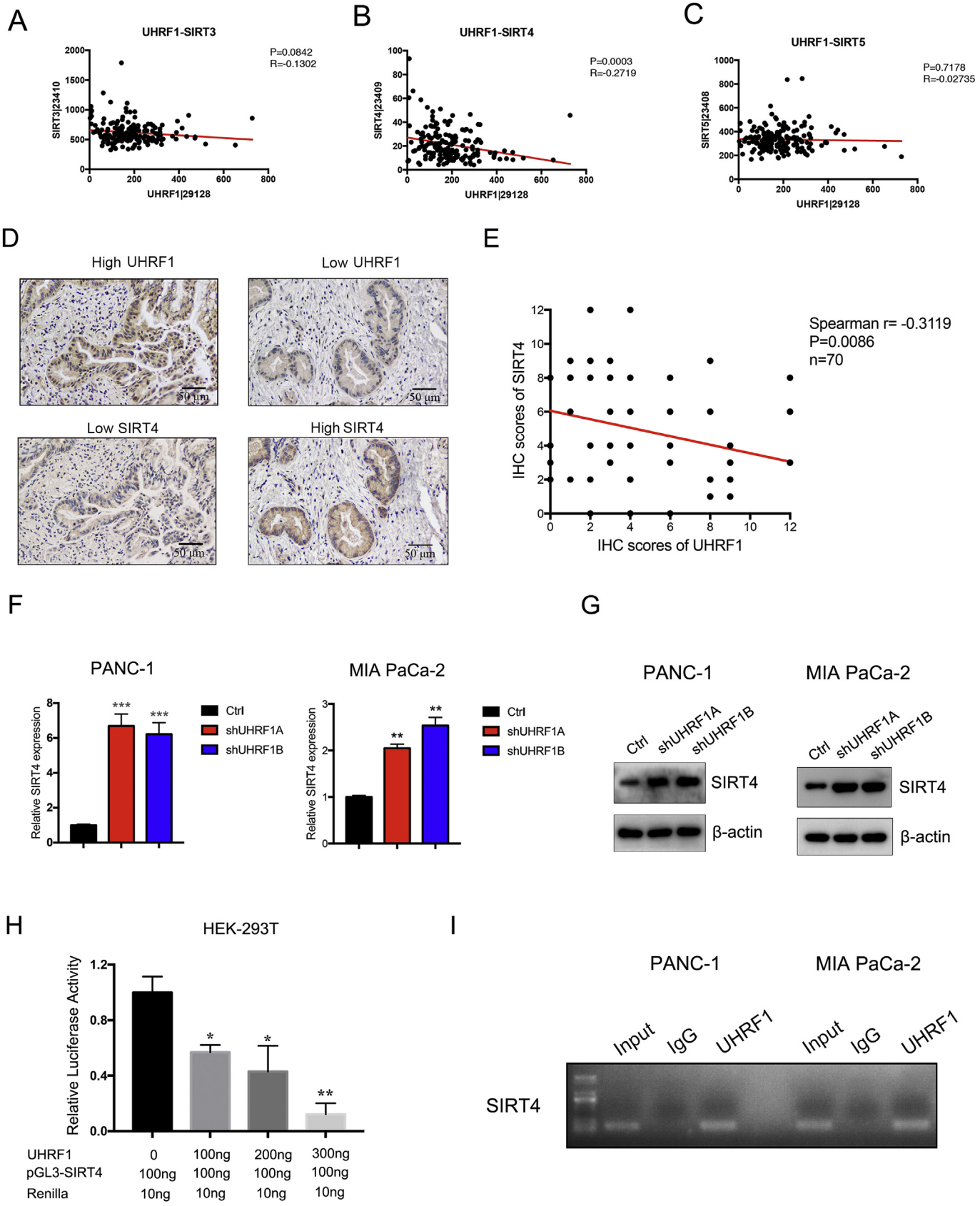


**Fig. 4. UHRF1 negatively correlated with SIRT4 expression in pancreatic cancer patients and regulated SIRT4 expression in pancreatic cancer cells.** (A–C) UHRF1 negatively correlated with SIRT4 mRNA levels in the TCGA cohort of pancreatic cancer patients but had no obvious correlation with SIRT3 and SIRT5. (D) Patients with higher levels of UHRF1 displayed lower SIRT4 expression. (E) UHRF1 negatively correlated with SIRT4 expression in pancreatic cancer patients as demonstrated by immunohistochemical staining and scoring. (F) Silencing UHRF1 expression increased SIRT4 expression in mRNA levels in PANC-1 and MIA PaCa-2 cells. (G) Knockdown of UHRF1 increased SIRT4 protein levels in PANC-1 and MIA PaCa-2 cells. (H) UHRF1 inhibited SIRT4 promoter luciferase activity. (I) ChIP results proved that UHRF1 could occupy the genomic region on SIRT4 promoter.

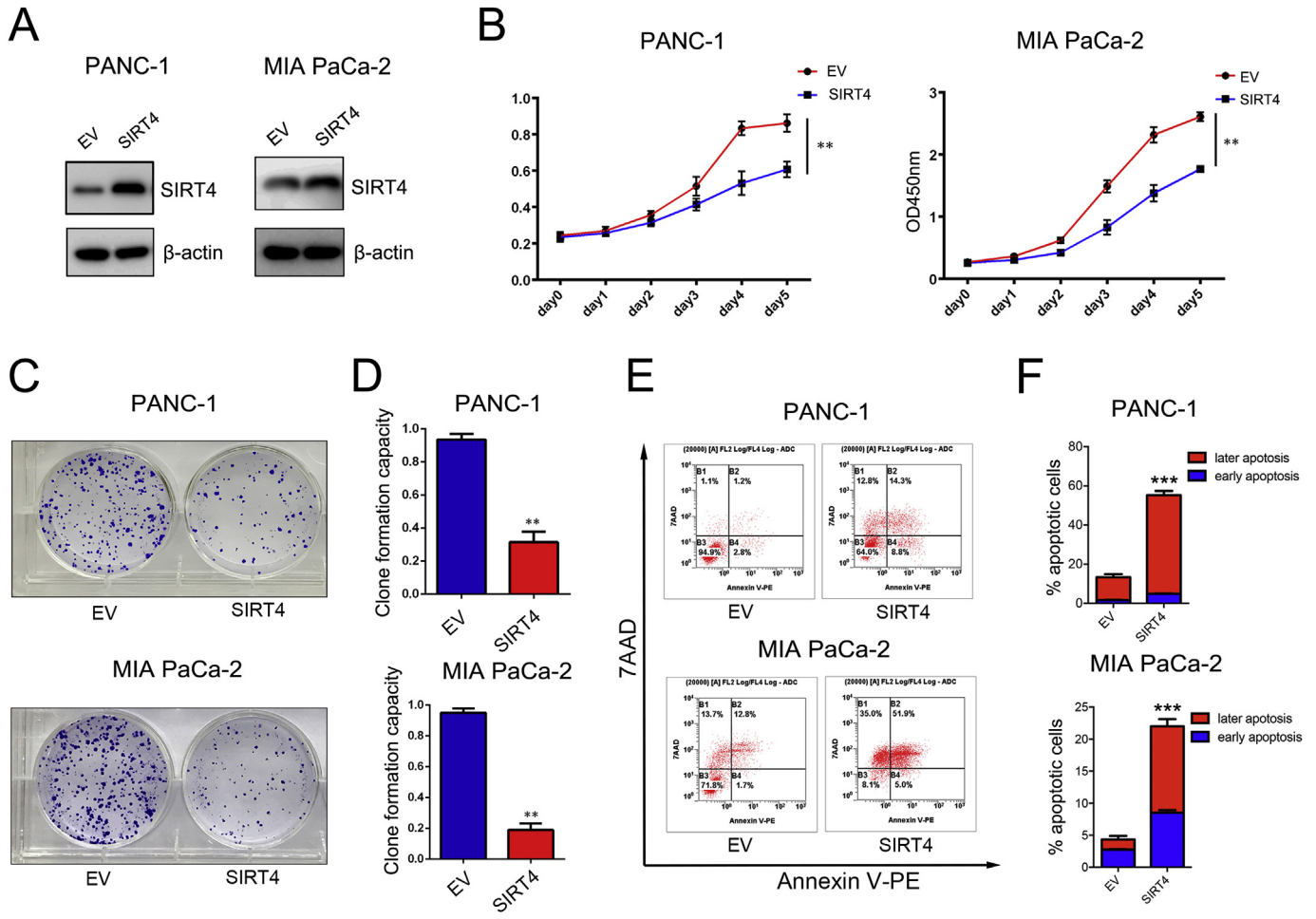


**Fig. 5. SIRT4 is a tumor suppressor in pancreatic cancer.** (A) SIRT4 was overexpressed in PANC-1 and MIA PaCa-2 cells and validated by Western blotting. (B) SIRT4 decreased cell viability of PANC-1 and MIA PaCa-2 cells measured by CCK-8 proliferation assay. (C and D) SIRT4 decreased colony formation capacity of PANC-1 and MIA PaCa-2 cells. (E and F) SIRT4 overexpression increased cell apoptosis of PANC-1 and MIA PaCa-2 cells.

capacity of PANC-1 and MIA PaCa-2 cells (Fig. 5C and D). We confirmed the role of SIRT4 in cell apoptosis. Overexpression of SIRT4 increased apoptosis of PANC-1 and MIA PaCa-2 cells (Fig. 5E and F). Therefore, SIRT4 suppresses pancreatic cancer cell proliferation and functions as a tumor suppressor.

### *3.6. SIRT4 negatively regulated aerobic glycolysis and suppressed HIF1α in pancreatic cancer*

To test the role of SIRT4 in regulating glycolysis in pancreatic cancer cells, we performed glycolytic measurement with Seahorse Extracellular Flux Analyser. As observed, overexpression of SIRT4 inhibited ECAR in PANC-1 and MIA PaCa-2 cells, indicating its negative role in aerobic glycolysis regulation (Fig. 6A and B). Moreover, when SIRT4 was overexpressed, the OCR increased accordingly, supporting its positive role in mitochondrial respiration (Fig. 6C and D). We measured the protein levels of HIF1α in SIRT4-overexpressing PANC-1 and MIA PaCa-2 cells. Western blotting demonstrated that the protein level of HIF1α decreased in the presence of SIRT4 overexpression (Fig. 6E). Moreover, our results demonstrated that in UHRF1 negatively correlated with SIRT4 expression, while positively correlated with HIF1α in HPDE, PANC-1 and MIA PaCa-2 cells (Fig. S3). Expression of glycolytic genes, including GLUT1, HK2 and LDHA, was also decreased at mRNA and protein levels (Fig. 6F and G). To confirm the *in vitro* observations, we analyzed the expressional correlation between SIRT4 with GLUT1, HK2 or LDHA in pancreatic cancer patients in the TCGA cohort. SIRT4 negatively and significantly correlated with these glycolytic genes (Fig. 6H–J). Moreover, we also demonstrated silencing SIRT4 expression could attenuate the decrease in cell viability and aerobic glycolysis in PNAC-1 cells(Fig. S4). Collectively, these results demonstrated that SIRT4 was a negative regulator of aerobic glycolysis that mediated the impact of UHRF1 on cell proliferation and glycolytic metabolism in pancreatic cancer cells.

In conclusion of the whole study, we demonstrated that higher levels of UHRF1 predicted worse prognosis of pancreatic cancer patients. *In vitro* cell lines demonstrated that UHRF1 positively regulated cell proliferation and aerobic glycolysis. UHRF1 suppressed expression of SIRT4, which was a tumor suppressor and negative regulator of aerobic glycolysis in pancreatic cancer cells (Fig. 6K). The present study uncovered UHRF1/SIRT4 axis as a novel predictive and treatment target for pancreatic cancer, and further studies are required to identify novel strategies by targeting the axis to improve pancreatic cancer overall survival.

## 4. Discussion

Despite progress in prognosis and treatment of pancreatic cancer, no significant progress has been made in improving survival; thus, there is an urgent need for a better understanding of the biology of pancreatic cancer [24]. Recent years have witnessed the progression and importance of metabolism reprogramming in oncogenesis and progression of cancer and metabolism reprogramming is regarded as one of the hallmarks of cancer [25,26]. This is especially true for pancreatic cancer, due to the limited oxygen and nutrient supply caused by severe

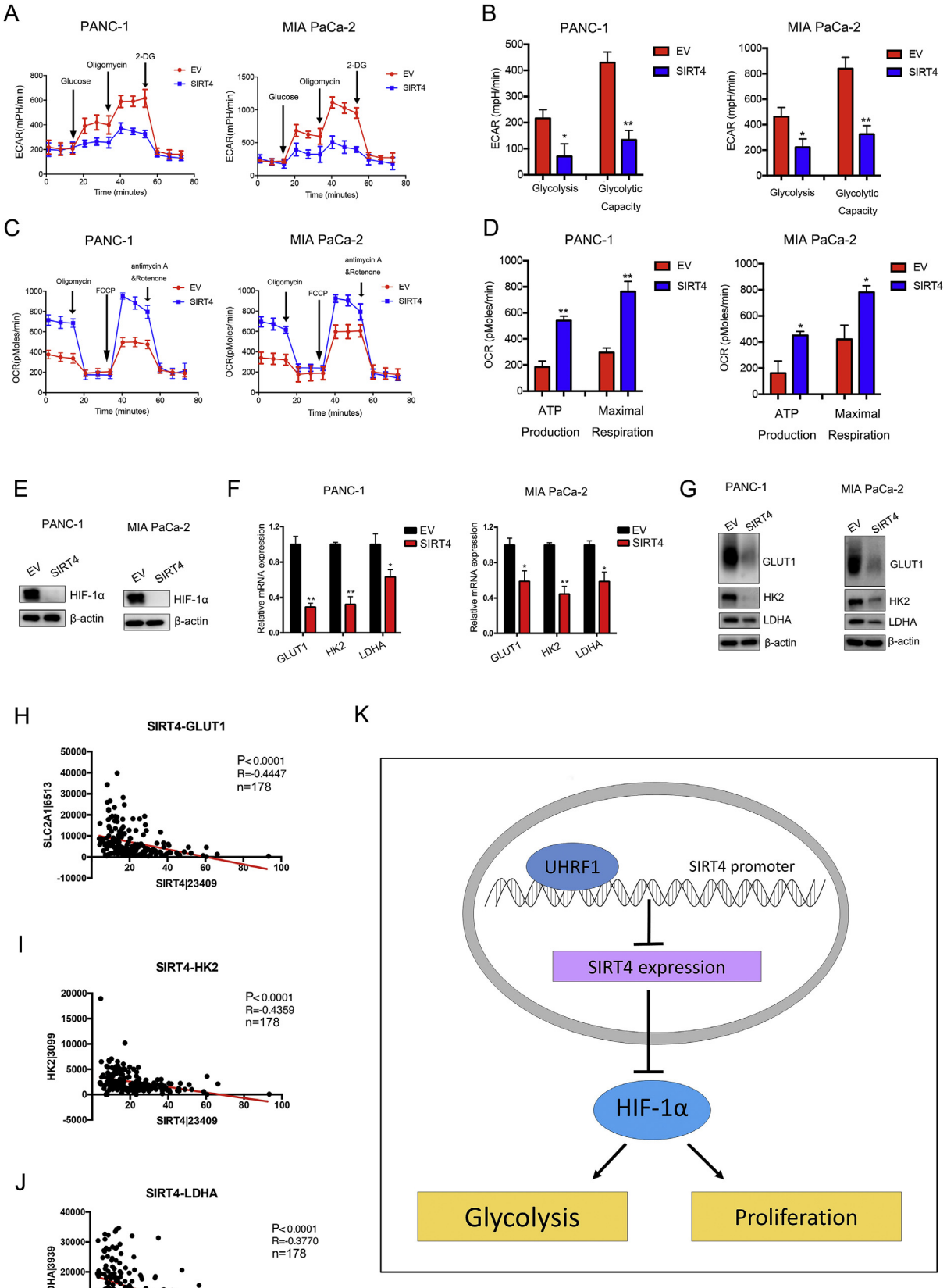


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**Fig. 6. SIRT4 negatively regulated aerobic glycolysis and suppressed HIF1α in pancreatic cancer.** (A and B) SIRT4 overexpression decreased glycolysis and glycolytic capacity of PANC-1 and MIA PaCa-2 cells measured by ECAR. (C and D) OCR demonstrated that SIRT4 overexpression increased mitochondrial respiration of PANC-1 and MIA PaCa-2 cells. (E) SIRT4 introduction into PANC-1 and MIA PaCa-2 cells decreased protein levels of HIF1α. (F and G) SIRT4 overexpression attenuated expression of GLUT1, HK2 and LDHA at mRNA and protein levels. (H–J) SIRT4 negatively correlated with GLUT1, HK2 and LDHA expression in pancreatic cancer patients from the TCGA cohort. (K) Schematic representation of the working model of the study.

hypoxic microenvironment and desmoplasia of pancreatic cancer cells. Therefore, understanding the basis of pancreatic cancer from the metabolic aspect will provide novel insights [27]. Our present study demonstrated that elevated expression of UHRF1 predicted worse prognosis of pancreatic cancer, by analyzing the TCGA pancreatic cancer cohort. Mechanistic studies illustrated that UHRF1 positively regulated aerobic glycolysis via suppression of SIRT4, a mitochondrial tumor suppressor. We also demonstrated that SIRT4 played negative roles in pancreatic cancer proliferation and aerobic glycolysis, which have seldom been discussed before.

The impact of UHRF1 on pancreatic cancer prognosis is consistent with a previous study that demonstrated that UHRF1 expression is higher in tumor samples than normal paratumor samples. Costello reported that UHRF1 was elevated in pancreatic cancer and stimulated growth and protected pancreatic cancer cells from stress through increasing the Keap1–Nrf2 pathway [16]. Tuveson reported that oncogene-induced Nrf2 transcription promotes reactive oxygen species (ROS) detoxification and tumourigenesis, which are all recognized positive elements in aerobic glycolysis control [28]. However, no studies have reported the direct role of UHRF1 in aerobic glycolysis. Our results indicate for the first time that UHRF1 is a positive regulator of aerobic glycolysis in pancreatic cancer. By analysis of the TCGA expression data, we confirmed the positive regulation of glycolytic genes by UHRF1, which was also confirmed by *in vitro* experiments in pancreatic cancer cell lines.

To investigate the mechanism accounting for UHRF1 in aerobic glycolysis, we focused on the mitochondrial tumor suppressor genes, including SIRT3, SIRT4 and SIRT5. Since the beginning of the century, the mammalian SIRT protein family has received much attention for its vital roles in metabolism and ageing [29,30]. Among them, SIRT3, SIRT4 and SIRT5 have mitochondrial targeting sequences, and their subcellular localization to this organelle has been confirmed experimentally. Mounting evidence has pointed out that these SIRT members are tumor suppressors [31]. SIRT3 is a major mitochondrial deacetylase, and many of its targets have important roles in metabolic homeostasis [32,33]. SIRT3 affects defence against oxidative stress by protecting cells from ROS, and decreased SIRT3 expression in cancer cells induces ROS production, which leads to HIF1α stability and enhanced aerobic glycolysis [34,35]. SIRT4 is abundantly expressed in pancreatic β cells and is involved in the regulation of insulin secretion; however, its precise enzymatic functions remain unclear [36]. Haigis reported that SIRT4 has tumour-suppressive activity and regulates the cellular metabolic response to DNA damage by inhibiting mitochondrial glutamine metabolism [37]. The roles of SIRT5 remain elusive in cancer, but it possesses some redundant functions with SIRT3 and SIRT4 in metabolism regulation and insulin secretion [38,39]. Expression of SIRT3 and SIRT4 is decreased by promoter chromatin modifications, and based on the fundamental role of UHRF1 in chromatin modification, we speculate that UHRF1 might promote aerobic glycolysis via suppression of these metabolism-related tumor suppressors [40–42]. We analyzed correlation in expression between UHRF1 and SIRT3 or SIRT4 in the TCGA database. We discovered a significant negative correlation between UHRF1 and SIRT4 expression. This correlation was confirmed experimentally. A previous study demonstrated that C-terminal-binding protein (CtBP) and cAMP-responsive element binding (CREB)2 regulate expression of SIRT4 as transcription factors [23,43]. CtBP and CREB2 are nuclear proteins and regulate the expression of target genes by chromatin modification [44,45]. Thus, we propose that UHRF1 might be the chromatin modifier responsible for SIRT4 silencing. Subsequent transcription analysis confirmed that UHRF1 is a chromatin modifier for SIRT4 transcriptional silencing in pancreatic cancer. The role of UHRF1 in chromatin modification is not sequence specific; thus, there is need to search for transcription factors that could bind SIRT4 promoter region, and at the same time interact with UHRF1 in pancreatic cancer.

Previous studies have demonstrated that decreased SIRT4 expression promotes poor prognosis in breast cancer, colorectal cancer, and esophageal squamous cell carcinoma; however, its role in pancreatic cancer has seldom been studied [46–48]. Our present study demonstrated that SIRT4 exhibits tumor suppressive functions *in vitro* in pancreatic cancer cells. To uncover the underlying mechanism, we examined its impact on HIF1α. The impact of SIRT4 on HIF1α was confirmed by TCGA dataset analysis and experimentally, although the underlying direct mechanism of SIRT4 in regulating HIF1α has not been discussed. This may due to the pivotal role of SIRT4 in mitochondrial behaviors. Piekorz reported that SIRT4 is associated with ROS production [49]. Increased ROS production inhibits the catalytic activity of prolyl hydroxylase, which is a negative regulator of HIF1α protein stability [50]. Although research on SIRT4 mainly focuses on glutamine metabolism, its role in glucose metabolism in cancer has seldom been studied. The present study reported for the first time that SIRT4 is a negative regulator of aerobic glycolysis in pancreatic cancer. This is also in line with the study of Goetzman, who reported that in high glucose conditions, SIRT4 exhibited an anti-Warburg effect [51]. Although pancreatic cancer is characterized by dense stroma, which restrict nutrient and oxygen supply, pancreatic cancer patients usually have hyperglycaemia caused by damage to insulin function. Thus, it is plausible that as observed in HEK293T cells, SIRT4 also functions as a negative regulator of aerobic glycolysis.

Like all the other cancers, pancreatic cancer cells rely on fuel sources for homeostasis and proliferation and become addicted to aerobic glycolysis. And enhanced aerobic glycolysis was mediated by up-regulations of glycolytic genes. For example, GLUT1 is one of the major members of the glucose transporter family. Its main function is to transport glucose into cells. It itself acts as a rate-limiting factor in glucose transport and therefore plays a crucial role in tumor energy metabolism. Pancreatic cancer cells proliferate rapidly and are in a microenvironment rich in interstitial, so the interior of the tumor is in a hypoxic state. Compared with aerobic respiration, the amount of adenosine triphosphate (ATP) produced by glycolysis under anaerobic conditions is extremely small, so tumor cells need to express GLUT1 in large quantities to meet their energy requirements. Increased expression of GLUT1 can accelerate cells. The uptake of glucose provides sufficient feedstock for glycolysis to promote ATP production. Therefore, understanding the basis of pancreatic cancer metabolism and develop strategies to target glycolytic genes may provide novel therapeutic avenues [52]. In the present study, we established UHRF1 as a novel regulator of aerobic glycolysis and SIRT4 might be its downstream target, forming a UHRF1/SIRT4 axis to increase expression of glycolytic genes and enhance aerobic glycolysis. The present suggests the feasibility of targeting UHRF1/SIRT4 axis to inhibit aerobic glycolysis and reverse malignancy of pancreatic cancer.

## Conflicts of interest

The authors have no conflict of interest to declare.

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## Appendix A. Supplementary data

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