

# SIRT3 Inhibits Cholangiocarcinoma Cell Proliferation by Targeting HIF1 $\alpha$ to regulate the EMT Signaling Pathway

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## Research Article

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# Abstract

## Background

Cholangiocarcinoma (CCA), is a rare biliary adenocarcinoma associated with poor outcomes. Deacetylase Sirtuin-3 (SIRT3), a histone deacetylase (HDAC), has been considered to be associated with various cancers and can be a potential new target for CCA. We intended to identify the target of SIRT3 and explore the mechanism of SIRT3 in CCA.

## Methods

The expression levels of SIRT3 and hypoxia-inducible factor-1 $\alpha$  (HIF1 $\alpha$ ) in CCA tissues and cell lines were examined by RT-qPCR. CCK-8 and EdU methods were used to detect cell proliferation in CCA. To assess the levels of proteins related to cell proliferation and epithelial-mesenchymal transition (EMT) process, western blot analysis was conducted. Co-immunoprecipitation and deacetylation assays were used to explore HIF1 $\alpha$  protein acetylation, stability and the relationship between SIRT3 and HIF1 $\alpha$  in CCA cells.

## Results

SIRT3 showed low expression in CCA tissues and cells. SIRT3 overexpression inhibited cell proliferation and EMT process. Moreover, the interaction between SIRT3 and HIF1 $\alpha$  was confirmed and HIF1 $\alpha$  expression was negatively regulated by SIRT3. Furthermore, we also found that HIF1 $\alpha$  was more easily degraded and showed a reduction in stability through deacetylation via SIRT3 knockdown. In rescue assays, HIF1 $\alpha$  also reversed the inhibitory effect of SIRT3 on cell proliferation and the EMT process.

## Conclusions

SIRT3 suppressed cell proliferation and the EMT process in CCA by targeting HIF1 $\alpha$ .

## Trial registration

Samples were obtained only after the patient has given informed consent according to the established plan approved by the Ethics Committee of The First Affiliated Hospital of Anhui Medical University.

# Introduction

Cholangiocarcinoma (CCA) is a primary liver malignancy, initially occurring in the intrahepatic biliary tract<sup>1</sup>. According to the existing statistic, the morbidity and mortality of CCA are increasing sharply each year due to its late clinical presentation, high conversion rate and poor prognosis<sup>2</sup>. CCA is often diagnosed at an advanced stage due to limited diagnostic methods<sup>3,4</sup>. Surgery is the choice for most patients, however, because of the rapid progression and metastasis of CCA tumors, surgical treatment is not appropriate for all patients<sup>5</sup>. In addition, radiotherapy or chemotherapy is still not ideal for improving the prognosis of patients with CCA<sup>6,7</sup>. Thus, new therapeutic targets or strategies are urgently needed<sup>8</sup>.

Sirtuin-3 (SIRT3), is a nicotinamide adenine dinucleotide (NAD<sup>+</sup>)-dependent deacetylase in mitochondria <sup>9</sup>. SIRT3 regulates cell survival, oxidative stress, apoptosis, metabolism, and through deacetylation <sup>10</sup>. SIRT3 plays an important role in tumor promotion or inhibition in numerous cancers <sup>11-14</sup>. However, the possible biological role of SIRT3 in CCA and its potential molecular mechanism were not available in previous studies. Although previous study has reported the anti-Warburg role of SIRT3 and has confirmed that SIRT3 inhibits the CCA progression by regulating the downstream pathway HIF1 $\alpha$ /PDK1/PDHA1 <sup>15</sup>. The Warburg effect represents the process of increased glucose uptake and aerobic glycolysis, which leads to the production of lactic acid, which becomes an early phenotype of cancer cells <sup>16,17</sup>. The specific underlying regulatory mechanism of SIRT3 on CCA inhibition remains unclear.

Hypoxia inducible factor 1 $\alpha$ , (HIF1 $\alpha$ ), has been found to regulate the expression of glycolytic-related genes <sup>18</sup>. Recently increasing evidence shows that HIF1 $\alpha$  is involved in malignant disease progression <sup>19,20</sup>. For example, increased HIF-1 $\alpha$  levels are related to a poor prognosis in breast cancer <sup>21</sup>. HIF1 $\alpha$  protein was highly expressed in prostate, lung, and colon cancer <sup>22</sup>. In cervical cancer, the deacetylation of HIF1 $\alpha$  is increased by SIRT2, which reduces HIF1 $\alpha$  degradation and increases HIF1 $\alpha$  stability by further ubiquitination <sup>23</sup>. Thus, in this study, we hypothesized that HIF1 $\alpha$  was modified by SIRT3 deacetylation to make it more susceptible to degradation and inhibit the proliferation of CCA. To test our hypothesis, we further explored the molecular mechanism and functional significance of SIRT3 to provide a novel research strategy for CCA treatment.

## Materials And Methods

### Cell culture

The CCA cell lines (HuCCT1, RBE and HCCC-9810) and a normal intrahepatic biliary epithelial cell (HIBEpiC) were used in this study (Chulabhorn Research Institute, Thailand). These CCA cell lines were maintained in RPMI-1640 supplemented with 10% FBS (Gibco, Grand Island, USA). A humidified incubator with 5% CO<sub>2</sub> was used to culture the cells at 37°C.

### Cell transfection

pcDNA3.1 targeting SIRT3, HIF1 $\alpha$ , and the negative control pcDNA3.1 were purchased from RiboBio, Guangzhou, China. The SIRT3 and HIF1 $\alpha$  plasmids were from obtained from the Zhao Lab of Fudan University (Shanghai, China). CCA cells were transfected with pcDNA3.1-SIRT3, sh-SIRT3, and pcDNA3.1-HIF1 $\alpha$  using the Lipofectamine RNAiMax reagent (Invitrogen).

### Clinical specimens

Clinical tissue specimens of CCA from 20 patients were histopathologically diagnosed at The First Affiliated Hospital of Anhui Medical University (Hefei, China). Samples were obtained only after the

patient has given informed consent according to the established plan approved by the Ethics Committee of The First Affiliated Hospital of Anhui Medical University.

### **Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis**

TRIzol reagent (Invitrogen, USA) was used to extract Total RNA from cells or tissues. Total RNA was reverse transcribed to cDNA by Reverse Transcription Kit (Takara, Dalian, China). Three replicates of each sample were amplified and analyzed with a Roche Light-Cycler (Roche, Basel, Switzerland). Results were normalized using GAPDH expression. The  $2^{-\Delta\Delta C_t}$  method was used to calculate relative expression level. The primers used were as follows: SIRT3 forward, 5'-AGG GAC GAT TAT TAA AGG TGG A-3' and reverse, 5'-TAC ATC CTG CAG GGA AAG C-3'; HIF1 $\alpha$  forward, 5'-CCC ATT CCT CAC CCA TCA AAT A -3' and reverse, 5'-CTT CTG GCT CAT ATC CCA TCA A-3'; GAPDH forward, 5'-GGG AAA CTG TGG CGT GAT-3' and reverse, 5'-GAG TGG GTG TCG CTG TTG A-3'.

### **Cell counting Kit-8 (CCK-8) assay**

CCA cells were seeded into 96-well plates at the density of  $2 \times 10^3$  cells/well. Next, cells were cultured for 24, 48, or 72 h in an incubator with 5% CO<sub>2</sub>. After 10  $\mu$ L of CCK-8 solution at 37°C for 2.5 h was added to each well, a microplate reader was applied to determine sample absorbance at 450 nm. The optical density (OD) value was measured, and the experiments were repeated three times.

### **5-Ethynyl-2'-deoxyuridine (EdU) assay**

Cell proliferation was determined by EdU assay using a KeyFluor488 Click-iTEdU kit (KeyGEN, Nanjing, China) following the manufacturer's instructions. About  $5.0 \times 10^3$  cells/well were seeded in a 96-well plate, and then transfected with pcDNA3.1-NC, pcDNA3.1-SIRT3, pcDNA3.1-SIRT3+pcDNA3.1-HIF1 $\alpha$ . After transfection, EdU solution was added and the cells were incubated for 2 h at 37°C, and then fixed with 4% polyformaldehyde. Cells were then stained with DAPI. The cells were then visualized in three randomly chosen visual fields using the Zeiss fluorescence photomicroscope (Carl Zeiss, Oberkochen, Germany).

### **Western blot analysis**

CCA cells were lysed with RIPA buffer (Beyotime, Shanghai, China). In order to separate the proteins from the sample buffer, 12% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel was added, and then transferred onto a PVDF membrane, which was blocked with 5% nonfat milk for 1 h. The primary antibodies were provided by Abcam Company, including anti-SIRT3 (ab217319, 1:1000), anti-GAPDH (ab8245, 1:10000), anti-PCNA (ab92552, 1:1000), anti-Ki67 (ab15580, 1:1000), anti-CDK2 (ab32147, 1:1000), anti-E-cadherin (ab40772, 1:10000), anti-N-cadherin (ab76011, 1:5000), anti-Slug (ab27568, 1:1000), anti-Twist (ab50887, 1:1000), anti-HIF1 $\alpha$  (ab270520, 1:1000) and anti-Tubulin (ab6160, 1:10000). The appropriate secondary antibodies were used at 1:2000 for all antibodies. Data were analyzed using ECL detection system (Pierce, Rockford, IL, USA).

### **Co-Immunoprecipitation assay**

CCA cells were collected and dissolved in 500 µl co-IP buffer containing a mixture of protease inhibitors (Sigma-Aldrich). Cells were prepared for extracting total proteins. Next, 1-2 µg HIF1α antibody was added into 300 µL cell lysates at 4°C for 30 minutes. Then, proteins were incubated with the beads at 4°C overnight. On the following day, the beads were washed for three times, and then, the proteins were prepared for western blotting.

### **Deacetylation assay**

HIF-1α and GFP proteins were expressed in HuCCT1 cells. In the deacetylation assay, the acetylated HIF-1α or SIRT3 immunoprecipitates were resuspended in 100 µl deacetylation buffer and 1 mM NAD. The reactions were pretreated with 10 mM nicotinamide for 10 min and incubated for 2 h at 30 °C. Next, beads were placed on ice for 15 min and boiled with sample buffer. The supernatant was analyzed by Western blotting with anti-acetyllysine antibody.

### **Statistical analysis**

Statistical analyses were performed with SPSS 19.0 software. The results presented as means ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by LSD test was performed for multiple comparisons, and the student's *t* test was used for comparisons between two groups. The data were followed by Tukey's post hoc test.  $p < 0.05$  was considered statistically significant.

## **Results**

### **SIRT3 inhibits cell proliferation and EMT process**

To investigate the role of SIRT3 in CCA, we first used RT-qPCR to analyze the expression of SIRT3 in 20 pairs of CCA tissues and 20 adjacent normal tissues, which showed that SIRT3 expression was lower in CCA tissues than in adjacent normal tissues (Fig. 1A). Decreased expression of SIRT3 was observed in HuCCT1, RBE, HCCC9810 cells, compared with HIBEpiC cells (Fig. 1B). Then we overexpressed SIRT3 and found elevated expression level of SIRT3 in HuCCT1 and RBE cells via RT-qPCR analysis (Fig. 1C). Declined cell viability induced by overexpressed SIRT3 was observed using a CCK-8 assay (Fig. 1D). The EdU incorporation assays also demonstrated that overexpression of SIRT3 reduced HuCCT1 and RBE cell proliferation (Fig. 1E). Similarly, SIRT3 overexpression reduced the protein levels of PCNA, Ki67, CDK2, which were associated with cell proliferation in CCA cells (Fig. 1F). Western blotting also revealed that the SIRT3 overexpression increased E-cadherin protein level. On the contrary, proteins like N-cadherin, Slug, Twist with strong EMT capacity were suppressed (Fig. 1G). Based on the above findings, it can be concluded that SIRT3 was downregulated in cholangiocarcinoma, and SIRT3 overexpression inhibited cell proliferation and EMT process.

### **SIRT3 directly interacts with HIF1α in CCA cells**

To explore the interaction between SIRT3 and HIF1α, co-immunoprecipitation (Co-IP) assays were performed. We found that SIRT3 and HIF1α precipitated each other as revealed by co-

immunoprecipitation (Co-IP) assays, and the correlation between SIRT3 and HIF1 $\alpha$  was initially identified (Fig. 2A). Protein level of HIF1 $\alpha$  in lysed protein samples was observed, and we found that the protein level of HIF1 $\alpha$  was reduced in SIRT3 overexpression group, while the protein level of HIF1 $\alpha$  was increased in SIRT3 knockdown group in CCA cells (Fig. 2B). We analyzed the expression of HIF1 $\alpha$  in 20 pairs of CCA tissues and 20 pairs of adjacent normal tissues by RT-qPCR, and the results showed that HIF1 $\alpha$  was highly expressed in CCA tissues compared with adjacent normal tissues (Fig. 2C). The expression protein levels of HIF1 $\alpha$  were also upregulated in CCA cell lines (Fig. 2D). Furthermore, HIF1 $\alpha$  and SIRT3 expression showed a significant negative correlation in CCA tissues (Fig. 2E). Thus, these data suggested that SIRT3 contributed to proliferation by directly modulating HIF1 $\alpha$  in CCA.

### **SIRT3 induces degradation of HIF1 $\alpha$ via deacetylation**

To further detect the relationship between SIRT3 and HIF1 $\alpha$ , HIF1 $\alpha$  was deacetylated by SIRT3 in HuCCT1 cells. SIRT3 knockdown showed high protein level of HIF1 $\alpha$  as well as the HIF1 $\alpha$  acetylation level, which indicated that SIRT3 induced HIF1 $\alpha$  deacetylation (Fig. 3A). Then, we suppressed HIF1 $\alpha$  synthesis by CHX (Cycloheximide) and HIF1 $\alpha$  proteasomal degradation by MG132. In HuCCT1 cells, HIF1 $\alpha$  degraded faster after SIRT3 overexpression, and the degradation of HIF1 $\alpha$  was blocked by MG132 (Fig. 3B). Similarly, SIRT3 knockdown decreased the HIF1 $\alpha$ -wt and HIF1 $\alpha$ -mut protein stability, while sh-SIRT3 had no effect on the GFP and K709A (Fig. 3C). The results taken together indicated that HIF1 $\alpha$  was more easily to be degraded and showed a reduction in stability through deacetylation via SIRT3 knockdown.

### **Overexpression of HIF1 $\alpha$ reverses the inhibition of SIRT3 on cell proliferation and EMT process**

To test the overexpression efficiency, we overexpressed HIF1 $\alpha$  and found elevated expression levels of HIF1 $\alpha$  in CCA HuCCT1 and RBE cells by RT-qPCR (Fig. 4A). The CCK-8 cell viability assay showed that HIF1 $\alpha$  overexpression significantly reversed the inhibitory effects of SIRT3 overexpression on cell proliferation in CCA cells (Fig. 4B-C). EdU assay also showed that overexpression of HIF1 $\alpha$  partially reversed the reduced proliferation of CCA cells induced by SIRT3 overexpression (Fig. 4D). The overexpression of HIF1 $\alpha$  reversed the decreased levels of proliferation-related proteins (PCNA, Ki67, CDK2) induced by SIRT3 overexpression (Fig. 4E). In addition, western Blotting also showed that HIF1 $\alpha$  overexpression reversed the promotive effect of SIRT3 overexpression on the protein and expression levels of E-cadherin, while HIF1 $\alpha$  upregulation also reversed the suppressive effect of SIRT3 overexpression on the protein and expression levels of N-cadherin, Slug and Twist (Fig. 4F). These results further suggested that HIF1 $\alpha$  overexpression reversed the inhibitory SIRT3 on cell proliferation and EMT process.

## **Discussion**

Sirtuin 3 (SIRT3) was found to have the most powerful mitochondrial deacetylase activity and is able to target key proteins for the normal function and metabolism of organelles<sup>24</sup>. Previous studies have found

that SIRT3 acted as a tumor promoter in the development of some types of cancer, such as esophageal cancer<sup>25</sup>, breast cancer<sup>26</sup>, gastric cancer<sup>27</sup>, colon cancer<sup>28</sup>. On the contrary, SIRT3 has also been described as a tumor suppressor in some cancers<sup>29,30</sup>. However, there are very few studies on SIRT3 in CCA. In our study, SIRT3 was lowly expressed in CCA tissues and cells. In addition, SIRT3 overexpression inhibited cell proliferation and EMT process. Next, we began to study the specific regulation mechanism of SIRT3 in CCA.

Previously, studies have shown that SIRT3 expression is closely associated with the expression of various HIF1 $\alpha$ -dependent genes in breast cancer patients<sup>31</sup>. In our study, we further confirmed the correlation between SIRT3 and HIF1 $\alpha$  in CCA. HIF1 $\alpha$  expression was negatively correlated with SIRT3 expression in CCA tissues, suggesting that SIRT3 contributed to cell proliferation by regulating HIF1 $\alpha$ .

Acetylation is a key posttranslational modification and is also considered to be a key metabolic enzyme<sup>32,33</sup>. Notably, in cervical cancer, SIRT2 increases HIF1 $\alpha$  deacetylation, which reduces HIF1 $\alpha$  degradation and increases HIF1 $\alpha$  stability through further ubiquitination<sup>23</sup>. Deacetylases have also been investigated as potential therapeutic targets for CCA<sup>34</sup>. Our study confirmed that SIRT3 can indeed control glycolytic metabolism by modifying the stability and activity of HIF1 $\alpha$  via deacetylation. We found that HIF1 $\alpha$  modified with deacetylation was more easily degraded, which downregulated the expression level of HIF1 $\alpha$  and decreased the stability of HIF1 $\alpha$ . These results suggested that SIRT3 directly inhibited the function of HIF1 $\alpha$ . Subsequently, we performed a rescue assay in which HIF1 $\alpha$  overexpression reversed the suppressive effect of SIRT3 overexpression on cell proliferation and EMT process. Therefore, the decrease of HIF1 $\alpha$  stability can enhance the inhibitory effect of SIRT3 on CCA cell proliferation and EMT process. In subsequent experiments, we will explore the effects of deacetylation and ubiquitination on the SIRT3/HIF1 $\alpha$  axis and the mechanism of SIRT3 in various stages of tumor development.

In conclusion, SIRT3 exerted its inhibitory role on the proliferation and EMT process of CCA cells via regulating HIF1 $\alpha$ . This finding may provide new research strategy for the CCA treatment.

## **Declarations**

### **Acknowledgement**

We appreciate all participants who contributed to the study

### **Conflicts of interest**

The authors declared no competing interests in this study.

### **Funding Sources**

There is no funding to report for the research.

### **Ethics approval and consent to participate**

Samples were obtained only after the patient has given informed consent according to the established plan approved by the Ethics Committee of The First Affiliated Hospital of Anhui Medical University.

### **Consent for publication**

Not applicable.

### **Availability of data and materials**

The datasets used during the current study are available from the corresponding author on reasonable request.

### **Authors' contributions**

Yihang Zhao and Yaxian Kuai conceived and designed the experiments. Yihang Zhao, Yaxian Kuai, Jianhua Xu, Yufang Cui, Juan Wu, Mingming Zhang, Lei Xu, Lixing Zhou, Bin Sun and Yang Li carried out the experiments. Yihang Zhao, Yaxian Kuai, Bin Sun and Yang Li analyzed the data. Yihang Zhao, Yaxian Kuai, Bin Sun and Yang Li drafted the manuscript. All authors agreed to be accountable for all aspects of the work. All authors have read and approved the final manuscript.

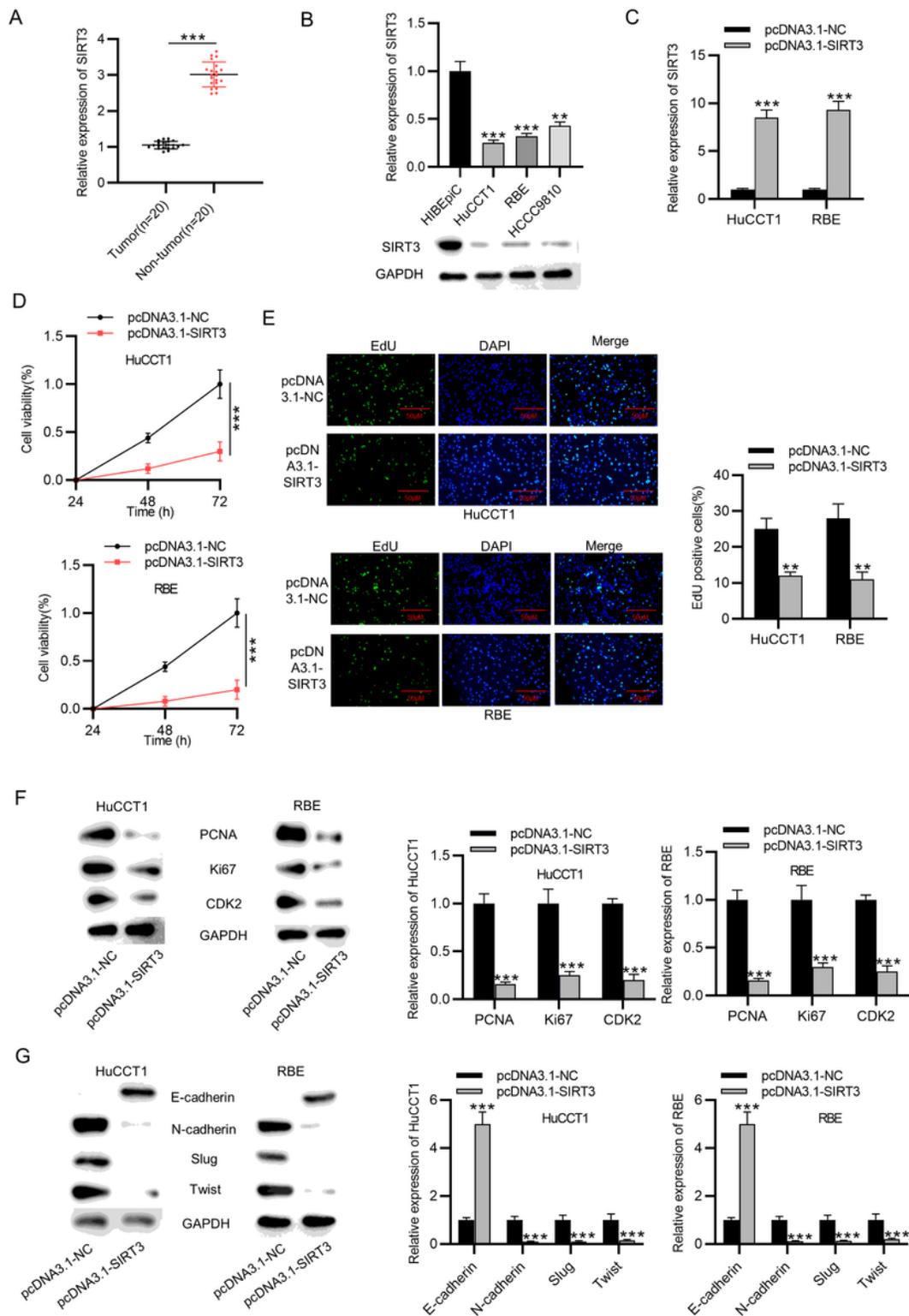
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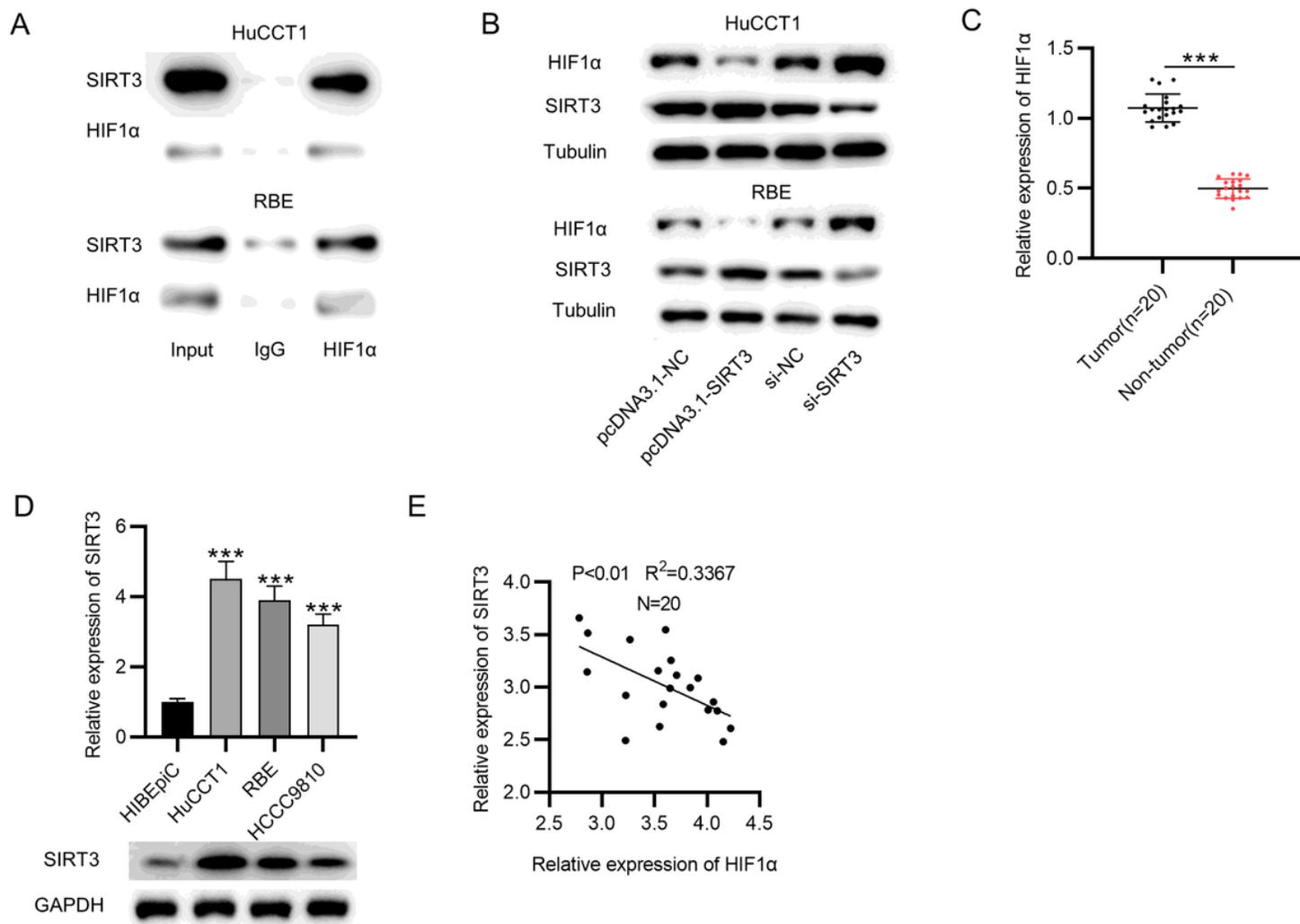
## Figures



**Figure 1**

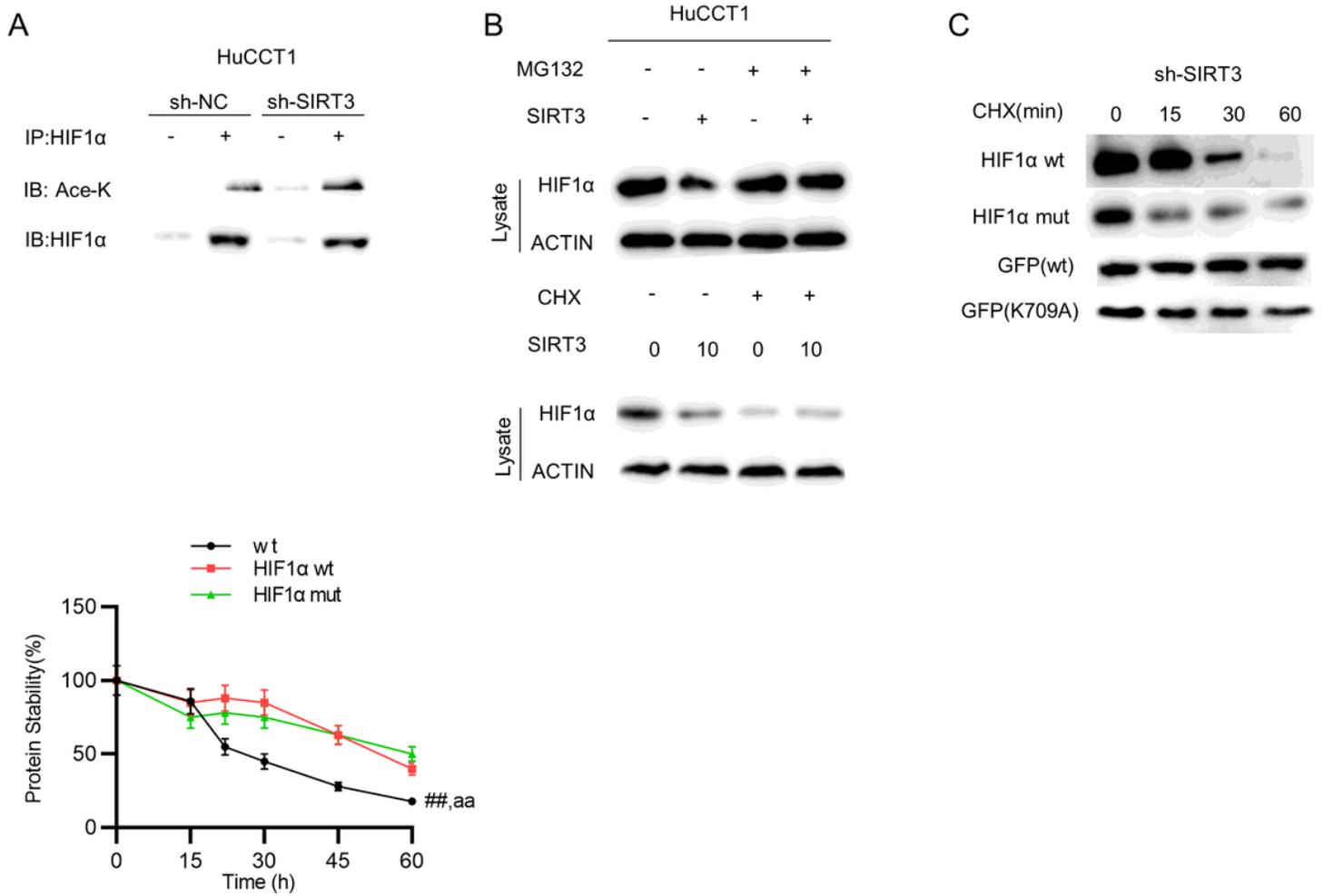
SIRT3 inhibits cell proliferation and EMT process (A) Expression of SIRT3 in 20 CCA tissues and 20 adjacent normal tissues was examined by RT-qPCR. (B) Expression levels of SIRT3 in CCA cell lines were detected by RT-qPCR. (C) RT-qPCR was used to detect the overexpression efficiency of SIRT3 in HuCCT1 and RBE cells. (D) CCK-8 assay was conducted to detect the effect of SIRT3 overexpression on viability of HuCCT1 and RBE cells. (E) Cell proliferation of HuCCT1 and RBE cells was detected by EdU assay. (F)

Representative images of immunoblotting and quantitative analysis of PCNA, Ki67 and CDK2 were examined by western blot and RT-qPCR. (G) Expression and protein levels of E-cadherin, N-cadherin, Slug, and Twist were examined by western blot and RT-qPCR. \* $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .



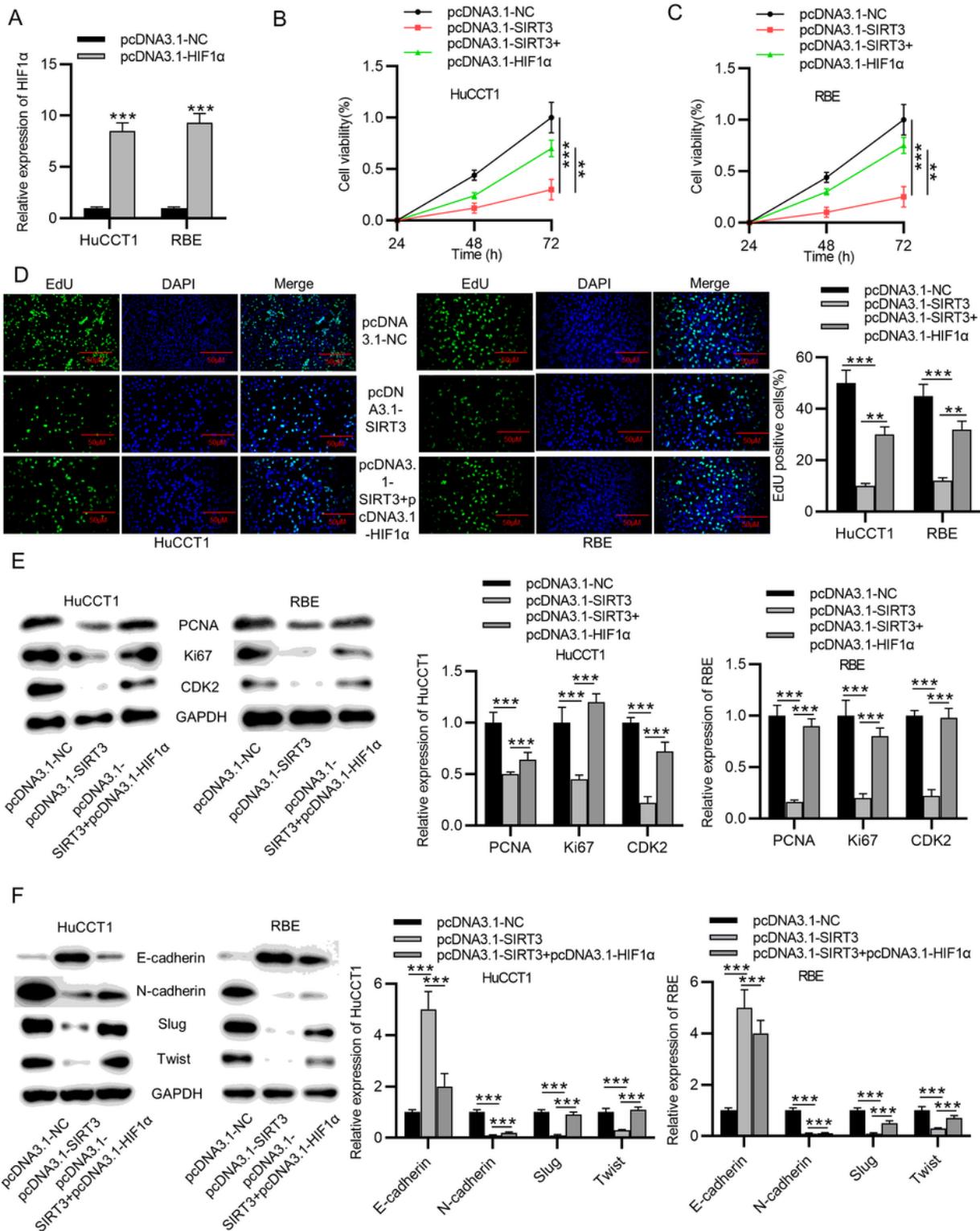
**Figure 2**

SIRT3 directly interacts with HIF1α in CCA cells (A) Co-immunoprecipitation (Co-IP) experiments were performed to identify the interactions between SIRT3 and its interacting protein HIF1α. (B) Expression level of HIF1α in lysed protein samples after the treatment was examined by western blot. (C) Expression of HIF1α in CCA tissues was examined by RT-qPCR. (D) Expression of HIF1α in HIBEoiC, HuCCT1, RBE and HCCC9810 cell lines was examined by RT-qPCR. (E) Correlation between HIF1α and SIRT3 expression was detected in CCA tissues. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .



**Figure 3**

SIRT3 induces degradation of HIF1α via deacetylation (A) HuCCT1 cells were co-transfected with HIF1α and SIRT3 knockdown and followed by western blot analysis. (B) HuCCT1 cells were harvested and lysated and visualized by western blotting after were MG132 and CHX treatment. (C) Protein stabilities of HIF1α-wt and HIF1α-mut were measured and expressed by western blotting and line graphs. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .



**Figure 4**

Overexpression of HIF1α reverses the inhibition of SIRT3 on cell proliferation and EMT process (A) Efficiency of HIF1α overexpression in HuCCT1 and RBE cells was examined by RT-qPCR. (B-D) CCK-8 and EdU assay were conducted to detect the viability and proliferation of HuCCT1 and RBE cells, respectively. (E) Representative images of immunoblotting and quantitative analysis of PCNA, Ki67 and CDK2 were

shown by western blotting and RT-qPCR. (F) EMT related protein levels were detected by western blot. \*p< 0.05, \*\*p< 0.01, \*\*\*p< 0.001.