The E3 Ubiquitin Ligase RBCK1 Promotes The Invasion and Metastasis of Hepatocellular Carcinoma By Destroying The PPARγ/PGC1α Complex

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Abstract

Background: The disruption of tumour cell metabolism can inhibit tumour metastasis, indicating that aerobic glycolysis is central to tumour development. However, the key factors responsible for mediating aerobic glycolysis in hepatocellular carcinoma (HCC) remain unknown.

Methods: This study investigated the function and clinical significance of RBCK1 protein expression in HCC. We analyzed RBCK1 expression from the TCGA-LIHC dataset and surgical specimens of 216 HCC patients. The correlation between the clinical characteristics and prognosis was also determined. Furthermore, over-expression and knockdown experiments of RBCK1 were developed to explore their effects on HCC cell migration, invasion and aerobic glycolysis. Moreover, a molecular mechanism of RBCK1 promotes HCC metastasis was explored.

Results: Here, we observed that RBCK1 expression was significantly upregulated in HCC tissues. Our clinical study showed that high RBCK1 expression significantly correlated with poor tumour survival and distant invasion. Functional assays using HCC cells revealed that RBCK1 promoted migration and invasion by enhancing the Warburg effect, and that GLUT1 was critical for RBCK1-mediated aerobic glycolysis. Furthermore, RBCK1-mediated regulation of WNT/β-catenin/GLUT1 pathway-induced HCC cell migration and aerobic glycolysis was dependent on the destruction of the PPARγ/PGC1α complex. Mechanistically, RBCK1 promoted PPARγ ubiquitination and degradation, causing RBCK1 overexpression to enhance the transcriptional activity of WNT/β-catenin. This consequently upregulated the expression of GLUT1-mediated aerobic glycolysis in HCC cells, promoting tumour cell metastasis and invasion.

Conclusion: Altogether, our findings identify a mechanism used by HCC cells to survive the nutrient-poor tumour microenvironment and also provide insight into the role of RBCK1 in HCC cell adaptation to metabolic stresses.

Background

Aerobic glycolysis, also known as the Warburg effect, is one of the most characteristic metabolic phenotypes of cancer cells, including hepatocellular carcinoma (HCC), where it plays an important role [1, 2]. Aerobic glycolysis is mainly affected by carcinogenic signals, such as PI3K/Akt and signalling [3, 4]. It is important to note that a metabolic disorder of tumour cells can inhibit metastasis, which indicates that aerobic glycolysis is at the core of tumour growth and survival [5]. Therefore, a better understanding of the mechanisms between cell metabolism and metastasis is essential for the development of new therapies, especially in liver cancer and other diseases, as the liver is an important organ responsible for several unique metabolic functions.

Glucose transporter 1 (GLUT1) belongs to the facilitative cell surface glucose transporter family, the members of which regulate glucose transport across the cell membrane [6]. It is well known that GLUT1 is overexpressed in several types of cancers [7, 8]. Accumulating evidence suggests that GLUT1 could promote cell proliferation and metastasis, thus playing a key role in various types of cancer, including
hepatocellular carcinoma, breast cancer, and kidney cancer [9–11]. Many studies indicate that SIRT1/GLUT1 axis promotes bladder cancer progression via regulation of glucose uptake [12]. In breast cancer, knocking down GLUT1 in ER-positive breast cancer cell lines rendered cells sensitive to tamoxifen therapy and even restored sensitivity to the drug in tamoxifen-resistant cells [13]. However, the mechanism underlying the regulation of GLUT1-mediated aerobic glycolysis in HCC metastasis remains unclear.

The ubiquitin-proteasome system (UPS) is an important regulator of cell signalling and proteostasis, both essential to various cellular processes [14]. As one of the most widespread and frequent cellular post-translational modifications, ubiquitination is also essential for normal cellular functions [15, 16]. Post-translational modification by E3 ubiquitin ligase could modulate the functions of target proteins, as well as their fate and intracellular mechanisms [17, 18]. Emerging evidence has identified E3 ubiquitin ligase as a key regulator of cancer [19]. Recently, RANBP2-type and C3HC4-type zinc finger-containing 1 (RBCK1), an E3 ubiquitin ligase by an N-terminal ubiquitin like (UBL) domain, an Npl4-type zinc finger (NZF) domain and a catalytic Cterminal RBR domain, which has important roles in cancer development [20]. Growing evidence indicates that RBCK1 is reported to be involved in immune regulation, antiviral signaling, iron and xenobiotic metabolism, as well as cancer[20, 21]. RBCK1 can directly interact with numerous proteins implicated in an extensive range of cellular processes, including the cell cycle, and transcription. For instance, Liu et al [22]. have demonstrated that RBCK1 modulated chemosensitivity in colorectal cancer (CRC), and could be served as a promising therapeutic target for CRC prevention. Yu et al [23]. have demonstrated that RBCK1 is overexpressed in human renal cell carcinoma, highlighting its potential as a therapeutic target for human cancers. RBCK1 also promotes the progression of lung adenocarcinoma by destabilising the tumour suppressor PKCζ [24]. Further, RBCK1 mRNA levels have been found to be elevated in HCC tissues upon referring to The Cancer Genome Atlas (TCGA) and GSE datasets. These studies suggest that RBCK1 may play an important role in HCC tumorigenesis and development. However, the precise role and underlying signalling cascade of RBCK1 in the progression of HCC remain unclear.

In this study, we first demonstrated that high RBCK1 expression levels were associated with poor prognosis in patients with HCC, and revealed the molecular mechanism of RBCK1 in the metabolism and progression of HCC. RBCK1 promoted HCC cell metastasis by enhancing the Warburg effect via GLUT1, and the mechanism underlying RBCK1-mediated regulation of WNT/β-catenin/GLUT1 pathway-induced HCC cell migration and aerobic glycolysis is dependent on destroyed PPARγ/PGC1α complex. Collectively, our data suggest that RBCK1 may act as a new candidate for therapeutic targets in HCC.

**Methods**

**Patients and specimens**

Cancerous and non-cancerous tissues were obtained from 216 patients with primary liver cancer at the Department of General Surgery, the Second Affiliated Hospital of Nanchang University. Pathologists
confirmed that all specimens were from normal tissues. This study was approved by the clinical research ethics committee of the Second Affiliated Hospital of Nanchang University, and all subjects agreed to provide samples for research purposes. Table 1 summarises the clinical characteristics of all patients.

Table 1
Relationship between RBCK1 expression and clinicopathological features

| Parameters                  | n  | RBCK1 expression | P value |
|-----------------------------|----|------------------|---------|
|                             |    | Low (n = 70)     | High (n = 146) |
| Age (years)                 |    |                  |         |
| ≤ 65                        | 90 | 31               | 59      |
| ≥ 65                        | 126| 39               | 87      |
| Sex                         |    |                  |         |
| Female                      | 113| 33               | 80      |
| Male                        | 103| 37               | 66      |
| Tumor size (cm)             |    |                  |         |
| ≤ 5                         | 129| 47               | 82      |
| ≥ 5                         | 87 | 23               | 64      |
| TNM                         |    |                  |         |
| T1-T2                       | 110| 47               | 63      |
| T3-T4                       | 106| 23               | 83      |
| Distant metastasis          |    |                  |         |
| No                          | 90 | 38               | 52      |
| YES                         | 126| 32               | 94      |
| Stage                       |    |                  |         |
| I-II                        | 96 | 41               | 55      |
| III-IV                      | 120| 29               | 91      |
| Differentiation             |    |                  |         |
| Well                        | 123| 45               | 78      |
| Moderate/Poor               | 93 | 25               | 68      |

Cell lines and culture
Four HCC cell lines (HCCLM3, Hep3B, SMMC7721, MHCC97h and SK-Hep-1) as well as normal human hepatocytes (HL-7702) were obtained from Rockville, USA. The cells were cultured in RPMI 1640 or DMEM (Gibco) supplemented with 10% FBS, 100 U mL\(^{-1}\) penicillin, and 100 mg mL\(^{-1}\) streptomycin at 37°C and 5% CO\(_2\).

**Western blot analysis**

Cell lysates were separated using 4–12% Bis-Tris gels (Life Technologies, USA) and transferred to a PVDF membrane. The cell membrane was sealed with 5% skimmed milk in TBST buffer, and incubated with the corresponding antibody. The antibodies used were anti-RBCK1 (Abcam), GLUT1 (Abcam), PPAR\(\gamma\) (Abcam), PGC1\(\alpha\) (Abcam), \(\beta\)-catenin (CST), anti-UB (Abcam), and anti-tubulin (Santa Cruz), all at a 1:1000 dilution.

**Immunohistochemical (IHC) staining**

HCC tissues were fixed, embedded, sectioned, and deparaffinised. A few of the dewaxed sections were subjected to haematoxylin and eosin (H&E) staining. IHC staining was performed according to the manufacturer's instructions using the Dako EnVision™ system (Agilent Technologies, USA). The cells were incubated with anti-RBCK1 and anti-GLUT1 (both at a 1:200 dilution) for 30 min.

**shRNA plasmids and constructs**

shRNA-mediated double stranded RNA (shRNA) silenced by RBCK1 (shRBCK1), \(\beta\)-catenin (sh\(\beta\)-catenin), or GLUT1 (shGLUT1), was synthesised by a gene pharmaceutical company in Shanghai, China. The full-length cDNA of human RBCK1, \(\beta\)-catenin, or GLUT1 was synthesised using gene pharmaceutical technologies and ligated to the pcDNA3.1 vector to generate p-RBCK1, p-\(\beta\)-catenin, or p-GLUT1, respectively. A blank vector was used as negative control. These shRNA plasmids and constructs were transfected with Lipofectamine 2000 transfection reagent (Invitrogen, USA) according to the manufacturer’s instructions.

**Cell migration and invasion assays**

Transwell assays were routinely used to assess the migration and invasion of HCC cell lines, with a few modifications\(^{27}\). For cell invasion assays, a layer of matrix gel was precoated on the upper part of the polycarbonate membrane.

**In vivo metastasis assay**

For *in vivo* metastasis assays, 1×10\(^6\) cells in 100 µL of phosphate-buffered saline were injected subcutaneously into the flanks of nude mice. Once the subcutaneous tumors reached 1 to 2 cm in diameter, they were removed and cut into pieces with a volume of approximately 1 mm\(^3\), and then the pieces were implanted in the livers of the nude mice (6 in each group, male BALB/c-\(\text{n}\)/\(\text{n}\), 6–8 weeks). The mice were sacrificed 6 weeks after tumor implantation. The livers and lungs were then processed and embedded in paraffin. The animal work was approved by the animal experimental ethics committee of
the Second Affiliated Hospital of Nanchang University, and was carried out according to the Guide for the Care and Use of Experimental Animals.

**Determination of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR)**

The Extracellular Flux Analyzer XF96 (Seahorse Bioscience, Billerica, MA, USA) was employed to measure cellular glycolysis capacity and cellular mitochondrial respiration, using the XF Cell Mito Stress Test Kit and Glycolysis Stress Test Kit (Seahorse Bioscience), respectively, according to the manufacturer’s instructions.

**Co-immunoprecipitation (Co-IP) and ubiquitination assays**

Immunoprecipitation analysis was performed as previously described. For in vivo ubiquitination experiments, the HA-Ubiquitin plasmid was stably transfected into RBCK1-knockout human hepatoma cells or control cells. Two days after transfection, cells were treated with 10 mmol L\(^{-1}\) MG132 for 10 h, to prevent proteasome degradation. The lysate was extracted using 2 mg of HA-tag or IgG antibody. The eluent was separated by SDS-PAGE and western blotting was performed with antibodies against RBCK1, PPAR\(\gamma\), and tubulin, respectively. For *in vitro* ubiquitination assays, the standard reaction mixture (25 µL) consisted of 20 mM HEPES NaOH (pH 7.5), 50 mM NaCl, 0.02 mg mL\(^{-1}\) BSA, 1 mM DTT, 5 mM MgCl\(_2\), 1 mM ATP, His-PPAR\(\gamma\) (1 pmol as tetramer), E1 (0.85 pmol), UbcH5c (1.25 pmol), FBXO9 (0.8 pmol), and Ub (174 pmol). The reaction mixture was prepared on ice and incubated at 30°C for 10 min, unless otherwise specified. SDS buffer was added to terminate the reaction and western blotting was performed using PPAR\(\gamma\) and tubulin antibodies, accordingly.

**Statistical analysis**

All results are shown as mean ± SD and at least three independent experiments were analysed using the Graphpad Prism 5 software (USA). We used the Kaplan-Meier method to generate survival curves, and the logrank test was used to measure statistical significance. The differences among the groups were analysed using the double tailed t-test and ANOVA. Data were considered statistically significant when p < 0.05.

**Results**

1. **High expression of RBCK1 is correlated with poor outcomes in patients with HCC**

TCGA (n = 424) cohort analyses revealed the upregulation of RBCK1 in HCC, and high expression of RBCK1 in HCC patients was positively correlated with stage and distant invasion (Fig. 1a, b and c). To further determine RBCK1 expression in HCC, qRT-PCR analyses were performed on HCC tissues and their corresponding adjacent tissues. Results showed that the average fold change of RBCK1 mRNA expression in HCC tissues compared with adjacent non-tumour-bearing tissues (Fig. 1d and e). Moreover,
analysis of the results obtained from western blotting (Fig. 1f and g) and IHC staining (Fig. 1h) revealed that the RBCK1 protein was upregulated in HCC tissues, compared with adjacent non-tumour-bearing tissues. These results indicated that RBCK1 expression is significantly upregulated in HCC tissues.

Evaluations of the correlations between RBCK1 overexpression and HCC clinicopathologic parameters revealed no significant association between RBCK1 expression and tumour size, age or histological type, but a significant correlation with TNM ($p < 0.002$), distant metastasis ($p = 0.013$) and clinical stage ($p = 0.006$) (Table 1). Additionally, the 216 HCC patients were divided into two groups based on the results of the immunohistochemically analysis: high RBCK1 expression group ($n = 146$) and low RBCK1 expression group ($n = 70$). Kaplan-Meier survival analysis showed that HCC patients with high RBCK1 expression levels exhibited poor overall survival (OS) ($p = 0.025$), poor disease-free survival (DFS) ($p = 0.0034$), compared to patients with low RBCK1 levels (Fig. 1i and j). This prognosis effect was also observed in the TCGA ($n = 425$) cohorts (Fig. S1). It should be noted that the results of multivariate Cox regression analysis indicate that RBCK1 overexpression was one of the independent predictive factors for detecting poor outcome in HCC patients (Table 2). Collectively, these data suggest that RBCK1 was upregulated in HCC tissues and associated with an unfavourable prognosis in HCC patients.
Table 2
Univariate and multivariate analyses of overall survival in HCC patients

| Parameters               | Univariate analysis |          |          |          | Multivariate analysis |          |          |
|--------------------------|---------------------|----------|----------|----------|-----------------------|----------|----------|
|                          | HR                  | 95% CI   | P value  | HR       | 95% CI                | P value  |
| Age (≥ 65 vs ≤65)        | 1.437               | 0.731–2.826 | 0.736     | –        | –                     | –        |
| Sex (Female vs Male)     | 1.851               | 0.584–2.927 | 0.724     | –        | –                     | –        |
| Tumor size (≤5 vs ≥6)    | 1.637               | 1.153–5.176 | 0.226     | –        | –                     | –        |
| Differentiation (Well vs Moderate/Poor) | 1.724               | 1.652–4.16 | 0.278     | –        | –                     | –        |
| TNM stage (T1-T2 vs T3-T4) | 2.607               | 1.415–5.542 | 0.012*    | 1.673   | 1.441–4.534           | 0.031*   |
| Distant metastasis (No vs Yes) | 1.381               | 0.716–3.765 | 0.031*    | 1.721   | 1.419–2.917           | 0.089    |
| Stage (I-II vs III-IV)   | 0.749               | 0.654–1.867 | 0.017*    | 1.432   | 0.983–3.837           | 0.037*   |
| RBCK1 expression (High vs Low) | 3.847               | 2.546–5.629 | 0.001*    | 2.231   | 1.736–4.841           | 0.019*   |

2. RBCK1 accelerates the metastasis of HCC cells in vitro and in vivo

To investigate the potential biological function of RBCK1 in HCC development, we first determined RBCK1 expression in HCC cell lines. qRT-PCR and western blot results showed that RBCK1 was significantly upregulated in HCC cells, compared to that in normal HL-7702 cell line (Fig. 2a and b). Based on the RBCK1 expression levels in HCC cell lines, we next established stable models of RBCK1 knockdown in HCCLM3 cell lines, as well as stable models of RBCK1 overexpression in Hep3B cell lines (Fig. 2c). Migration and invasion assays revealed that the mobility and invasiveness of HCC cells was markedly
inhibited by RBCK1 knockdown, but significantly promoted by RBCK1 overexpression, compared with control cells (Fig. 2d and e). Similarly, RTCA assay results also showed that RBCK1 knockdown notably suppressed the metastatic ability of HCCLM3 cells, and RBCK1 overexpression promoted the metastatic ability of Hep3B cells (Fig. S2). As EMT is significantly associated with the metastatic abilities of cancer cells, we examined the effects of RBCK1 expression on the EMT phenotype of HCC cells. As shown in Fig. 2f and g, immunofluorescence assays indicate that RBCK1 knockdown increased the epithelial marker, but decreased the mesenchymal marker in HCCLM3 cells. The stable RBCK1 knockdown can therefore inhibit HCC invasion and metastasis.

We further examined the effects of RBCK1 on HCC metastasis by establishing an orthotopic liver tumour model in nude mice. The experiment included shNC and shRBCK1 groups. Histological analysis showed the development of intrahepatic metastasis in five cases from the shNC group, compared to only one case in the HCCLM3-shRBCK1 group (Fig. 2h and j). In addition, H&E-stained serial lung sections revealed that the number of HCC lung micrometastases significantly decreased in the shRBCK1 group (Fig. 2i). In contrast, overexpression of RBCK1 increased the number of intrahepatic and lung metastatic nodules (Fig. 2k). Collectively, these results indicate that the stable knockdown of RBCK1 can inhibit HCC invasion and metastasis both in vitro and in vivo, while also acting as a tumour oncogene candidate during HCC progression and metastasis.

3. RBCK1 promotes HCC progression by enhancing the Warburg effect

E3 ubiquitin ligase contributes to reprogrammed metabolism in the progression of several types of cancer. As the Warburg effect is a well-characterised metabolic shift that ubiquitously occurs in tumour cells, including HCC, we explored the role of RBCK1 in HCC glucose metabolism. RBCK1 knockdown also dramatically decreased the cellular levels of glucose-6-phosphate (G6P), glucose consumption, lactate production, and ATP in HCCLM3 cells (Fig. 3a), while RBCK1 overexpression generated opposite trends in Hep3B cells (Fig. 3f). To further validate the impact of RBCK1 on glycolysis in HCC, ECAR, which reflects overall glycolytic flux, was measured. RBCK1 knockdown was shown to significantly decrease the glycolytic rate and capacity of HCCLM3 cells (Fig. 3b and c), whereas RBCK1 overexpression increased ECAR in Hep3B cells (Fig. 3g and h). Results obtained from the measurement of OCR, an indicator of mitochondrial respiration, revealed an increase in HCCLM3/shRBCK1 cells (Fig. 3d and e), whereas RBCK1 overexpression produced a decrease in Hep3B cells (Fig. 3i and j). Moreover, these responses were also exhibited in SMCC7721/shRBCK1 and SK-Hep-1/p-RBCK1 cells (Fig. S3).

To investigate whether the Warburg effect was responsible for the progression of HCC cells, HCCLM3/shRBCK1 and Hep3B/p-RBCK1 cells were treated with 2-DG at different concentrations (0, 4, or 8 mM) for 24 h. Results showed that, in HCCLM3/shRBCK1 and Hep3B/p-RBCK1 cells, 2-DG significantly inhibited glycolysis in a dose-dependent manner (Fig. 3k and l). The migratory and invasive ability of HCCLM3/shRBCK1 and Hep3B/p-RBCK1 cells was also decreased in a dose-dependent manner (Fig. 3m and n). In order to demonstrate that glycolysis modulates HCC migration and invasion, cells were cultured in medium containing galactose instead of glucose, thereby reducing the glycolytic flux and forcing the cells to rely on oxidative phosphorylation. We observed that this reduced glycolytic flux greatly abrogated
the increased migratory and invasive ability of Hep3B cells induced by RBCK1 overexpression (Fig. 3o). These findings indicate that RBCK1 suppresses oxidative phosphorylation while promoting aerobic glycolysis (Warburg effect) in HCC cells, but promotes migration and invasion by enhancing the Warburg effect in HCC cell lines in vitro.

4. RBCK1 promotes the Warburg effect via GLUT1 in HCC cells

Previous studies have demonstrated that GLUT1 plays an important role in glycolysis [6]. We explored whether RBCK1 regulated GLUT1 expression, by initially observing the expression of GLUT1 in RBCK1-knockdown or -overexpressing HCC cells. Western blotting results showed that RBCK1 knockdown significantly decreased GLUT1 expression in HCCLM3 cells (Fig. 4a). Conversely, RBCK1 overexpression markedly increased GLUT1 expression in Hep3B cells (Fig. 4b). Further, the upregulation of GLUT1 attenuated the loss of GLUT1 expression in HCCLM3/shRBCK1 cells (Fig. 4c). Meanwhile, the rescue experiments indicate that restoration of GLUT1 expression abolished the reduced metastasis ability of HCC cells induced by RBCK1 silence (Fig. 4d and e). Importantly, the in vivo tumour metastatic assay revealed that overexpression of GLUT1 rescued the decreased incidence of intrahepatic and lung metastasis of HCCLM3/shRBCK1 cells (Fig. 4f). Moreover, investigations into whether RBCK1 increased glycolysis via GLUT1 expression revealed that an upregulation of GLUT1 expression rescued the RBCK1-mediated reduction in cellular G6P, glucose consumption, lactate production, and ATP levels in HCC cells (Fig. 4g). Meanwhile, RBCK1 knockdown decreased ECAR and OCR in HCC cells, whereas a simultaneous overexpression of GLUT1 attenuated the decrease in glycolytic rate and capacity (Fig. 4h-k).

Next, we assessed the effect of decreased GLUT1 expression on RBCK1 and GLUT1 protein levels, as well as on cell migration and invasion abilities, in RBCK1-overexpressing Hep3B cells. Western blotting analyses showed that RBCK1 overexpression significantly increased GLUT1 expression, whereas the knockdown of GLUT1 expression dramatically inhibited the RBCK1-induced increase in GLUT1 expression in Hep3B cells (Fig. 4l). Meanwhile, GLUT1 downregulation significantly reduced RBCK1-enhanced cell migration and invasion (Fig. 4m and n). Furthermore, in vivo metastatic assay results showed that GLUT1 downregulation decreased the incidence of intrahepatic and lung metastasis in the Hep3B-RBCK1 group (Fig. 4o). Moreover, investigations into whether RBCK1 increased glycolysis via GLUT1 expression revealed that knockdown of GLUT1 expression rescued the RBCK1-mediated increase in cellular G6P, glucose consumption, lactate production, and ATP levels in HCC cells (Fig. 4p). Meanwhile, RBCK1 overexpression increased ECAR and OCR in HCC cells, whereas a simultaneous knockdown of GLUT1 attenuated the increase in glycolytic rate and capacity (Fig. 4q-t). Collectively, these findings indicated that GLUT1 is a functional downstream target of RBCK1 in the regulation of aerobic glycolysis, and that it is critical in RBCK1-mediated tumour progression.

5. RBCK1-induced activation of GLUT1 is mediated by WNT/β-catenin signalling

To further clarify the mechanism by which RBCK1 regulates GLUT1 in HCC, the effect of RBCK1 on the global gene expression patterns of HCCLM3 cells was assessed at the transcriptome level via RNA sequencing (RNA-seq) (Fig. 5a). Gene set enrichment analysis (GSEA) was performed to determine the
effects of transcriptomic changes on biological functions and pathways. The WNT signalling pathway was significantly positively correlated with RBCK1 in HCCLM3 cells (Fig. 5a). As GLUT1 is a target gene of WNT/β-catenin, we speculated that RBCK1 regulated GLUT1 via the WNT/β-catenin signalling pathway. To test this hypothesis, we measured changes in β-catenin expression in RBCK1-knockdown HCCLM3 cells. Western blotting analyses showed that total and nuclear β-catenin protein expression decreased with decreasing RBCK1 expression in HCCLM3 cells (Fig. 5b). The TOP-Flash reporter luciferase assay showed that RBCK1 knockdown decreased the transcriptional activity of TCF4 in MHCC97H cells, compared with the shNC control (Fig. 5c). In contrast, RBCK1 overexpression generated opposite effects in Hep3B cells (Fig. 5d and e). We further determined that β-catenin upregulation rescued the decrease in GLUT1 expression, cell migration, and cell invasion induced by RBCK1 knockdown (Fig. 5f-i).

To verify that RBCK1 regulates GLUT1 expression through the WNT/β-catenin signalling pathway, we measured the levels of GLUT1 and β-catenin in the presence of the WNT/β-catenin pathway inhibitors XAV-939. Consistently, XAV-939 inhibited the mRNA and protein levels of GLUT1 in HCCLM3 cells (Fig. 5j and k). Transwell assay showed that XAV-939 markedly decreased RBCK1-induced cell migration and invasion (Fig. 5l). XAV-939 leads to decrease in cellular G6P, glucose consumption, lactate production, and ATP levels in HCC cells (Fig. 5m). Meanwhile, XAV-939 inhibited ECAR in HCC cells (Fig. 5n). Meanwhile, the rescue experiments indicate that XAV-939 attenuated the enhanced metastasis ability and Warburg effect of HCC cells induced by RBCK1 overexpression (Fig. 5o-t). Consistently, β-catenin silence abolished the increased metastasis ability and Warburg effect of HCC cells induced by RBCK1 overexpression (Fig. S4). Taken together, RBCK1 promotes the metastasis of HCC cells via GLUT1-mediated Warburg effect through activation of WNT/β-catenin signalling.

6. RBCK1 destroyed the PPARγ/PGC1α complex to activate the WNT/β-catenin pathway and Warburg effect in HCC cells

To clarify the mechanism through which RBCK1 regulates the WNT/β-catenin signalling pathway in HCC cells, we first determined whether there was a direct interaction between the RBCK1 and β-catenin proteins. Co-IP showed that no direct interaction existed between these proteins (Fig. 6a). The PPARγ/PGC1α complex induces the inhibition of the canonical WNT/β-catenin pathway and contributes to glucose homeostasis. Therefore, we speculated that RBCK1 regulated WNT/β-catenin via the destroyed PPARγ/PGC1α complex. To test this hypothesis, we first determined whether RBCK1 influenced the activation of the β-catenin/GLUT1 pathway via the destroyed PPARγ/PGC1α complex. Changes in β-catenin, GLUT1, PGC1α, and PPARγ expression, as well as in the PPARγ/PGC1α complex, were measured in RBCK1-knockdown HCC cells. Results showed that RBCK1 knockdown in HCCLM3 cells significantly increased the levels of PPARγ expression and PPARγ/PGC1α complex, decreased β-catenin and GLUT1 expression, but produced no change in PGC1α protein levels (Fig. 6b and c). By contrast, RBCK1 overexpression in Hep3B cells significantly decreased the levels of PPARγ protein expression and PPARγ/PGC1α complex, increased β-catenin and GLUT1 expression, while also producing no change in PGC1α protein levels (Fig. 6d and e). In addition, the PPARγ mRNA level in HCC cells remained unaffected.
by changes in RBCK1 expression (Fig. 6f). The PPARγ/PGC1α complex is therefore involved in RBCK1-mediated regulation of the β-catenin/GLUT1 pathway.

To verify that RBCK1 regulates the β-catenin/GLUT1 pathway through the destroyed PPARγ/PGC1α complex, PPARγ was knocked down in RBCK1-downregulated HCC cells. PPARγ downregulation inhibited the decrease in β-catenin and GLUT1 expression, cell migration, and aerobic glycolysis observed in RBCK1-knockdown HCCLM3 cells (Fig. 6g-i). By contrast, PPARγ upregulation enhanced the increase in β-catenin and GLUT1 expression, cell migration, and aerobic glycolysis observed in RBCK1-upregulated HepG2 cells (Fig. 6j-m). To determine whether the RBCK1-induced anti-Warburg effect was dependent on the PPARγ/PGC1α complex, we treated HCC cells with the PPARγ inhibitor GW9662. As expected, GW9662 reversed the decreased ECAR levels induced by RBCK1 knockdown (Fig. 6n). Importantly, IHC staining (Fig. 6o) revealed that compared with adjacent non-tumour-bearing tissues, the RBCK1, Ki67, β-catenin, GLUT1 and Vimentin protein were upregulated, whereas PPARγ and PGC1α were downregulated in HCC tissues. These results indicated that the RBCK1-mediated regulation of β-catenin/GLUT1 pathway-induced HCC cell migration and aerobic glycolysis is therefore dependent on the destroyed PPARγ/PGC1α complex.

7. RBCK1 destroyed the PPARγ/PGC1α complex by promoting the ubiquitination and degradation of PPARγ

RBCK1 has the ability to interact with different substrates in order to exert its effects. We observed whether RBCK1, PPARγ, and PGC1α directly interacted in HCC cells. Co-IP analysis detected endogenous RBCK1 and PPARγ in the immunoprecipitate, indicating an interaction between RBCK1 and PPARγ, but no direct interaction between RBCK1 and PGC1α (Fig. 7a and b). Moreover, GST-pull down assay indicate that RBCK1 could bind to PPARγ in vitro system (Fig. 7c and d). These findings confirmed that RBCK1 directly bind with PPARγ in HCC cells, and that RBCK1 destroyed the PPARγ/PGC1α complex by regulating PPARγ expression.

We then assessed the mechanisms by which RBCK1 regulates PGC1α. Consistent with findings from a previous study, which showed PPARγ degradation via the UPS, treatment with the proteasome inhibitor MG132 led to significant accumulation of endogenous PPARγ protein in HCC cells (Fig. 7e and f). Moreover, the data indicate that efficient ubiquitination of His-PPARγ was detected in the presence of E1, E2 (UBCH5c), FBXO9 (an E3 ubiquitin ligase for PPARγ), and Ub (Fig. 7g). PPARγ is therefore also degraded by the UPS in HCC cells. Next, we determined whether RBCK1 could directly mediate PPARγ ubiquitination. Interestingly, PPARγ poly-ubiquitination was substantially increased by ectopic RBCK1 expression, but decreased by RBCK1 knockdown (Fig. 7h and i). The data also showed that mutations in all the Lys sites of PPARγ abolished its poly-ubiquitination (Fig. 7j). As expected, mutation of the Lys48 site on ubiquitin (Ub) almost completely abolished RBCK1-mediated PPARγ ubiquitination, whereas the K63R mutation produced no effect (Fig. 7k). Consistent with the ubiquitination results, a degradation dynamics assay showed that the half-life of exogenously expressed PPARγ was significantly increased in RBCK1-overexpressing HCC cells, compared with that in control cells (Fig. 7l and m). These data showed
that RBCK1 mediates Lys48-linked poly-ubiquitination of PPARγ, which leads to PPARγ degradation in the proteasome. Collectively, these results indicate that RBCK1 destroyed the PPARγ/PGC1α complex by promoting PPARγ ubiquitination and degradation (Fig. 8).

**Discussion**

HCC is one of the most common malignant tumours in the world, and the second leading cause of cancer-related deaths [25]. Despite major improvements in diagnosis and treatment methods, metastasis remains the main cause of treatment failure and death [26]. Although modern multidisciplinary nursing strategies have been applied to treat tumour metastasis, the overall five-year survival rate remains at 25–39%, with the recurrence rate of patients with advanced liver cancer being approximately 80% [27]. Metastasis of a malignant tumour is often the main reason for treatment failure in patients with liver cancer [28]. Moreover, the molecular mechanism of HCC metastasis is still unclear. In the present study, we determined that high RBCK1 expression predicts poor HCC prognosis, and that RBCK1 accelerates the metastasis of HCC through enhanced GLUT1-mediated Warburg effect, by destroying the PPARγ/PGC1α complex.

As a key regulator of unfolded protein response in the development of cancer, RBCK1 was initially identified during the screening of progesterone regulatory genes in human breast cancer cells [29]. RBCK1 overexpression can promote the progression of lung adenocarcinoma, CRC and Renal cell carcinoma [22, 23, 30]. Several studies have highlighted the involvement of RBCK1 in all aspects of cancer biology, and reported that many of its molecular functions are consistent with its role in cancer [20]. However, there is no information on the specific role or molecular mechanism of RBCK1 in HCC. Here, we found that RBCK1 expression was upregulated and associated with poor prognosis in patients with HCC. Additionally, RBCK1 expression was correlated with tumour size and vascular invasion. The ectopic expression of RBCK1 markedly promoted HCC migration and invasion both *in vitro* and *in vivo*. Moreover, our results revealed that RBCK1 could promote aerobic glycolysis and inhibit mitochondrial respiration in HCC cells. These findings are important not only to better understand the biological functions of RBCK1 in cancer, but also for assessing the potential of RBCK1 as a therapeutic target.

Aerobic glycolysis, also known as the Warburg effect, is the most characteristic metabolic phenotype of cancer cells, including HCC. The Warburg effect is due to the oncogene activation and overexpression of glucose transporters or enzymes from the glycolysis pathway [31]. The destruction of tumour cell metabolism can inhibit metastasis, indicating that aerobic glycolysis is at the core of tumour growth and survival [5]. Therefore, it is very important to obtain an in-depth understanding of the relationship between cell metabolism and tumour metastasis, in order to develop new treatment methods against liver cancer. We describe here a novel mechanism wherein RBCK1 promotes HCC invasion and metastasis by upregulating a GLUT1-mediated Warburg effect. First, our data indicated that high RBCK1 and GLUT1 levels were observed in HCC tissues, compared with adjacent non-tumour-bearing tissues. We also found that RBCK1 inhibition reduced GLUT1 expression and decreased aerobic glycolysis and metastasis in HCC *in vitro* and *in vivo*. Moreover, GLUT1 upregulation rescued the decreased aerobic glycolysis and
lung metastasis induced by RBCK1 knockdown, whereas GLUT1 inhibition significantly decreased RBCK1-enhanced aerobic glycolysis and metastasis. These findings suggested that GLUT1 is a functional downstream target of RBCK1 in the regulation of aerobic glycolysis, and is critical for RBCK1-mediated tumour metastasis.

As a downstream target of WNT/β-catenin signalling, GLUT1 plays important roles in glycolysis and chemotherapeutic resistance in HCC[4, 32]. Here, we determined that decreases in GLUT1 expression, cell migration, and invasion induced by RBCK1 knockdown were rescued by β-catenin upregulation. The WNT/β-catenin pathway inhibitors XAV-939 or ICG-001 notably decreased RBCK1-induced cell migration and invasion. Furthermore, RBCK1 knockdown in HCC cells significantly increased PPARγ expression and the PPARγ/PGC1α complex, but decreased β-catenin and GLUT1 expression, while producing no change in PGC1α protein levels. The downregulation of PPARγ inhibited the decreases in β-catenin and GLUT1 expression, cell migration, and aerobic glycolysis observed in RBCK1-knockdown HCC cells. Our findings demonstrated for the first time that RBCK1-mediated regulation of WNT/β-catenin/GLUT1 pathway-induced HCC cell migration and aerobic glycolysis is dependent on the destroyed PPARγ/PGC1α complex.

Finally, we investigated the mechanism by which RBCK1 destroyed the PPARγ/PGC1α complex. Post-translational modification by E3 ubiquitin ligase could modulate the functions of target proteins, their fate, as well as their intracellular mechanisms [19]. RBCK1 interacts with different substrates in order to exert its effects. Studies have reported that the ubiquitin–proteasome-mediated degradation of PPARγ is the critical mechanism by which PPARγ levels are regulated in cells [33]. Similarly, our results suggest, for the first time, that RBCK1 destroyed the PPARγ/PGC1α complex by promoting the ubiquitination and degradation of PPARγ. This conclusion is based on the following observations. Firstly, RBCK1 directly binds with PPARγ in HCC cells, as indicated by Co-IP analyses. Secondly, efficient ubiquitination of His-PPARγ occurred in the presence of E1, E2 (UBCH5c), FBXO9 (an E3 ubiquitin ligase for PPARγ), and Ub. Next, PPARγ poly-ubiquitination was increased by RBCK1 overexpression, but decreased by RBCK1 knockdown. Mutations in all the Lys sites of PPARγ abolished PPARγ poly-ubiquitination. Finally, mutation of the Lys48 site of ubiquitin completely abolished RBCK1-mediated PPARγ ubiquitination, whereas the K63R mutation on Ub produced no effect.

**Conclusion**

In summary, our study generated the initial evidence associating high levels of RBCK1 expression with poor prognosis in patients with HCC, and revealed the molecular mechanism of RBCK1 during the metabolism and progression of HCC. We also showed that RBCK1 promoted HCC cell metastasis by enhancing the Warburg effect via GLUT1. More importantly, RBCK1-mediated regulation of WNT/β-catenin/GLUT1 pathway-induced HCC cell migration and aerobic glycolysis is dependent on destroyed PPARγ/PGC1α complex. Based on these findings, RBCK1 has the potential to serve as a candidate biomarker for the future diagnosis and treatment of HCC.

**Abbreviations**
HCC, hepatocellular carcinoma; UPS, ubiquitin-proteasome system; CRC, colorectal cancer; TCGA, The Cancer Genome Atlas; H&E, haematoxylin and eosin; IHC, Immunohistochemical; shRNA, shRNA-mediated double stranded RNA; OCR, oxygen consumption rate; ECAR, extracellular acidification rate; Co-IP, Co-immunoprecipitation; OS, overall survival; DFS, disease-free survival; G6P, glucose-6-phosphate; RNA-seq, RNA sequencing; GSEA, Gene set enrichment analysis; Ub, ubiquitin; 2-DG, 2-deoxyglucose; AA, antimycin A; CHX, cycloheximide; GLUT1, Glucose transporter 1; RBCK1, RANBP2-type and C3HC4-type zinc finger-containing 1.

Declarations

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Ethics approval and consent to participate

Inclusion of human participants, and use of human data and human tissue in this study were approved by the Ethics Committee of the Second Affiliated Hospital of Nanchang University. The use of animals in this study was approved by the animal research committee in the Second Affiliated Hospital of Nanchang University.

Consent for publication

The author and participants are agree for publication.

Availability of data and material

All data generated or analyzed during this study are included in this published article. Additional datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

All authors read and approved the final version of the manuscript, and the authors declare that they have no competing interests.

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Authors’ contributions


ZX, JS and WMZ performed majority of the experiments and wrote the paper. XXL, YYD and SHZ participated in analysis and interpretation of data. DBX and XY helped in collecting samples. XGP and LFC conceived the study concept and designed the experiments. All authors read and approved the final manuscript.

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Figures
Figure 1

RBCK1 expression was over-expressed in metastatic HCC and closely correlated with poor prognosis of patients. A and B, RBCK1 mRNA expression profiles in TCGA liver cancer dataset. C, Association with RBCK1 expression and clinicopathologic characteristics in HCC patients in TCGA cohort. D-G, Expression levels of RBCK1 mRNA and protein in clinical HCC tissues and their adjacent noncarcinoma normal tissues were investigated by qRT-PCR (D and E) and Western blot assay (F and G), respectively. **P<0.01. H, Immunohistochemistry was employed to identify the expression of RBCK1 protein in HCC tissues and
RBCK1 promoted the migration and invasion of HCC cells in vitro and accelerated the metastasis of HCC cells in vivo. A and B, protein and mRNA levels of RBCK1 in HCC cell lines and normal human hepatocytes (HL-7702). C, Western blot and qRT-PCR analyses were used to detect the expression level of RBCK1 in HCCLM3 and Hep3B cells stably transfected with the RBCK1-silenced or -overexpressing
plasmid. *P<0.05, **P<0.01. D, Transwell migration and Transwell invasion assays of HCCLM3 cells transfected with RBCK1 knockdown vector. *P<0.05, **P<0.01. E, Transwell migration and Transwell invasion assays of Hep3B cells transfected with RBCK1 overexpression plasmid. F and G, Confocal microscopy analysis of the E-cadherin and Vimentin in RBCK1 knockdown or overexpression HCC cells. The red and green signal represents the staining of the corresponding protein, and the blue signal represents the nuclear DNA staining by DAPI. H and J, Fluorescence of metastases generated in metastasis model of orthotopic liver transplantation with stably transfected HCC cells or control cell was monitored, n = 8. I and K, H&E staining of the paraffin-embedded sections of intrahepatic and lung metastatic nodules. n=8/group.
Figure 3

RBCK1 promotes the migration and invasion of HCC cells by modulating the Warburg effect. A, Cellular G6P levels, glucose consumption, lactate production, and ATP levels in HCCLM3/shRBCK1 cells. Three independent experiments were performed. *P<0.05, **P<0.01. B and C, ECAR data showing the glycolytic rate and capacity in RBCK1-silenced HCC cells. Glucose (10 mM), the oxidative phosphorylation inhibitor oligomycin (1.0 μM), and the glycolytic inhibitor 2-deoxyglucose (2-DG, 50 mM) were sequentially injected.
into each well at the indicated time points. All measurements were normalized to the cell number calculated using crystal violet assay at the end of the experiment. *P<0.05. D and E, OCR results showing the basal respiration and maximum respiration in RBCK1-silenced HCC cells. Oligomycin (1.0 μM), the mitochondrial uncoupler carbonyl cyanide p-trifluoromethoxy phenylhydrazone (FCCP, 1.0 μM), and the mitochondrial complex I inhibitor rotenone plus the mitochondrial complex III inhibitor antimycin A (Rote/AA, 0.5 μM) were sequentially injected. All measurements were normalized to the cell number calculated using crystal violet assay at the end of the experiment. *P<0.05, **P<0.01. F, Cellular G6P levels, glucose consumption, lactate production, and ATP levels in Hep3B/p-RBCK1 cells. G and H, ECAR data showing the glycolytic rate and capacity in RBCK1-overexpressing HCC cells. *P<0.05, **P<0.01. I and J, OCR results showing the basal respiration and maximum respiration in RBCK1-overexpressing HCC cells. *P<0.05, **P<0.01. K and L, Lactate production in HCCLM3/shRBCK1 (K) or Hep3B/p-RBCK1 cells (L) in presence of 2-DG. *P<0.05, **P<0.01. M and N, Effects of 2-DG on the migration and invasion of HCCLM3/shRBCK1 (M) or Hep3B/p-RBCK1 cells (N). *P<0.05, **P<0.01. O, Culturing cells in medium containing galactose but no glucose annulled the effect of RBCK1 overexpression on cell migration and invasion in Hep3B cells.
Figure 4

The effects of RBCK1 in HCC cells partially depended on modulating GLUT1 expression. A, western blotting and qRT-PCR analyses of GLUT1 expression levels in HCCLM3/shRBCK1 cells. *P<0.05, **P<0.01. B, western blotting and qRT-PCR analyses of GLUT1 expression levels in Hep3B/p-RBCK1 cells. *P<0.05, **P<0.01. C, Western blotting was used to detect the expression of RBCK1 and GLUT1 in the different groups. Tubulin was a loading control. D and E, the quantification of Transwell assays in the
different groups. *P<0.05, **P< 0.01. F, Representative pictures (right) and quantification (left) of intrahepatic and lung metastases in the different groups of nude mice. n=8. G, Cellular G6P levels, glucose consumption, lactate production, and ATP levels in the indicate group. H-K. ECAR (H and J) and OCR (I and K) were measured in the indicate group. *P<0.05. L, Western blotting was used to detect the expression of RBCK1 and GLUT1 in the indicate group. Tubulin was a loading control. M and N, the quantification of Transwell assays in the indicate group. *P<0.05. O, Representative pictures (right) and quantification (left) of intrahepatic and lung metastases in the different groups of nude mice. n=8. P, Cellular G6P levels, glucose consumption, lactate production, and ATP levels in the indicate group. Q-T. ECAR (Q and S) and OCR (R and T) were measured in the indicate group. *P<0.05.
Figure 5

RBCK1 promotes the GLUT1-mediated Warburg effect via WNT/β-catenin signaling pathway. A, Heatmap illustrates genes with at least 2-fold change in transcript levels between HCCLM3 cells with RBCK1 knockdown and control (left); Gene set enrichment analysis (GSEA) indicate a significant change of WNT/β-catenin, oxidative phosphorylation and PPAR signaling (right) induced by RBCK1. B and D, the total and nuclear protein levels of β-catenin were assessed by Western blotting in RBCK1-silencing...
HCCLM3 cells (B) or RBCK1-overexpression Hep3B cells (D). Tubulin and Histone 3 were used as a loading control, respectively. C and E, the relative luciferase activity levels in cells transfected with TOP-flash and FOP-flash vectors in in RBCK1-silencing HCCLM3 cells (C) or RBCK1-overexpression Hep3B cells (E) are shown. *P<0.05, **P< 0.01. F and G, western blotting and qRT-PCR analyses of RBCK1, β-catenin and GLUT1 expression levels in the indicate group. Tubulin was a loading control. *P<0.05, **P<0.01. H, the quantification of Transwell assays in the indicate group. *P<0.05, **P< 0.01. F, Quantification of intrahepatic and lung metastases in the different groups of nude mice. n=8. J and K, Western blotting and qRT-PCR showing the expression level of RBCK1, β-catenin and GLUT1 in HCCLM3 cells transfected with shRBCK1 or XAV-939. Tubulin was a loading control. *P<0.05. L, the quantification of Transwell assays in HCCLM3 cells transfected with shRBCK1 or XAV-939. *P<0.05, **P< 0.01. M, Cellular G6P levels, glucose consumption, lactate production, and ATP levels in HCCLM3 cells transfected with shRBCK1 or XAV-939. *P<0.05, **P< 0.01. N, ECAR was measured in HCCLM3 cells transfected with shRBCK1 or XAV-939. O and P, Western blotting and qRT-PCR showing the expression level of RBCK1, β-catenin and GLUT1 in RBCK1-overexpression Hep3B cells transfected with XAV-939. Tubulin was a loading control. *P<0.05. Q and R, the quantification of Transwell assays in RBCK1-overexpression Hep3B cells transfected with XAV-939. *P<0.05, **P< 0.01. S, Cellular G6P levels, glucose consumption, lactate production, and ATP levels in RBCK1-overexpression Hep3B cells transfected with XAV-939. *P<0.05, **P< 0.01. T, ECAR was measured in RBCK1-overexpression Hep3B cells transfected with XAV-939.
Figure 6

RBCK1 activated the WNT/β-catenin pathway and Warburg effect in HCC cells by destroying the PPARγ/PGC1α complex. A, The Co-IP assay showed no interaction between RBCK1 and β-catenin. B, Western blotting showing the expression level of RBCK1, PPARγ, PGC1α, β-catenin and GLUT1 in RBCK1-silencing HCCLM3 cells. Tubulin was a loading control. C, co-IP combined Western blotting assay showing the expression level of PPARγ and PGC1α in RBCK1-silencing HCCLM3 cells. Tubulin was a
loading control. D, Western blotting showing the expression level of RBCK1, PPARγ, PGC1α, β-catenin and GLUT1 in RBCK1-overexpression Hep3B cells. Tubulin was a loading control. E, co-IP combined Western blotting assay showing the expression level of PPARγ and PGC1α in RBCK1-overexpression Hep3B cells. Tubulin was a loading control. F, qRT-PCR showing the expression level of RBCK1 and PPARγ in RBCK1-silencing HCCLM3 cells. G, Western blotting showing the expression level of RBCK1, PPARγ, β-catenin and GLUT1 in RBCK1-silencing HCCLM3 cells transfected with shPPARγ. Tubulin was a loading control. H, the quantification of Transwell assays in RBCK1-silencing HCCLM3 cells transfected with shPPARγ. *P<0.05.

I, Cellular G6P levels, glucose consumption, lactate production, and ATP levels in RBCK1-silencing HCCLM3 cells transfected with shPPARγ. *P<0.05, **P<0.01. J, Western blotting showing the expression level of RBCK1, PPARγ, β-catenin and GLUT1 in RBCK1-overexpression Hep3B cells transfected with p-PPARγ. Tubulin was a loading control. K and L, the quantification of Transwell assays in RBCK1-overexpression Hep3B cells transfected with p-PPARγ. *P<0.05. M, Cellular G6P levels, glucose consumption, lactate production, and ATP levels in RBCK1-silencing HCCLM3 cells transfected with shPPARγ. *P<0.05, **P<0.01. N, ECAR was measured in RBCK1-silencing HCCLM3 cells transfected with shPPARγ. O, Immunohistochemistry was employed to identify the expression of RBCK1, Ki67, PPARγ, PGC1α, β-catenin, GLUT1 and Vimentin protein in HCC tissues and their adjacent noncarcinoma normal tissues. Scale bar, 50 μm.
RBCK1 interacts and destabilizes PPARγ by promoting PPARγ ubiquitination and degradation in HCC cells. A, Co-IP showing direct binding of endogenous RBCK1 and PPARγ in HCCLM3 cells. B, Co-IP showing that endogenous RBCK1 and PPARγ were not directly bound. C and D, GST-pull down assay showing RBCK1 and PPARγ were directly bound in HEK293 cells. E and F, HCC cells were treated with MG132 (Z-Leu-Leu-Leu-CHO, 15 μmol/L) for the indicated times, and levels of PPARγ were determined. G, the ubiquitination level of HA-PPARγ were detected in the presence of E1, E2 (UBCH5c), FBXO9 and Ub in HEK293 cells. H and I, Knockdown or overexpression of RBCK1 altered the ubiquitination of PPARγ. The cells in each group were treated with proteasomal inhibitor MG132. Cell lysates were prepared and subjected to immunoprecipitation with anti-PPARγ antibody. The level of ubiquitin-attached PPARγ was detected by western blotting with anti-Ub antibody. J, Ubiquitination of wild-type PPARγ or the K-to-R mutant (mutations in all Lys site of PPARγ gene) in HEK293 cells. K, Measurement of PPARγ.
ubiquitination type in HEK293 cells. L and M, HCC cells were transfected with plasmid encoding HA-PPARγ either with or without the Flag-RBCK1 plasmid. Then, the cells were subjected to cycloheximide (CHX) (20 μmol/L) exposure at the indicated times, and the degradation of PPARγ was detected with anti-HA antibody.

**Figure 8**

Proposed model by which E3 ubiquitin ligase RBCK1 promotes GLUT1-mediated Warburg effect by destroying the PPARγ/PGC1α complex.

**Supplementary Files**

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