RBCK1 promotes the stabilization of HBx by linear ubiquitination to drive the progression of HBV-associated hepatocellular carcinoma

yongyin li[1], Peng Chen[1], Zheyu Dong[1], Yuxin Zhou[1], Qiuyue Ye[1], Junling Chen[1], Jianzhong Cai[1], Yiyan Huang[1], Jiayue Yang[1], Yaoting Feng[1], Liangxing Chen[1], and Libo Tang[1]

[1]Southern Medical University

January 18, 2023

Abstract

Background & Aims: Linear ubiquitin chain assembly complex (LUBAC) has been reported to participate in cancer progression, but its role in hepatocellular carcinoma (HCC) remains unknown. This study aimed to investigate the functions and potential tumorigenic mechanisms of LUBAC components in HBV-associated HCC. **Methods:** The expression of LUBAC components (*RBCK1*, *RNF31*, and *Sharpin*) and Met1-linked ubiquitination (M1-Ubi) and their correlation with prognosis were detected. The biological functions of RBCK1 in HBV-associated HCC were investigated *in vitro* and *in vivo*. The regulation of RBCK1 on the HBx protein expression was analyzed by cycloheximide chase assays, coimmunoprecipitation, and ubiquitin assays. **Results:** We found that the expression of LUBAC components and M1-Ubi was significantly upregulated in HCC and correlated with poor prognosis. Interestingly, subgroup analysis revealed that *RBCK1*, not *RNF31* or *Sharpin*, was exclusively overexpressed in HBV-associated HCC compared to non-HBV-associated HCC. Upregulated *RBCK1* expression was associated with larger tumor size, higher AFP level, and poor prognosis in HBV-associated HCC cohort. Functionally, RBCK1-knockdown suppressed cell growth and migration, and also inhibited the progression of xenografted tumors in HBV-associated HCC mouse model. Mechanistically, RBCK1 interacted with HBx to promote its stabilization by increasing M1-Ubi ubiquitination and reducing K48-linked ubiquitination. Furthermore, clinical analysis confirmed a positive correlation between RBCK1 and HBx, and the co-expression of which predicted poor prognosis for HCC patients. **Conclusion:** RBCK1 is an oncogenic gene to promote tumor progression and may serve as a potential target for HBV-associated HCC.

Title page

Title

RBCK1 promotes the stabilization of HBx by linear ubiquitination to drive the progression of HBV-associated hepatocellular carcinoma

Running title

RBCK1 drives HBV-associated HCC progression

Authors:

Peng Chen[1, *], Zheyu Dong[1, *], Yuxin Zhou[1, *], Qiuyue Ye[1, *], Junling Chen[1], Jianzhong Cai[1, 2], Yiyan Huang[1, 2], Jiayue Yang[1, 2], Yaoting Feng[1, 2], Liangxing Chen[1, 2], Libo Tang[1, +], Yongyin Li[1, +]

*Authors share co-first authorship.

+Authors share co-corresponding authors.

Affiliations

[1] State Key Laboratory of Organ Failure Research, Guangdong Provincial Key Laboratory of Viral Hepatitis Research, Department of Infectious Diseases, Nanfang Hospital, Southern Medical University, Guangzhou, China;

[2] The First School of Clinical Medicine, Southern Medical University, Guangzhou, China.

Correspondence

Yongyin Li, Department of Infectious Diseases, Nanfang Hospital, Southern Medical University, No. 1838 North Guangzhou Avenue, Guangzhou 510515, China; Phone: +86-20-8278783; Fax: +86-20-82787830; E-mail: yongyinli@foxmail.com;

Libo Tang, Department of Infectious Diseases, Nanfang Hospital, Southern Medical University, No. 1838 North Guangzhou Avenue, Guangzhou 510515, China; Phone: +86-20-62787428; Fax: +86-20-62787428; E-mail: tanglibosmu@foxmail.com;

Abstract

Background & Aims: Linear ubiquitin chain assembly complex (LUBAC) has been reported to participate in cancer progression, but its role in hepatocellular carcinoma (HCC) remains unknown. This study aimed to investigate the functions and potential tumorigenic mechanisms of LUBAC components in HBV-associated HCC.

Methods: The expression of LUBAC components (*RBCK1* ,*RNF31* , and *Sharpin*) and Met1-linked ubiquitination (M1-Ubi) and their correlation with prognosis were detected. The biological functions of RBCK1 in HBV-associated HCC were investigated*in vitro* and *in vivo* . The regulation of RBCK1 on the HBx protein expression was analyzed by cycloheximide chase assays, coimmunoprecipitation, and ubiquitin assays.

Results: We found that the expression of LUBAC components and M1-Ubi was significantly upregulated in HCC and correlated with poor prognosis. Interestingly, subgroup analysis revealed that *RBCK1* , not *RNF31* or *Sharpin* , was exclusively overexpressed in HBV-associated HCC compared to non-HBV-associated HCC. Upregulated*RBCK1* expression was associated with larger tumor size, higher AFP level, and poor prognosis in HBV-associated HCC cohort. Functionally, RBCK1-knockdown suppressed cell growth and migration, and also inhibited the progression of xenografted tumors in HBV-associated HCC mouse model. Mechanistically, RBCK1 interacted with HBx to promote its stabilization by increasing M1-Ubi ubiquitination and reducing K48-linked ubiquitination. Furthermore, clinical analysis confirmed a positive correlation between RBCK1 and HBx, and the co-expression of which predicted poor prognosis for HCC patients.

Conclusion: RBCK1 is an oncogenic gene to promote tumor progression and may serve as a potential target for HBV-associated HCC.

Keyword

RBCK1; Linear ubiquitin chain assembly complex, Hepatitis B virus X protein linear ubiquitination, Hepatitis B virus-associated hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) represents the seventh-most frequently occurring cancer and the second-most common cause of cancer mortality worldwide [1]. Although the effect of the preventive vaccine for newborns and antiviral treatments, hepatitis B virus (HBV) remains the most important global risk factor of HCC. Previous studies have proved that the hepatitis B X protein (HBx) is essential for viral replication; meanwhile, it also involves in regulating gene transcription, intracellular signal transduction, genotoxic stress response, protein degradation, cell cycle control, apoptosis, and behaving as a prominent oncogenic driver for HBV-associated HCC (HBV-HCC) [4, 5]. Of note, it also promotes the metastasis of HCC by regulating

molecules associated with the migration and invasion of tumor cells [6, 7]. Several studies on HBx transgenic mice have investigated the hepatocarcinogenic effects of HBx and found that only those with high HBx levels developed HCC [8, 9]. HBx hijacking the ubiquitin-proteasome system (UPS) was considered a central theme around virus-induced oncogenesis [10]. The versatile UPS components, in turn, regulated the stability of HBx via direct or indirect mechanisms [11]. HBx is an unstable protein that is ubiquitinated and rapidly degraded by the proteasome pathway to maintain a very low intracellular level [12, 13]. Clinical reports have presented HBx as a possible diagnostic marker by demonstrating its expression in 40% of sera and 85% of liver tissue samples from patients with HCC [14]. However, the underlying driven factors and mechanisms that maintain that stability of HBx for HBx-induced carcinogensis are poorly understood.

Ubiquitination is an essential post-translational modification that controls most cellular processes, including cell cycle progression, DNA damage response, gene transcription, receptor transport, and protein stability [15]. Distinct from the biochemically inter-ubiquitin linkages through the seven internal lysine residues of ubiquitin, the linear ubiquitin chain (also called Met1-linked polyubiquitination, M1-Ubi) was recently found to assemble via peptide-bond formation between the α-amino group (α-NH2) of the N-terminal methionine (Met1) of one ubiquitin and the carboxyl group of the C-terminal glycine of another ubiquitin [16]. Linear ubiquitin chain assembly complex (LUBAC), the sole E3 ubiquitin ligase known to assemble linear ubiquitin chains specifically, is composed of RBCK1 (haemoxidized IRP2 ubiquitin ligase 1L; also known as RBCK1), RNF31 (HOIL1-interacting protein; also known as RNF31), and Sharpin (SHANK-associated RH domain-interacting protein) [17]. Among these three subunits of LUBAC, RNF31 has a catalytic center in its RING2 domain responsible for assembling linear ubiquitin chains. In contrast, RBCK1 and SHARPIN have been recognized as accessory proteins for the process [16, 18]. RBCK1 and SHARPIN harbor Ub-like (UBL) domains that interact with the Ub-binding domains (UBDs) in RNF31 to form a stable complex, thereby exhibiting ubiquitin ligase activity to involve in biological functions including immune signaling, development in mice, protein quality control, Wnt signaling, and autophagy [19]. Increasing evidence shows that M1-Ubi is linked to cancer progression; however, the underlying mechanism for regulating HBV-associated HCC remains largely unknown.

In this study, we found that the expression of LUBAC and M1-Ub was upregulated in HCC and associated with poor prognosis. Interestingly, only RBCK1 was overexpressed and correlated with tumor malignancy and poor prognosis of HBV-associated HCC patients. Knocking down RBCK1 in HepG2.2.15 cell lines, which carried with an integrated 2.15-fold version of the HBV genome and stably produced HBV, significantly impaired cell growth and migration *in vitro* and inhibited tumor progression in an HBV-associated HBV mouse model. Mechanistically, RBCK1 interacted with HBx and promoted its stabilization by increased M1-Ubi and decreased K48-linked ubiquitination modification. Thus, our study suggests that RBCK1 mediating M1-Ubi of HBx is critical in the process of HBV-associated HCC development, which may be a novel target for HCC therapeutics.

Material and methods

HCC cell lines

Human HCC cell lines HepG2.2.15 and HepaG2 were obtained from Shanghai Institute for Biological Sciences (Shanghai, China). The cells were routinely cultured in the Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (ExCell, South Logan, Utah, USA) at 37 °C in a humidified atmosphere of 5% CO_2. The cell lines have been identified to be correct.

HCC tissue specimens

The current study was conducted on 147 paraffin-embedded, archived HCC specimens, which were histopathologically and clinically diagnosed at the Nanfang Hospital from 2015 to 2016 (Table S1). HCC tissues and paired adjacent non-tumor tissues were frozen in liquid nitrogen until further use. Adjacent non-tumor tissues were obtained from a standard distance (3 cm) from resected neoplastic tissues of patients with HCC who underwent surgical resection and confirmed by pathological evaluation. Prior patient consent and approval from the Institutional Research Ethics Committee of Nanfang Hospital were obtained for these

clinical materials for research purposes. It also conformed to the provisions of the Declaration of Helsinki.

Animals

C57BL/6 and BALB/c nude mice were purchased from the Laboratory Animal Center of Southern Medical University. Mice were housed in a temperature-controlled animal facility (20-22 °C) under 12:12h light/dark cycle. The mice had free access to water and food. All animal work was approved by the Ethical Committee for Experimental Animal Care of Southern Medical University (Guangzhou, China). The studies reported in this paper are in accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

Cell viability assay

Cell viability was evaluated using a cell counting kit-8 (CCK-8) (B34302, Bimake, Houston, TX 77014 USA) according to the manufacturer's instructions. Briefly, the indicated cells (4×10^3 per well) were inoculated in 96-well plates for 24, 48, 72, and 96 h, respectively. CCK-8 solution (10 μl) was added to each well, and the plates were incubated for 2 h. The absorbance at 450 nm was then analyzed and calculated.

Transwell migration assay

The transwell migration assay was performed using 8.0 μm pore polycarbonate membrane inserts (Corning, cat#3422) according to the manufacturer's instructions. Briefly, cells were resuspended by FBS-free starvation medium and then planted in the inserts, and growth medium was added outside the chamber in the wells of the plate. After 12 hours of incubation, the cells that go through the compartment were stained using Hoechst 33342 (Thermo Scientific, Waltham, MA). Then the stained cells were counted as the mean number of cells per field of view.

Cell cycle analysis

The cell cycle of the indicated cells was determined using the cell cycle detection Kit (BD) according to the manufacturer's instructions by a FACS Calibur machine using CellQuest software (BD Biosciences). The data were analyzed using FlowJo software.

RNA extraction and quantitative real-time polymerase chain reaction (qRT–PCR)

qRT–PCR was performed as described previously. The primer sequences used for RBCK1:Forward: 5-TGCTCAGATGCACACCGTC-3; Reverse: 5-CAAGACTGGTGGGAAGCCATA-3. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) Forward: 5-TGCACCACCAACTGCTTAGC-3; Reverse:5-GGCATGGACTGTGGTCATGAG-3. GAPDH was used as an internal control.

Western blot analysis

Western blot analysis was performed as described previously [20]. An anti-GAPDH antibody was used for the normalization of protein expression. Bands were detected using an ECL Detection Kit (Millipore, Germany) and visualized using the enhanced chemiluminescent imaging system (Sage Creation, Beijing, China) according to the manufacturer's instructions.

Immunoprecipitation

Briefly, cells were collected and lysed for 30 minutes on ice and centrifuged at 12000 rpm for 15 mins. Soluble lysates were incubated with the indicated antibodies overnight at 4, followed by incubation with Protein A/G magnetic beads (Bimake, Houston, TX 77014 USA) at 4 for another 2 hours. The immunocomplex was washed 5 times with cold immunoprecipitation wash buffer (150 mM NaCl, 10 mM HEPES pH 7.4, 0.1% NP-40) and boiled in 1× sodium dodecyl sulfate sample buffer for 5 minutes. The coprecipitate was resolved using SDS-PAGE and immunoblotted with specific antibodies. The protein bands were visualized using the enhanced chemiluminescent imaging system (Sage Creation, Beijing, China) according to the manufacturer's instructions.

Immunohistochemistry

Human HCC surgical specimens were fixed in 10% neutral formalin-fixed, dehydrated, and embedded in paraffin. Tissues were cut into 4 μm-thick sections and incubated in citrate buffer (pH 9.0) for 5 mins at 120; then, the endogenous peroxidase was blocked by 0.3% H_2O_2 for 15 mins at room temperature. The slides were incubated with 10% normal goat serum in PBS for 30 mins at 37 to block the non-specific binding sites, followed by incubation with appropriate primary antibodies overnight at 4, and then used the streptavidin–peroxidase kit (ZSGB-Bio, China) according to the manufacturer's instructions. Target protein expression levels were independently evaluated by two pathologists.

Gene set enrichment analysis (GSEA) and public data deposition

For GSEA, we obtained the GSEA software (version 4.03) from the website (http://software.broadinstitute.org/gsea/index.jsp). The data was downloaded from the TCGA LIHC cohort and GSE14520 cohort. The samples in the TCGA cohort were divided into two groups according to the expression of RBCK1. Then we obtained the GMT files (c2.all.v7.2.symbols.gmt [Curated]) from the molecular signatures database (http://www.gsea-msigdb.org/gsea/downloads.jsp). Based on gene expression profile and phenotype grouping, the minimum gene set was 10. The maximum gene set was 500, 1000 times resampling, P adj. value (adjust method: Benjamini-Hochberg) <0.05 and an FDR <0.25 were considered statistically significant.

Statistical analysis

Data were expressed as the mean ± standard deviation (SD). The Mann-Whitney U test was used when two groups were compared. The Kruskal-Wallis H test was used to compare more than two groups. Correlations between variables were assessed with Spearman s rank order correlation coefficient. Asterisk coding as indicated in the figure legends as *p < 0.05; **p < 0.01; ***p < 0.001. The patients were divided into two groups according to whether they exhibited a high (> the median) or low (< the median) RBCK1 expression level. Survival curves were analyzed by the Kaplan–Meier method, and a log-rank test was used to assess significance. Statistical parameters, including the number of biological replicates and repeat experiments, were reported in the figure legends. All calculations were performed using GraphPad prism 7.0 or the SPSS software package.

Results

The expression of LUBAC components and M1-Ubi is elevated and predicts poor prognosis in HCC.

To explore the role of linear ubiquitin chain assembly complex (LUBAC) and linear ubiquitin chain (also called Met1-linked polyubiquitination M1-Ubi), which is catalyzed explicitly by LUBAC in HCC, we first analyzed the expression of LUBAC components (*RBCK1* , *RNF31,Sharpin*) in the distinct stages of HCC development. Interestingly, we found that the expression of LUBAC components gradually increased in the development of HCC (Fig. 1A), indicating the oncogenic role of LUBAC. An analysis of the data from the TCGA LIHC cohort also confirmed the upregulated expression of LUBAC components in the tumor tissues compared with that in the normal control (Fig. 1B). In addition, IHC staining analysis from our HCC cohort (Fig. 1C) and proteinatlas dataset (Fig. 1D) revealed a higher expression of LUBAC components in tumor tissues compared with that in normal tissues. Next, to detect whether LUBAC-mediated M1-Ubi was upregulated in HCC with the elevation of LUBAC components expression, we performed immunofluorescent staining and found that the immunofluorescent density of M1-Ubi was significantly higher than that in the adjacent normal tissues (Fig. 1E). Kaplan-Meier (KM) analysis suggested that the upregulated expression of LUBAC components was associated with poor prognosis in HCC patients (Fig. 1F). Notably, while co-expression of *RBCK1, RNF31, Sharpin*was defined as a gene set to reflect the M1-Ubi level, the KM analysis revealed that high expression of the gene set led to a reduced overall survival (OS) time compared with that of the low expression group in the TCGA LIHC cohort (Fig. 1F). Collectively, the above results indicate the oncogenic role of LUBAC and M1-Ubi in HCC.

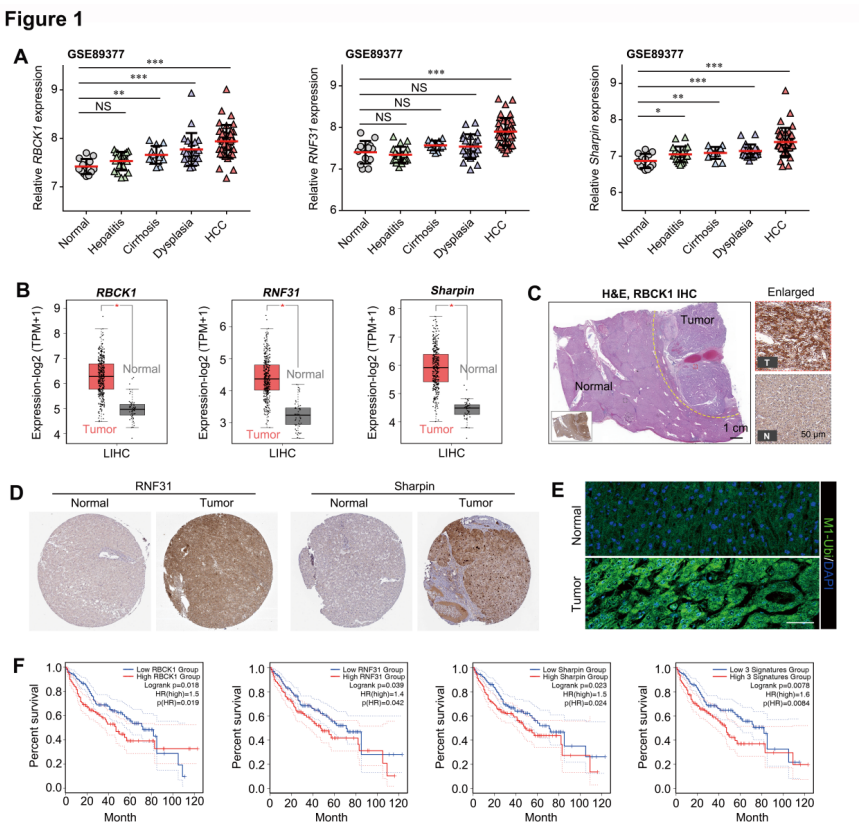


Figure 1 Linear ubiquitin chain assembly complex and M1-Ubi are upregulated in HCC and predict poor prognosis. (A) The expression of*RBCK1* , *RNF31* , and *Sharpin* mRNA levels in the distinct stages of HCC development in GSE89377 cohort. (B) The expression of *RBCK1* , RNF31, and *Sharpin* mRNA levels in the cohort. (C-D) IHC staining of RBCK1, RNF31, and Sharpin in our (C) or proteinatlas cohort (D). (E) Immunofluorescence staining of M1-Ubi. (F) Kaplan-Meier analysis for OS of two groups of HCC patients defined by low and high expression of *RBCK1* , *Sharpin, RNF31,* or co-expression of the above three genes in the TCGA LIHC cohort. Data were shown as Mean ± SD, **p* <0.05, ***p*<0.01, ****p* <0.001; Mann-Whitney U test. HCC, hepatocellular carcinoma; IHC, Immunohistochemistry; IF, immunofluorescence; LIHC, liver hepatocellular carcinoma; M1-Ubi, Met1-linked polyubiquitination; OS, overall survival; TCGA, the The Cancer Genome Atlas.

The LUBAC component RBCK1, not RNF31 or Sharpin, is upregulated in HBV-associated HCC and associated with poor prognosis.

As chronic HBV infection is one of the most important global risk factors of HCC, we tended to investigate the potential role of LUBAC in HBV-associated HCC. We first explored the expression of LUBAC components in HBV-associated HCC public datasets. Interestingly, we found that the expression of LUBAC components was significantly upregulated in HCC tissues compared to that in normal tissues (Fig. 2A, B). However, subgroup analysis revealed that only *RBCK1* but not*RNF31* /*Sharpin* showed an elevated level in HBV-associated HCC tissues compared with that in non-HBV-associated HCC tissues (Fig. 2A, B). Next, we explored the relationship between the expression of LUBAC components and tumor malignancy and patients' prognosis. As indicated in Figure 2C &D, we found that patients with higher*RBCK1* expression showed larger tumor size and elevated AFP compared to the control, which was not seen regarding *Sharpin* and*RNF31* expression. As for the survival analysis, we found that patients with high *RBCK1* expression but not*RNF31* /*Sharpin* expression predicted poor prognosis (Fig. 2E).

As the above results indicated the specific regulation of HBV on *RBCK1* expression, we verified the functional characteristics of RBCK1 in our HCC cohort and mouse models. We found that RBCK1 expression was significantly elevated in the tumor tissues when compared to that in non-tumor tissues (Fig. 3A). Meanwhile, the upregulated expression of RBCK1 was also observed in DEN/CCl_4-induced spontaneous HCC mouse model (Fig. 3B). In the HBV-associated HCC cohort, we found RBCK1 was upregulated in HBV-associated HCC compared with that in non-HBV-associated HCC (Fig. 3C). In the HBV mouse model by hydrodynamic injection with pAAV-HBV1.2 plasmid, we found that the expression of RBCK1 was elevated in the liver, the natural target organ of HBV infection, which further confirmed the regulation of HBV on RBCK1 expression (Fig. 3D). Similar to the public datasets, upregulated RBCK1 expression correlated with large tumor size and high AFP level (Fig. 3E), and predicted poor prognosis (Fig. 3F) in our total HCC and HBV-associated HCC cohort. Thus, these results indicate that HBV infection exerts profound effects on LUBAC via specifically regulating the expression of RBCK1.

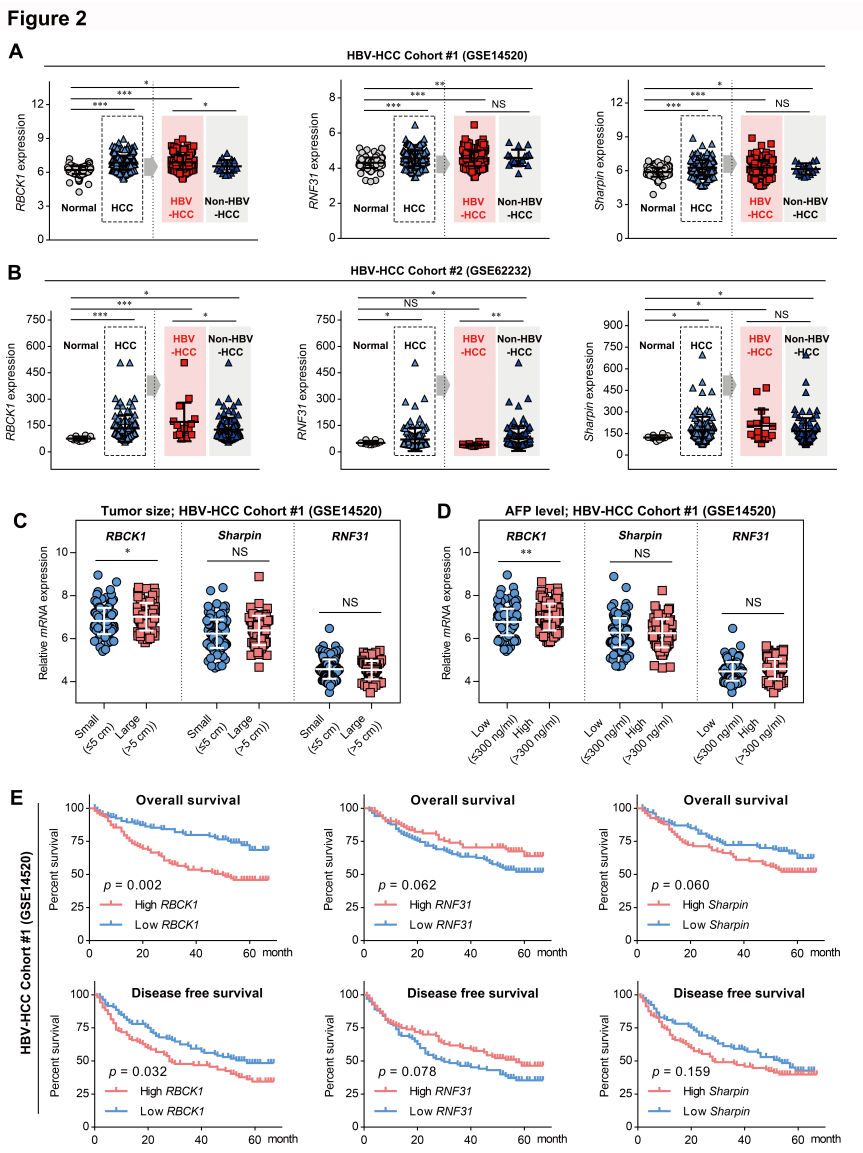


Figure 2 Upregulated expression of RBCK1 but not Shrapin/RNF31 is related to poor progno-

sis in HBV-associated HCC patients. (A-B) The expression of *RBCK1 /Sharpin /RNF31* in GSE14520 (A; Normal, n=217; HCC, n=228; non-HBV-HCC, n=17; HBV-HCC, n=211) and GSE62232 (B; Normal, n=10; HCC, n=110; non-HBC-HCC, n=94; HBV-HCC, n=16) cohort. (C-D) The association between RBCK1 expression and tumor size (C), and AFP level (D) in GSE14520 cohort. (E) Kaplan-Meier analysis for OS and DFS of two groups of HCC patients defined by low and high expression of RBCK1 in GSE14520 cohort. Data were shown as Mean ± SD, *p <0.05, **p <0.01. (A, B, C, D) Mann-Whitney U test, and (E) log-rank Mantel-Cox test. DFS, disease-free survival.

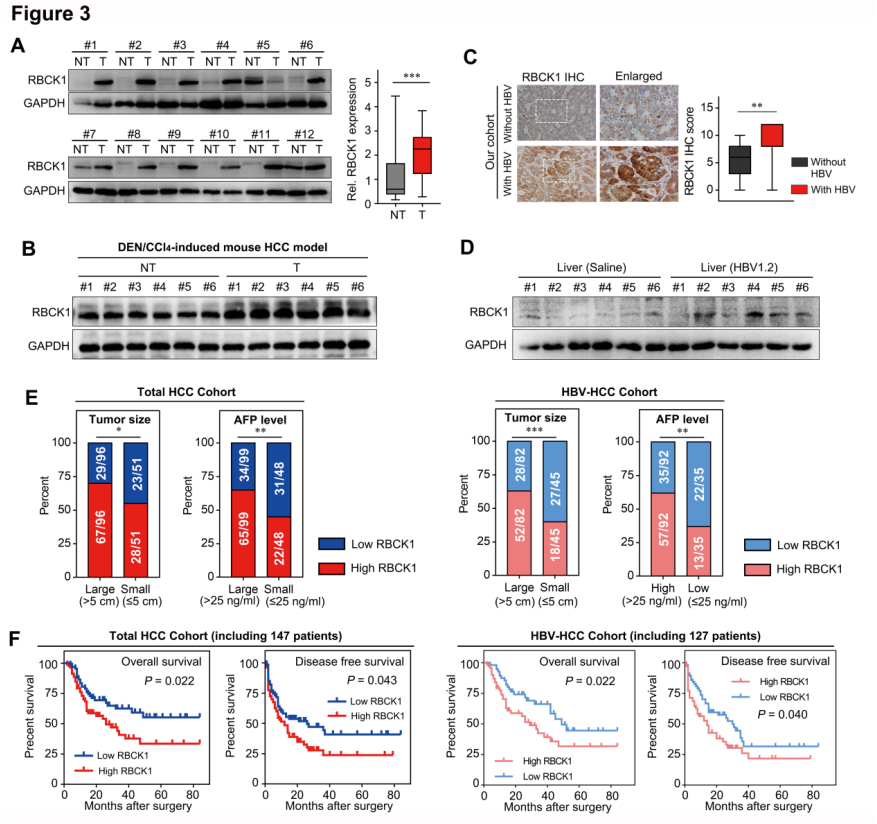


Figure 3 RBCK1 is upregulated by HBV infection and predicts poor prognosis in HCC. (A-B) RBCK1 expression in HCC patients' tissues (A) and DEN/CCl_4-induced mouse HCC tissues (B). (C) IHC staining of RBCK1 in HBV-associated HCC and non-HBV tissues. (D) Expression of RBCK1 in liver tissues from HBV mouse model by hydrodynamic injection with pAAV-HBV1.2 plasmid. (E) The association between RBCK1 expression and tumor size, and AFP level in total-HCC and HBV-associated HCC cohort. (F) Kaplan-Meier analysis of OS and DFS in HCC patients with distinct RBCK1 expression in our total-HCC and HBV-associated HCC cohort. Data were shown as Mean ± SD, *p<0.05, **p <0.01, ***p<0.001; (A, B) Mann-Whitney U test, and (C) log-rank Mantel-Cox test.

Targeting RBCK1 significantly inhibits the proliferation and migration of HBV-associated HCC *in vitro* and *in vivo*.

To elucidate the functional role of RBCK1 in HBV-associated HCC, we stably knocked down RBCK1 expression in HepG2.2.15 cells with lentivirus (Fig. 4A), which is carried with HBV. CCK8, EdU incorporation, and colony-forming assays showed that RBCK1 knockdown prominently inhibited the HepG2.2.15 cell proliferation ability (Fig. 4B-D). In addition, knocking down the expression of RBCK1 downregulated the percentage of cells in G1-phase, whereas it upregulated cells in S-phase (Fig. 4E). The above results indi-

cated that RBCK1-knockdown inhibited cell proliferation by promoting the S-phase arrest in the cell cycle. As previously reported, HBV infection confers HCC cells with high metastatic potential [6, 21]. Thus, we further investigated whether RBCK1 played a key role in this process. We found that RBCK1-knockdown significantly reduced the expression of mesenchymal markers, including N-cadherin, Vimentin, and Snail. The transwell assay and wound healing assay also showed that RBCK1-knockdown inhibited cell migration *in vitro* (Fig. 4F). Taken together, these results indicated that RBCK1 possesses oncogenic activities in HBV-associated HCC.

To verify the oncogenic role of RBCK1 in HBV-associated HCC *in vivo* , we established a subcutaneous nude mouse model with RBCK1-silenced HepG2.2.15 cells. We found that silencing of RBCK1 significantly alleviated tumor growth and resulted in an apparent decrease in tumor weights (Fig. 4G). Additionally, IHC staining demonstrated a reduction in the expression of proliferating cell marker Ki-67 and mesenchymal marker N-cadherin (Fig. 4H), which further confirmed the tumor-promoting effect of RBCK1.

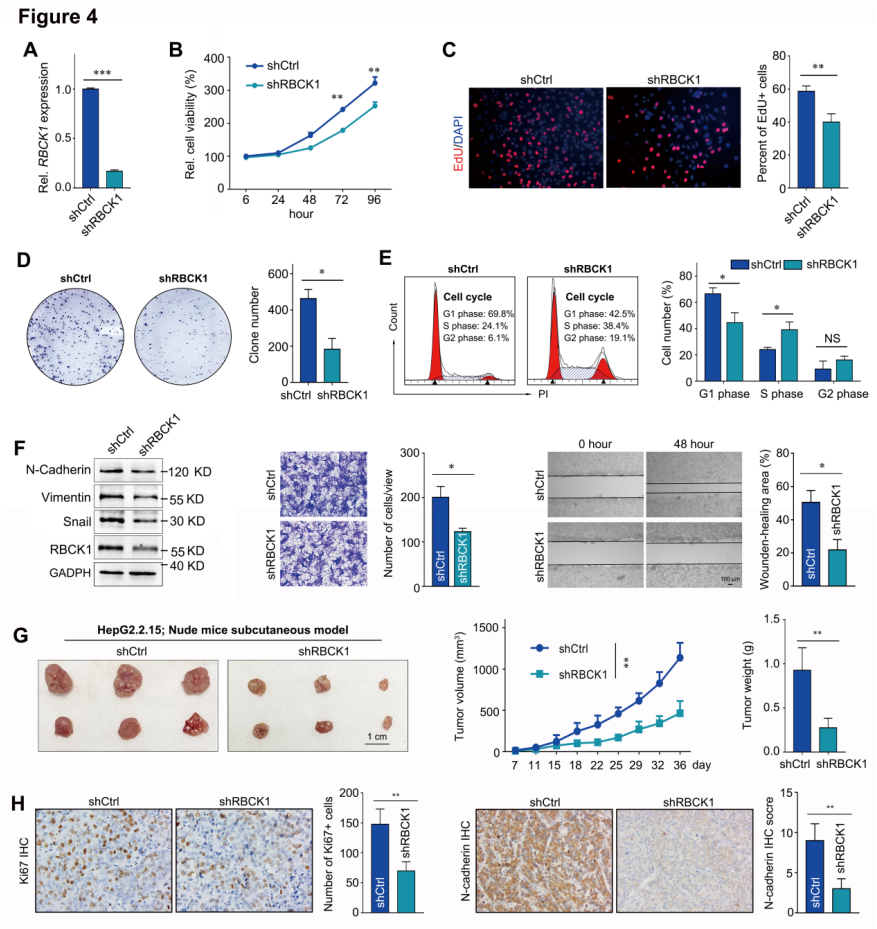


Figure 4 Knocking down the expression of RBCK1 inhibits the progression of HBV-associated HCC *in vitro* and *in vivo*.(A) QRT-PCR analysis of *RBCK1* mRNA expression in HepG2.2.15 cells infected with the RBCK1-knockdown lentivirus or the control lentivirus. (B-D) The effect of RBCK1 knockdown on cell proliferation was detected by CCK8 (B), EdU incorporation (C), and colony formation assays (D). (E) The distribution of different cell cycle phases in the RBCK1-knockdown HepG2.2.15 cells. (F) Western blot of mesenchymal markers expression (left), migration capacities indicated by transwell (middle), and wound healing (right) assays in HepG2.2.15 cells infected with the RBCK1-knockdown lentivirus or

the control lentivirus. (G) Subcutaneous xenograft tumors of the indicated cells, n=6 (left); tumor volumes were measured and recorded twice a week, and a growth curve was plotted (middle); the wet weight of the indicated xenograft tumors (right). (H) Representative images of IHC staining of Ki67 (left) and N-cadherin (right) in the xenograft tumors. Scale bars, 100μm. Data were shown as Mean ± SD, *p <0.05, **p <0.01, ***p <0.001. (A-D, F) Mann-Whitney U test.

RBCK1 interacts with HBx and promotes its stabilization via linear ubiquitination.

Recent evidence has proved that HBx is paramount in regulating multiple cellular processes in HBV-associated HCC, including cell proliferation and migration [6, 21, 22]. To explore whether RBCK1 participated in regulating the activation of HBx-related signal pathways, we performed Gene set enrichment analysis (GSEA) using the differential expression genes between high RBCK1 expression and low RBCK1 expression group. We found that HBx-related gene sets (WU_HBx_Targets_2_UP and WU_HBx_Targets_3_UP) were strongly enriched in high RBCK1 expression group in both TCGA LIHC and GEO cohorts (Fig. 5A). Meanwhile, knocking down RBCK1 in HepG2.2.15 cells significantly reduced the expression of HBx (Fig. 5B). As reported in the previous study, HBx protein could be rapidly degraded by the proteasome pathway, and thereby we speculated whether RBCK1, the component of LUBAC, regulated M1-Ubi of HBx and modulated its stability. By means of cycloheximide chase assays, we found that RBCK1-knockdown increased the degradation of HBx, whereas co-transfected HBx-Flag and RBCK1-His plasmids in HepG2 cells increased the stability of HBx-Flag (Fig. 5C, D). Furthermore, using ubiquitination assays, we noticed that RBCK1-knockdown increased K48-linked ubiquitination of HBx, which has been reported to mediate the proteasomal degradation of HBx [12, 23], but decreased the M1-Ubi (Fig. 5E), which may mediate the stability of HBx. Interestingly, we observed an interaction (Fig. 5F) and a co-localization (Fig. 5G) of RBCK1 with HBx. In addition, we found that RBCK1 interacted with HBx but not with the other HBV-related protein (HBc, LHBs, MHBs, SHBs) (Fig. 5H). Thus, these results suggest that RBCK1 specifically regulated the stability of the HBx protein by linear ubiquitination.

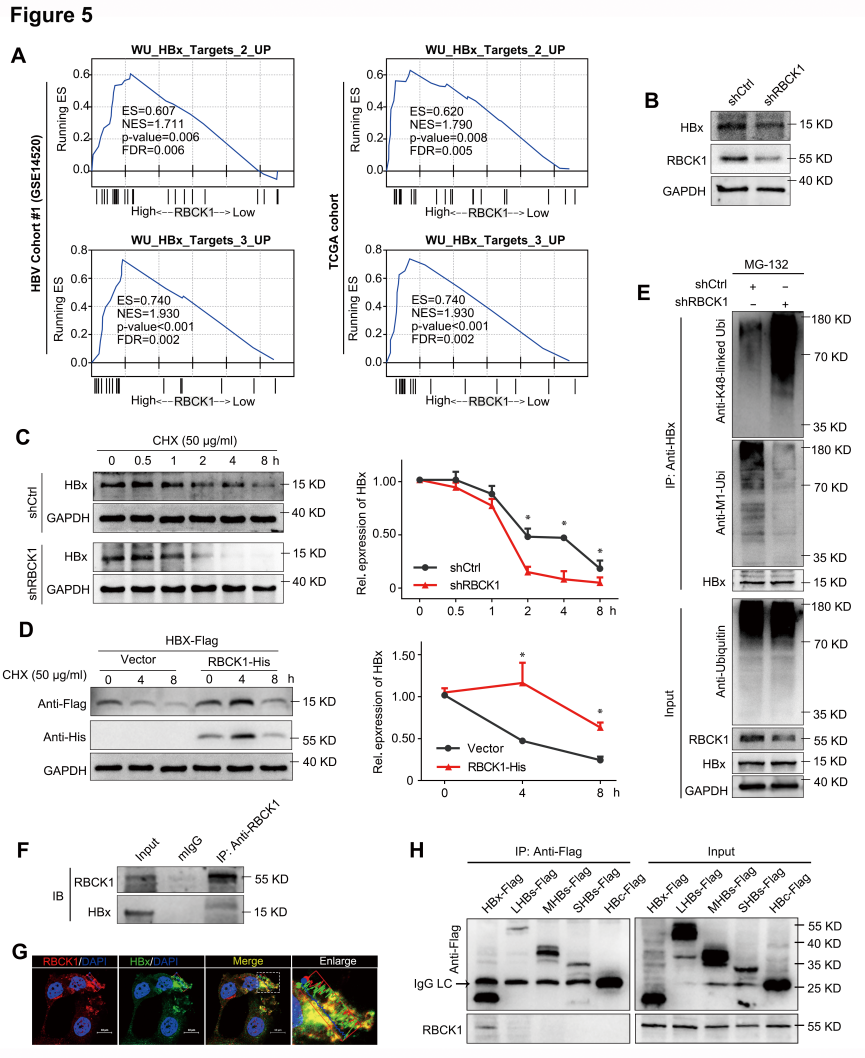


Figure 5 RBCK1 inhibits the degradation of HBx via linear ubiquitination. (A) GSEA for RBCK1 expression in HBx-related gene signatures (WU_HBx_Target_2_UP, WU_HBx_Target_3_UP) in the GEO and TCGA LIHC cohort. (B) Western blot analysis of indicated proteins in the RBCK1-knockdown and control cells. (C) Western blot of HBx in HepG2.2.15 cells incubated with cycloheximide at different time points. (D) Western blot of indicated tag protein detected in HepG2 cells with exogenous HBx-plasmid transfection and incubated with cycloheximide at different time points. (E) Co-IP for the K48-linked and Met1-linked ubiquitination of HBx in the RBCK1-knockdown and control cells after 8 hours 10 μM MG132 treatment. (F) Co-IP and blotting of anti-RBCK1 or anti-HBx in HepG2.2.15 cells. (G) IF staining of RBCK1 and HBx in HepG2.2.15 cells. (H) Co-IP and blotting of anti-RBCK1 or anti-Flag in HepG2 cells transduced with HBx-Flag, LHBs-Flag, MHBs-Flag, SHBs-Flag, HBc-Flag plasmids, respectively. Data were shown as Mean ± SD, $^{**}p$ <0.01. (C, D) Mann-Whitney U test. Co-IP, Co-immunoprecipitation assays; GSEA, Gene set enrichment analysis.

Co-expression of RBCK1 and HBx predicts poor prognosis in HCC patients.

We further explored the correlation of RBCK1 and HBx in the clinical samples. As expected, a positive correlation between the RBCK1 and HBx levels was obtained in our HBV-associated HCC cohorts (Fig. 6A), which verified the regulation of RBCK1 on HBx to promote the progression of HBV-associated HCC.

Meanwhile, we also observed a positive correlation between the *RBCK1* and *X* (a gene that encodes HBx protein) mRNA expression in the TCGA cohort (Fig. 6B). In addition, the Kaplan-Meier analysis suggested that the OS and DFS of HCC patients with high *X* expression were significantly worse than that of patients with low *X* expression (Fig. 6C). Moreover, high RBCK1 and X co-expression was associated with poor prognosis in HCC patients compared to those with low genes expression (Fig. 6D). Collectively, these results indicate that RBCK1 which mediates the stabilization of HBx is a potential target for HBV-associated HCC patients.

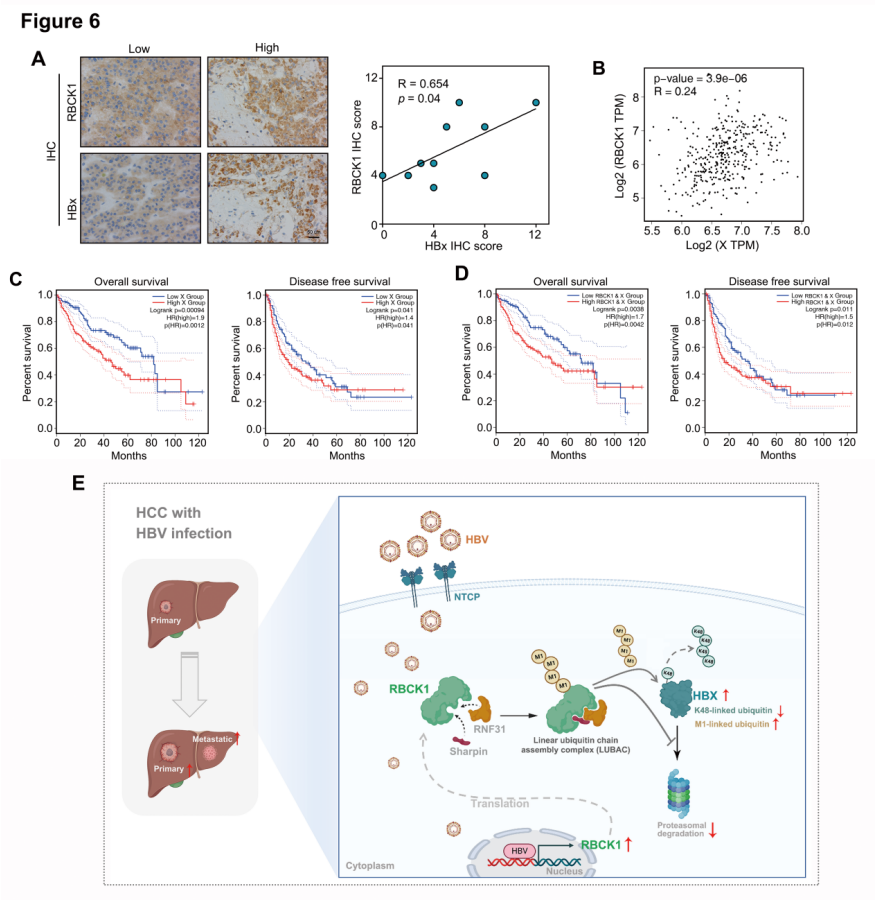


Figure 6 Co-expression of RBCK1 and HBx predicts poor prognosis for HCC patients. (A) Representative images of IHC staining of RBCK1 and HBx in HCC tissues, and the correlation between RBCK1 and HBx levels, n=10. (B) The correlation between *RBCK1* and *HBx*mRNA expression in TCGA LIHC cohort. (C) Kaplan-Meier analysis for OS and DFS of two groups of HCC patients defined by low and high expression of *HBx* in TCGA LIHC cohort. (D) Kaplan-Meier analysis for OS and DFS of two groups of HCC patients defined by low and high co-expression of *RBCK1* and *HBx* in TCGA LIHC cohort. (E) Graphical abstract of the study. (C, D) log-rank Mantel-Cox test.

Discussion

LUBAC-mediated M1-Ubi has been gradually studied since it was first found to be a new type of ubiquitination [24] and affected the progression of multiple cancers [19, 25, 26]. However, its role in HCC development and progression is poorly understood. In the present study, we revealed that the components of LUBAC (*RBCK1* ,*RNF31* , *Sharpin*) and M1-Ubi expression were upregulated in HCC tissues compared with that in the normal control, which predicted poor prognosis. However, we found that only the expression of*RBCK1*

but not *RNF31* or *Sharpin* was elevated in HBV-associated HCC tissues and related to poor prognosis in HBV-associated HCC cohorts. Targeting RBCK1 significantly inhibited the growth and metastasis of HBV-associated HCC cells *in vitro* and*in vivo* . Mechanistically, RBCK1 interacted with HBx to upregulate its M1-Ubi and inhibit its K48-linked ubiquitination, thereby reducing its degradation. Thus, our study proposed that RBCK1, a component of LUBAC and mediated HBx linear ubiquitination, was a potential target for HBV-associated HCC therapeutics (Fig. 6E).

About 700 RING finger family proteins have been identified, most of which contain the RING-In-Between-RING (RBR) domain. Unlike most E3 ubiquitin ligases that mediate the proteolytic poly-ubiquitination, recent studies revealed that several RING family ubiquitin ligases promoted atypical ubiquitination on their substrates. For example, RBCK could cooperate with RNF31 and Sharpin to form LUBAC to mediate the linear ubiquitination of IKKγ and promote the activation of NF-ϰB signaling [24]. More recently, it has been reported that LUBAC-mediated linear ubiquitination played a key role in driving the progression of cancers by promoting the activation of oncogenic signaling pathways. In prostate cancer (PCa), phosphatase and tensin homolog (PTEN) were reported to be modified by M1-Ubi at two sites, K144 and K197, which significantly inhibited PTEN phosphatase activity and thus accelerated PCa progression [26]. LUBAC was recruited to mediate the M1-Ubi of AGO2 to regulate the overall mRNA homeostasis of cancer cells under hypoxia [27]. Furthermore, the M1-Ubi of STAT1 could inhibit its binding to the type-I interferon receptor IFNAR2, thereby restricting STAT1 activation and controlling antiviral interferon signaling [28].

In the previous studies, it had been reported that the expression of RBCK1 and Sharpin was upregulated in tumor tissues [29, 30], and exerted oncogenic functions by stabilizing the expression RNF31 and promoting the transactivation of Versican expression; however, it was independent of their M1-Ubi activity. Thus, a more comprehensive study is needed on the potential functions of M1-Ubi and LUBAC components in HCC, especially in HBV-associated HCC. Our study revealed that LUBAC-mediated M1-Ubi and LUBAC components were significantly elevated in HCC tissues and correlated with poor prognosis. HBV is a primary etiology of chronic viral hepatitis and further leads to the occurrence of HCC. We performed analyses with the public and our cohorts to further explore the role of LUBAC-mediated M1-Ubi and its components in the HBV-associated HCC. However, we found that only *RBCK1,* rather than *RNF31* or *Sharpin,* was upregulated in the HBV-associated HCC compared to the non-HBV-associated HCC tissues, indicating the specific interaction between RBCK1 and the HBV components. In further exploration, upregulated expression of RBCK1 was found to be associated with high AFP levels, large tumor size, and poor prognosis in both public and our HBV-associated HCC cohorts. Moreover, inhibiting RBCK1 expression alleviated the growth and metastasis of HBV-carried HCC cells *in vitro* and *in vivo* .

RBCK1 with RANBP2 and C3HC4 zinc-finger is a 58 kDa protein that contains the N-terminal ubiquitin-like (UBL) domain, the Npl4 zinc finger (NZF) domain, and the catalytic carbon terminal RBR domain [31]. Recently, quite a few E3 ubiquitin ligases have exhibited abnormal expression in tumors, making them valuable diagnostic markers and drug targets [32]. Previous studies have revealed that publicly-available databases and *in vitro* analysis have determined that mRNA expression of *RBCK1* in HCC cells and tissues was significantly higher than those of normal counterparts [30, 33]. For factors upregulating the expression of RBCK1, Queisser MA *et al* . considered that the hypoxia-inducible factor regulated the expression of RBCK1 in breast cancer [25]. In addition, RBCK1 was identified as a copy number variation (CNV)-driven gene by screening out CNV-driven differentially expressed genes in liver cancer [34]. In the present study, we demonstrated that RBCK1 expression was upregulated in HBV-associated HCC tissues compared with that in non-HBV-associated HCC tissues, indicating a positive regulation of HBV on the expression of RBCK. Numerous gene expressions were regulated by HBV infection via various mechanisms, including the HBx pathway [35], N6 methyladenosine modification [36], DNA methylation [37] *et al* . Increasing evidence indicates that HBx, the transactivating factor of viral genes, is mainly involved in the regulation of HCC occurrence, proliferation, invasion, migration, and glucose metabolism. HBx interacts with cellular proteins to activate various signaling pathways or affect gene expression by epigenetic regulation. Nevertheless, how HBV regulated the expression of RBCK1, and whether RBCK1 in turn regulated the replication of HBV necessitate more exploration.

In summary, our results demonstrate that LUBAC-mediated M1-Ubi and the LUBAC components play a critical role in the development and progression of HCC. More importantly, the expression of RBCK1 is specifically upregulated and predicts poor prognosis in HBV-associated HCC. RBCK1 interacts with HBx to enhance its stability by increasing M1-Ubi and reducing the K48-linked ubiquitination, which promotes the growth and metastasis of HBV-associated HCC. Thus, Our finding provides new insights into the mechanism by which RBCK1 and LUBAC-medicated M1-Ubi promote the progression of HBV-associated HCC, and sheds light on the potential value of RBCK1-targeting therapeutic.

Author contributions

PC, ZYD, and YYL designed the study. PC, ZYD, YXZ, and QYY performed most of the experiments. PC and ZYD analyzed the data. ZYD, YXZ, and QYY collected the study data. PC, ZYD, LBT, and YYL wrote the manuscript. YYL and LBT reviewed the manuscript. YYL and LBT supervised the study.

Financial support

This work was supported by the National Natural Science Foundation of China (81971933 and 82022007), the Local Innovative and Research Teams Project of Guangdong Pearl River Talents Program (2017BT01S131), the National Key Research and Development Program of China (No.2022YFC2303600 and No.2022YFC2304800), and the Outstanding Youth Development Scheme of Nanfang Hospital, Southern Medical University (2020J003).

Conflict of interest

The authors have no conflict of interest to declare.

Reference

[1] Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018;68:394-424.

[2] Li J, Hu Y, Yuan Y, Zhao Y, Han Q, Liu C, et al. Repurposing of Antazoline Hydrochloride as an Inhibitor of Hepatitis B Virus DNA Secretion. Virol Sin 2021;36:501-509.

[3] Iannacone M, Guidotti LG. Immunobiology and pathogenesis of hepatitis B virus infection. Nat Rev Immunol 2022;22:19-32.

[4] Rizzo GEM, Cabibbo G, Craxi A. Hepatitis B Virus-Associated Hepatocellular Carcinoma. Viruses 2022;14.

[5] Levrero M, Zucman-Rossi J. Mechanisms of HBV-induced hepatocellular carcinoma. J Hepatol 2016;64:S84-S101.

[6] Xu X, Fan Z, Kang L, Han J, Jiang C, Zheng X, et al. Hepatitis B virus X protein represses miRNA-148a to enhance tumorigenesis. J Clin Invest 2013;123:630-645.

[7] Liu H, Yan Y, Lin J, He C, Liao H, Li H, et al. Circular RNA circSFMBT2 downregulation by HBx promotes hepatocellular carcinoma metastasis via the miR-665/TIMP3 axis. Mol Ther Nucleic Acids 2022;29:788-802.

[8] Gao Y, Feng J, Yang G, Zhang S, Liu Y, Bu Y, et al. Hepatitis B virus X protein-elevated MSL2 modulates hepatitis B virus covalently closed circular DNA by inducing degradation of APOBEC3B to enhance hepatocarcinogenesis. Hepatology 2017;66:1413-1429.

[9] Lee YY, Mok MT, Kang W, Yang W, Tang W, Wu F, et al. Loss of tumor suppressor IGFBP4 drives epigenetic reprogramming in hepatic carcinogenesis. Nucleic Acids Res 2018;46:8832-8847.

[10] Hu Z, Zhang Z, Doo E, Coux O, Goldberg AL, Liang TJ. Hepatitis B virus X protein is both a substrate and a potential inhibitor of the proteasome complex. J Virol 1999;73:7231-7240.

[11] Minor MM, Slagle BL. Hepatitis B virus HBx protein interactions with the ubiquitin proteasome system. Viruses 2014;6:4683-4702.

[12] Wan T, Lei Z, Tu B, Wang T, Wang J, Huang F. NEDD4 Induces K48-Linked Degradative Ubiquitination of Hepatitis B Virus X Protein and Inhibits HBV-Associated HCC Progression. Front Oncol 2021;11:625169.

[13] Wu Q, Zhang L, Xu X, Zhang Y, Shi J, Lin X, et al. Hepatitis B Virus X Protein Is Stabilized by the Deubiquitinating Enzyme VCPIP1 in a Ubiquitin-Independent Manner by Recruiting the 26S Proteasome Subunit PSMC3. J Virol 2022;96:e0061122.

[14] Wang WL, London WT, Lega L, Feitelson MA. HBxAg in the liver from carrier patients with chronic hepatitis and cirrhosis. Hepatology 1991;14:29-37.

[15] Senft D, Qi J, Ronai ZA. Ubiquitin ligases in oncogenic transformation and cancer therapy. Nat Rev Cancer 2018;18:69-88.

[16] Kirisako T, Kamei K, Murata S, Kato M, Fukumoto H, Kanie M, et al. A ubiquitin ligase complex assembles linear polyubiquitin chains. EMBO J 2006;25:4877-4887.

[17] Fuseya Y, Fujita H, Kim M, Ohtake F, Nishide A, Sasaki K, et al. The HOIL-1L ligase modulates immune signalling and cell death via monoubiquitination of LUBAC. Nat Cell Biol 2020;22:663-673.

[18] Ikeda F, Deribe YL, Skanland SS, Stieglitz B, Grabbe C, Franz-Wachtel M, et al. SHARPIN forms a linear ubiquitin ligase complex regulating NF-kappaB activity and apoptosis. Nature 2011;471:637-641.

[19] Jahan AS, Elbaek CR, Damgaard RB. Met1-linked ubiquitin signalling in health and disease: inflammation, immunity, cancer, and beyond. Cell Death Differ 2021;28:473-492.

[20] Chen P, Luo X, Dai G, Jiang Y, Luo Y, Peng S, et al. Dexmedetomidine promotes the progression of hepatocellular carcinoma through hepatic stellate cell activation. Exp Mol Med 2020;52:1062-1074.

[21] Xia L, Huang W, Tian D, Zhu H, Zhang Y, Hu H, et al. Upregulated FoxM1 expression induced by hepatitis B virus X protein promotes tumor metastasis and indicates poor prognosis in hepatitis B virus-related hepatocellular carcinoma. J Hepatol 2012;57:600-612.

[22] Yang L, Zou T, Chen Y, Zhao Y, Wu X, Li M, et al. Hepatitis B virus X protein mediated epigenetic alterations in the pathogenesis of hepatocellular carcinoma. Hepatol Int 2022;16:741-754.

[23] Tan G, Yi Z, Song H, Xu F, Li F, Aliyari R, et al. Type-I-IFN-Stimulated Gene TRIM5gamma Inhibits HBV Replication by Promoting HBx Degradation. Cell Rep 2019;29:3551-3563 e3553.

[24] Tokunaga F, Sakata S, Saeki Y, Satomi Y, Kirisako T, Kamei K, et al. Involvement of linear polyubiquitylation of NEMO in NF-kappaB activation. Nat Cell Biol 2009;11:123-132.

[25] Queisser MA, Dada LA, Deiss-Yehiely N, Angulo M, Zhou G, Kouri FM, et al. HOIL-1L functions as the PKCzeta ubiquitin ligase to promote lung tumor growth. Am J Respir Crit Care Med 2014;190:688-698.

[26] Guo Y, He J, Zhang H, Chen R, Li L, Liu X, et al. Linear ubiquitination of PTEN impairs its function to promote prostate cancer progression. Oncogene 2022;41:4877-4892.

[27] Zhang H, Zhao X, Guo Y, Chen R, He J, Li L, et al. Hypoxia regulates overall mRNA homeostasis by inducing Met(1)-linked linear ubiquitination of AGO2 in cancer cells. Nat Commun 2021;12:5416.

[28] Zuo Y, Feng Q, Jin L, Huang F, Miao Y, Liu J, et al. Regulation of the linear ubiquitination of STAT1 controls antiviral interferon signaling. Nat Commun 2020;11:1146.

[29] Tanaka Y, Tateishi K, Nakatsuka T, Kudo Y, Takahashi R, Miyabayashi K, et al. Sharpin promotes hepatocellular carcinoma progression via transactivation of Versican expression. Oncogenesis 2016;5:e277.

[30] Chen X, Ye Q, Zhao W, Chi X, Xie C, Wang X. RBCK1 promotes hepatocellular carcinoma metastasis and growth by stabilizing RNF31. Cell Death Discov 2022;8:334.

[31] Elton L, Carpentier I, Verhelst K, Staal J, Beyaert R. The multifaceted role of the E3 ubiquitin ligase HOIL-1: beyond linear ubiquitination. Immunol Rev 2015;266:208-221.

[32] Wang P, Dai X, Jiang W, Li Y, Wei W. RBR E3 ubiquitin ligases in tumorigenesis. Semin Cancer Biol 2020;67:131-144.

[33] Xu Z, Shao J, Zheng C, Cai J, Li B, Peng X, et al. The E3 ubiquitin ligase RBCK1 promotes the invasion and metastasis of hepatocellular carcinoma by destroying the PPARgamma/PGC1alpha complex. Am J Cancer Res 2022;12:1372-1392.

[34] Lu X, Ye K, Zou K, Chen J. Identification of copy number variation-driven genes for liver cancer via bioinformatics analysis. Oncol Rep 2014;32:1845-1852.

[35] Sekiba K, Otsuka M, Funato K, Miyakawa Y, Tanaka E, Seimiya T, et al. HBx-induced degradation of Smc5/6 complex impairs homologous recombination-mediated repair of damaged DNA. J Hepatol 2022;76:53-62.

[36] Kim GW, Imam H, Khan M, Mir SA, Kim SJ, Yoon SK, et al. HBV-Induced Increased N6 Methyladenosine Modification of PTEN RNA Affects Innate Immunity and Contributes to HCC. Hepatology 2021;73:533-547.

[37] Okamoto Y, Shinjo K, Shimizu Y, Sano T, Yamao K, Gao W, et al. Hepatitis virus infection affects DNA methylation in mice with humanized livers. Gastroenterology 2014;146:562-572.

Figure 1

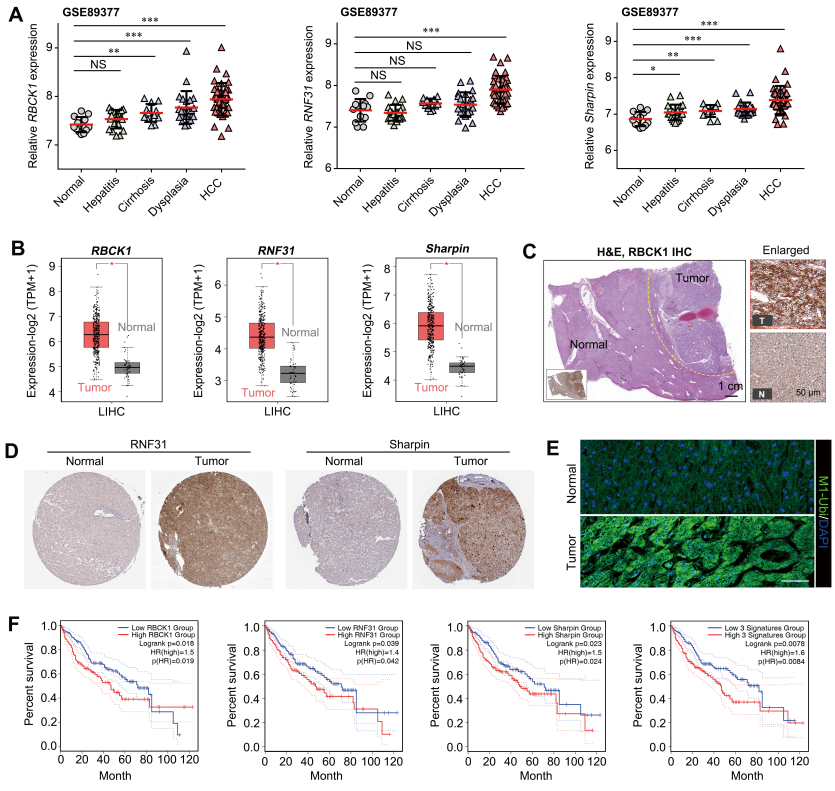


Figure 2

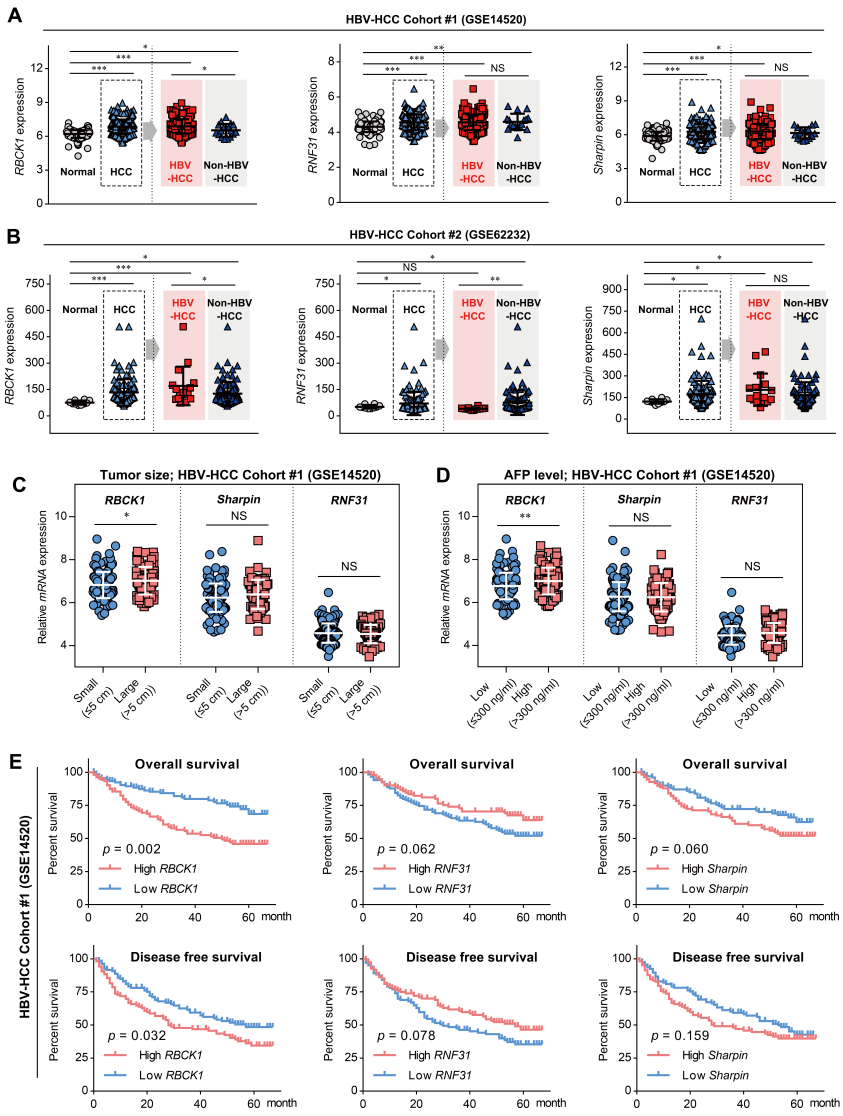


Figure 3

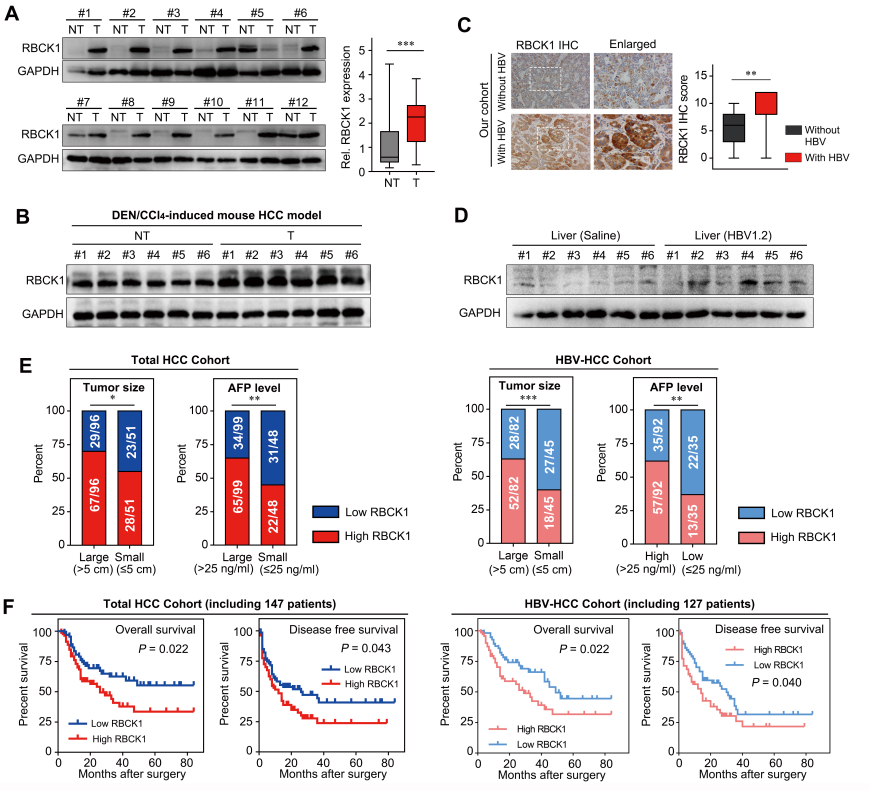


Figure 4

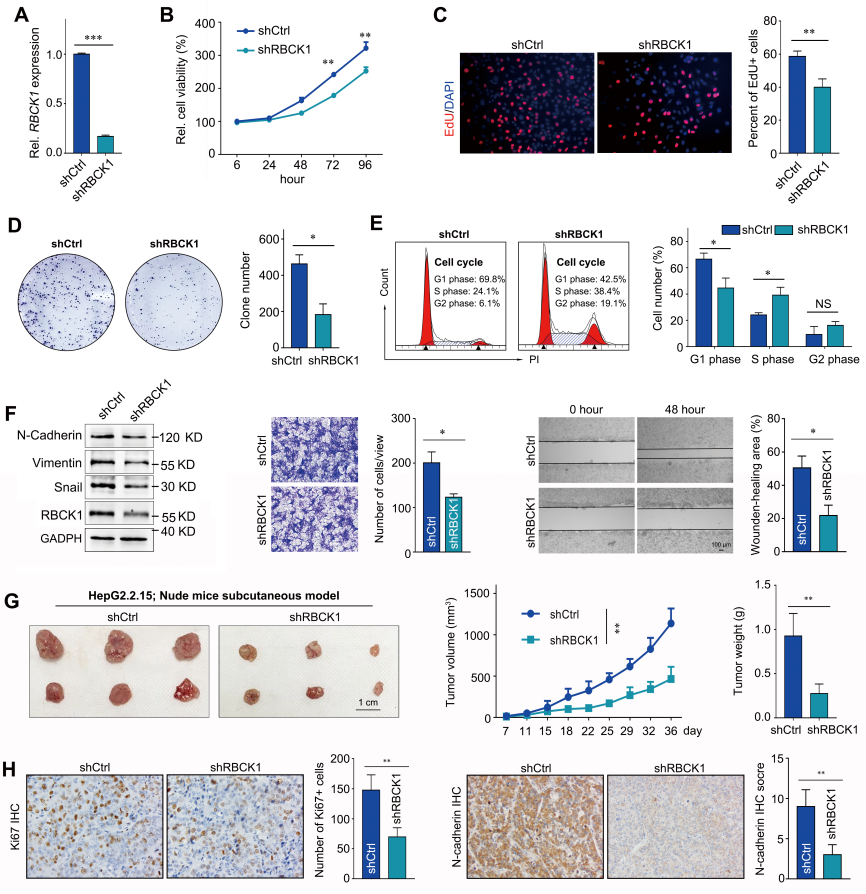


Figure 5

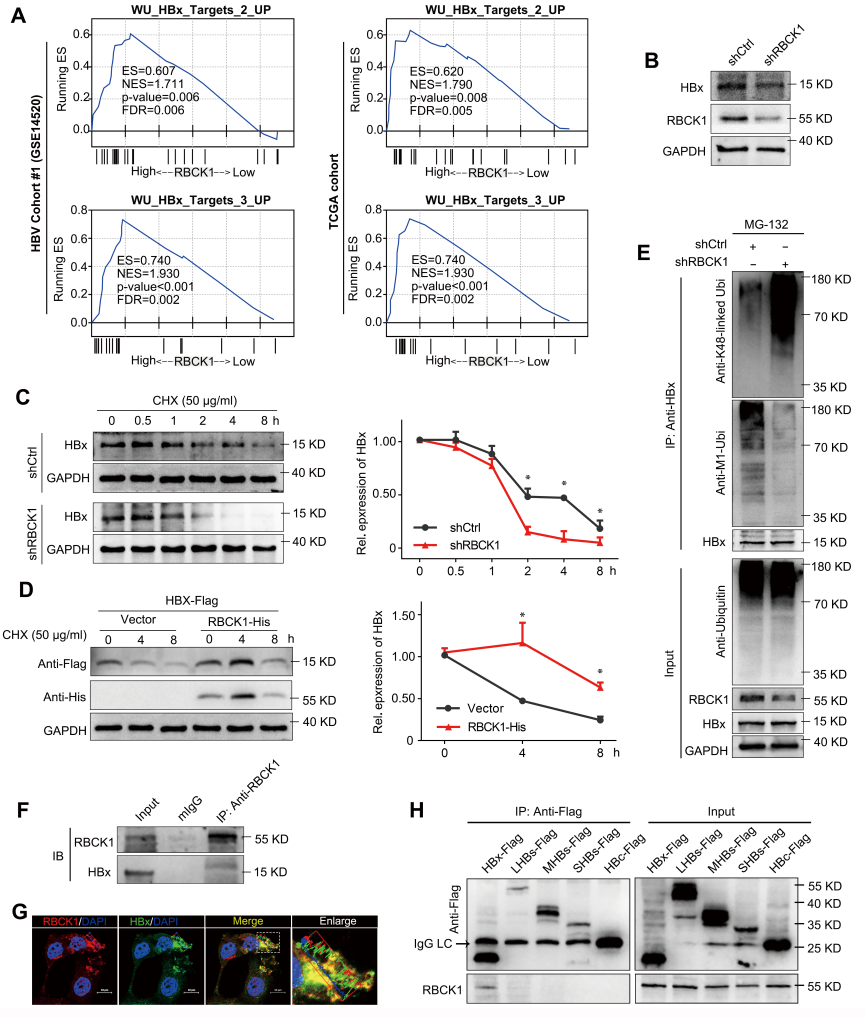
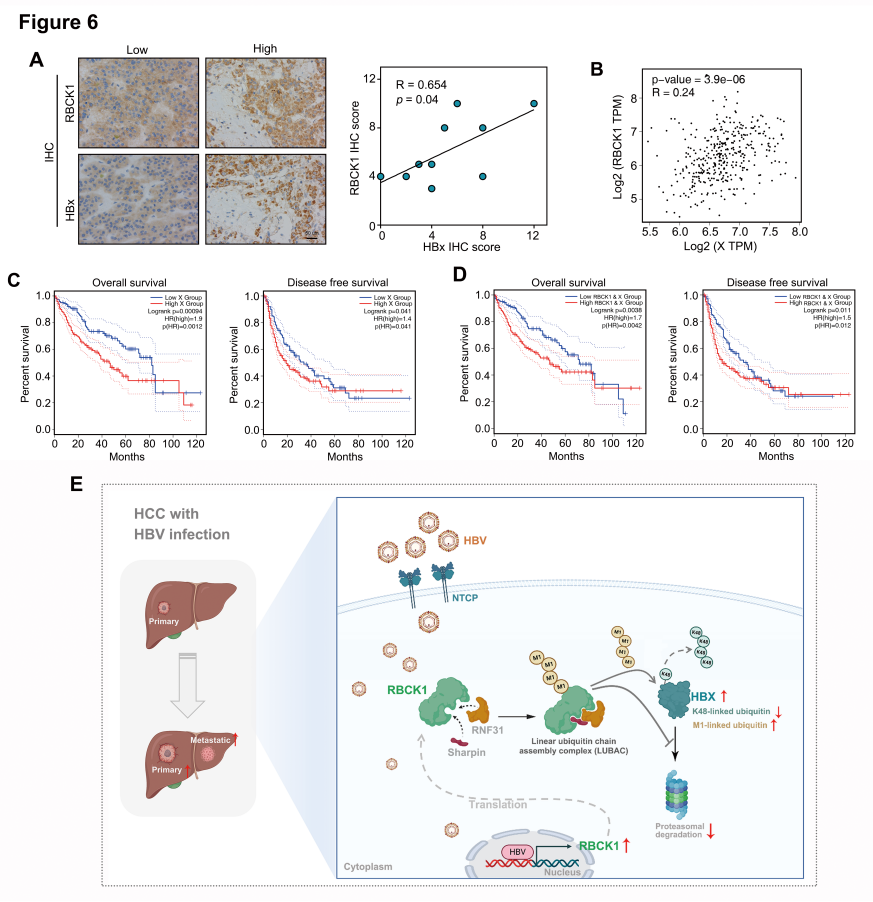


Figure 6



Hosted file

Supplementary Table 1.docx available at https://authorea.com/users/576939/articles/619516-rbck1-promotes-the-stabilization-of-hbx-by-linear-ubiquitination-to-drive-the-progression-of-hbv-associated-hepatocellular-carcinoma