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HKDC1 promotes liver cancer stemness under hypoxia through stabilizing β-catenin

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Abstract

Background and Aims: Hexokinases (HKs), a group of enzymes catalyzing the first step of glycolysis, have been shown to play important roles in liver metabolism and tumorigenesis. Our recent studies identified hexokinase domain containing 1 (HKDC1) as a top candidate associated with liver cancer metastasis. We aimed to compare its cell-type specificity with other HKs upregulated in liver cancer and investigate the molecular mechanisms underlying its involvement in liver cancer metastasis.

Approach and Results: We found that, compared to HK1 and HK2, the other 2 commonly upregulated HKs in liver cancer, HKDC1 was most strongly associated with the metastasis potential of tumors and organoids derived from 2 liver cancer mouse models we previously established. RNA in situ hybridization and single-cell RNA-seq analysis revealed that HKDC1 was specifically upregulated in malignant cells in HCC and cholangiocarcinoma patient tumors, whereas HK1 and HK2 were widespread across various tumor microenvironment lineages. An unbiased metabolomic profiling demonstrated that HKDC1 overexpression in HCC cells led to metabolic alterations distinct from those from HK1 and HK2 overexpression, with HKDC1 particularly impacting the tricarboxylic acid cycle. HKDC1 was prometastatic in HCC orthotopic and tail vein injection mouse models. Molecularly, HKDC1 was induced by hypoxia and bound to glycogen synthase kinase 3β to stabilize β-catenin, leading to enhanced stemness of HCC cells.

Conclusions: Overall, our findings underscore HKDC1 as a prometastatic HK specifically expressed in the malignant compartment of primary liver tumors,

Abbreviations: CAF, cancer-associated fibroblast; CCA, cholangiocarcinoma; HK, hexokinase; HKDC1, hexokinase domain containing 1; KO, knockout; OE, overexpression; PPTR, Prom1^{CreERT2}; Pten$^{flx/flx}$; Tp53$^{flx/flx}$; Rosa-ZsGreen; scRNA-seq, single-cell RNA sequencing; TCA, tricarboxylic acid; TME, tumor microenvironment.

Li Fan and Cheng Tian contributed equally to this work.

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thereby providing a mechanistic basis for targeting this enzyme in advanced liver cancer.

Keywords: cholangiocarcinoma, hepatocellular carcinoma, HKDC1, hypoxia, stemness, metabolome

INTRODUCTION

Hexokinases (HKs) are a family of enzymes that catalyzes the first step in glucose metabolism, the phosphorylation of glucose into glucose 6-phosphate.[1] There are 4 conventional HK isoforms: HK1, HK2, HK3, and HK4. They are similar in structure but different in their enzymatic efficacy and tissue specificity.[2] The liver, one of the most important metabolic organs of the body, predominantly uses HK4 (also known as glucokinase or GCK) to phosphorylate glucose. In HCC, HK4 is down-regulated, and HK1 and HK2 are upregulated to support tumorigenesis.[3–5] Hexokinase domain containing 1 (HKDC1) is a fifth human HK discovered recently through its association with gestational diabetes.[6–8] HKDC1 has a low HK activity compared to the other HKs and is nearly undetectable in the normal liver tissue.[9,10] Subsequent studies found that HKDC1 is significantly upregulated and is a protumorigenic player in HCC and other cancers.[11–14] Its impact on glucose update of cancer cells and the subcellular association with mitochondria-associated membranes have also been demonstrated.[15] What remains unexplored are the potential differences of HKDC1 compared to the other upregulated HKs regarding their lineage specificity and metabolic activity in HCC. HCC is well known for its complex tumor microenvironment (TME) consisting of various cell types with different functions and metabolic needs.

In this study, our group independently identified *Hkdc1* as a top upregulated gene in the metastatic tumors and organoids derived from an HCC genetic mouse model we previously established *Prom1*^{CreERT2}; *Pten*$^{flx/flx}$; *Tp53*$^{flx/flx}$; *Rosa-ZsGreen* (PPTR) mice.[16] Orthotopic tumors developed from PPTR tumor organoids gradually acquired cellular and molecular features of cholangiocarcinoma (CCA), the second most common primary liver cancer, and showed a significantly increased metastatic potential.[17] We followed up on these findings and compared the distribution of HKDC1 to that of HK1 and HK2 in HCC and CCA patient tumors. For functional and metabolic analyses, we used human HCC cell lines and orthotopic xenograft models, given the rarity of human CCA cell lines. Since PPTR tumor organoids exhibited strong liver cancer stem cell traits,[17] we studied the involvement of HKDC1 in cancer stemness. We examined its participation in WNT/β-catenin signaling because of the known importance of this pathway in supporting the stemness of both normal and malignant liver cells.[18,19]

METHODS

Mice

Animal protocols were approved by the St. Jude Animal Care and Use Committee, and the care and use of experimental animals complied with the animal welfare laws, guidelines, and policies. Kaplan-Meier animal survival curves were graphed using GraphPad Prism 10.

Liver cancer patient samples

Deidentified HCC and CCA patient samples were obtained under a protocol approved by the Institutional Review Boards at St. Jude Children's Research Hospital and The University of Tennessee.

Cell lines and culture

PLC/PRF/5 and Hep3B were purchased from the American Type Culture Collection (ATCC). Huh7 cells were a gift from Dr Jun Yang from the Department of Surgery at St. Jude Children's Research Hospital.

HKDC1 knockout by CRISPR/Cas9

HKDC1^{KO} cells were generated using the standard CRISPR/Cas9 technology by the Center for Advanced Genome Engineering at St. Jude's Children's Research Hospital. sgRNA: 5′-GGCCCTGGTCAATGACACCGNGG-3′.

RNAscope staining

RNAscope in situ hybridization of human and mouse *Hkdc1*, *Hk1*, *Hk2*, and *Hif1a* mRNA transcripts was performed on freshly cut paraffin sections according to the manufacturer's protocol (Advanced Cell Diagnostics) by Comparative Pathology Core at St. Jude's Children's Research Hospital.

Laduviglusib treatment and colony formation assay

5×10^3 PLC/PRF/5 cells were seeded in 6-well plates and cultured for 7 days with or without laduviglusib (HY-10182, 10 μM, MedChemExpress). Colonies were stained with crystal violet (#C581-25, Thermo Fisher Scientific), and ImageJ was used to measure colony growth area from 3 biological replicates.

RNA extraction, sequencing, and data analysis

The total RNA was extracted from cells using RNeasy Mini Kit (#74106, Qiagen) following the manufacturer's protocol. The total RNA library was constructed using the Illumina TrueSeq stranded mRNA library prep kit and sequenced using the HiSeq. 2000/2500 or NovaSeq. 6000 platform (2×101-bp pair-end reads).[20–24]

Metabolomics analysis by LC-MS/MS

Huh7 cell metabolites were analyzed by acidic pH reverse phase LC-MS/MS with a self-packed column (75 μm × 15 cm with 1.9 μm C18 resin from Dr. Maisch GmbH) coupled with Q Exactive HF Orbitrap MS in positive ion mode.[25] The data analysis was performed by the in-house software suite JUMPm.[26,27]

Statistical analysis

Student *t* test was used to evaluate differences between the 2 groups. A one-way ANOVA test was used to compare ≥ 3 groups. A p value of < 0.05 was considered statistically significant. Detailed methods can be found in the Supplemental Material, http://links.lww.com/HEP/I699.

RESULTS

HKDC1 is a top upregulated gene in metastatic tumors and organoids in the PPTR liver cancer mouse model

We previously generated a liver cancer mouse model by targeting a *Prom1*-expressing liver progenitor population with the dual loss of tumor suppressor genes *Pten* and *Tp53* (*Prominin1*^{CreERT2}; *Pten*$^{flx/flx}$; *Tp53*$^{flx/flx}$; *Rosa*^{ZsG}, or PPTR).[16] PPTR mice developed frequent primary tumors in the liver with histological features of HCC. However, metastases were rare in this model. We then generated multiple cancer organoid lines derived from the PPTR primary tumors, and they showed varying levels of tumorigenicity when orthotopically transplanted into the mouse liver. A subset of PPTR tumor organoids demonstrated a high metastatic potential in the orthotopic allograft model, while others failed to grow in vivo (Figure 1A).[17] Tumors in the PPTR allograft model acquired CCA features, potentially due to a hepatocyte-to-cholangiocyte trans-differentiation.[17] RNA-seq transcriptomic profiling of the PPTR genetic and allograft tumors, as well as the tumor organoids, was previously performed (GSE94583). In this study, comparative analyses were conducted to identify genes associated with enhanced metastasis in the allograft model. Similar analyses were performed for a hepatoblastoma mouse model we established by engineering an activating *Notch* mutation, *NICD* (Notch intercellular domain), into the *Prom1*-expressing liver progenitors (*Prominin1*^{CreERT2}; *Rosa*$^{NICD1/+}$; *Rosa*^{ZsG}, or PNR). Combining analyses from both mouse models enabled us to identify genes commonly associated with liver cancer metastasis.

We found that *Hkdc1*, a recently identified HK family member, was among the top genes significantly upregulated in the metastatic PNR and PPTR tumors and organoids (Figure 1B and Supplemental Figure S1, http://links.lww.com/HEP/I661). Although the other 2 HKs commonly upregulated in liver cancer, *Hk1* and *Hk2*, exhibited similar upregulation in the metastatic PPTR and PNR tumors, their association with the metastatic potential of the tumor organoid was less pronounced compared to *Hkdc1*. To validate these findings, we performed RNAscope in situ hybridization using primary and metastatic tumors from the PPTR genetic mouse model. *Hkdc1* mRNA was detected in the metastatic tumor at a much higher level than that of *Hk1* and *Hk2* (Figure 1C). Since Hkdc1 is structurally similar to HK1, we further compared their protein levels in the PPTR tumors and organoids through immunoblotting. We confirmed the increased levels of Hkdc1 protein in PPTR metastatic tumors (Figure 1D) and metastatic organoids (Figure 1E) but not that of HK1. Overall, among the HK family members, Hkdc1 showed the strongest association with metastasis in the PPTR mouse model. Transcriptomic data from metastatic tumors in liver cancer patients are limited. We analyzed a published HCC study with RNA-seq profiles of matched primary tumors and portal vein tumor thrombosis, a form of intrahepatic metastasis of HCC (GSE77509).[28] *HKDC1* expression was upregulated in the tumors compared to the tumor-adjacent normal liver controls. The 15 portal vein tumor thrombosis samples whose transcriptomes clustered with their primary tumors showed a further increase in *HKDC1* expression (Supplemental Figure S2A, http://links.lww.com/HEP/I661). Individually, 9 out of the 15 portal vein tumor thrombosis samples had higher *HKDC1* expression than their matched primary tumor (Supplemental Figure S2B, http://links.lww.com/HEP/I661). Although no statistics were performed due to the small sample size, these data suggest that *HKDC1* expression is positively

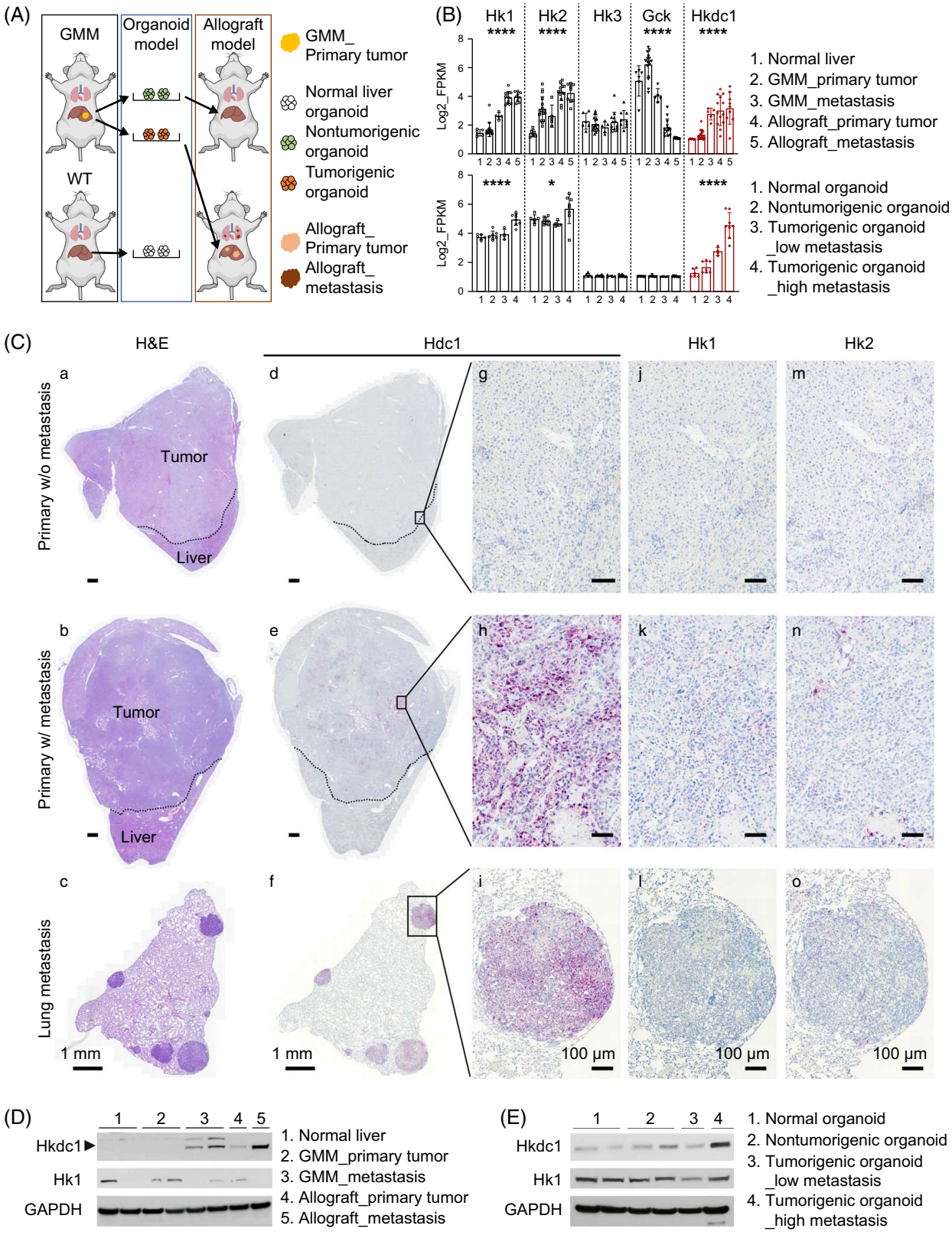

FIGURE 1 HKDC1 is upregulated in metastatic tumors and organoids in PPTR liver cancer mouse model. (A) A schematic illustration for the establishment of PPTR genetic mouse model, organoid model, and orthotopic allograft models (generated by BioRender). (B) Quantitative comparison of the gene expression of the 5 HKs in PPTR tumor tissues (n = 6, 16, 5, 13, and 9 for the 5 indicated groups, respectively) and organoids (n = 6, 7, 4, and 8 for the 4 indicated groups, respectively). One-way ANOVA test: *p* values: * < 0.05; **** < 0.0001. (C) H&E (a–c) and RNAscope staining of *Hkdc1* (d–i), *Hk1* (j–l), and *Hk2* (m–o) on the serial sections from the indicated tumors in the PPTR genetic mouse model. Images on the same column share the same scale bar. (D) Immunoblotting of Hkdc1 and Hk1 in the normal liver and indicated PPTR tumors. (E) Immunoblotting of Hkdc1 and Hk1 in the normal liver organoids and indicated PPTR tumor organoids. Abbreviations: H&E, hematoxylin and eosin; HKDC1, hexokinase domain containing 1; PPTR, Prom1^{CreERT2}; Pten$^{flx/flx}$; Tp53$^{flx/flx}$; Rosa-ZsGreen.

associated with tumor development in patients with HCC and likely further increases in HCC metastases.

HKDC1 upregulation is highly specific to malignant cells in patients with HCC and CCA

Expression of HKDC1 in human HCC and CCA has been previously reported.[18–20] However, liver cancer is known for its complex TME with the presence of various nonmalignant cell populations. The cell-type specificity of HKDC1 and other HKs has not been characterized, which could contribute to their potentially different contributions to liver tumorigenesis. Thus, we examined the expression of *HK1*, *HK2*, and *HKDC1* in malignant cells and nonmalignant cells using a previously reported single-cell RNA sequencing (scRNA-seq) of 46 HCC and CCA tumors.[29] We found that *HKDC1* expression was highly specific to the malignant cells, whereas *HK1* and *HK2* expression was detected in both malignant and nonmalignant compartments (Figure 2A). Consistently, tSNE analysis of all the 17,164 malignant cells and 35,625 nonmalignant cells combined from both cancer types in this data set showed that the majority of *HKDC1*$^+$ cells were malignant while *HK1* and *HK2* expression was predominantly found in nonmalignant cells (Figure 2B). To assess the expression of these genes in normal tissue and tumor, we further analyzed a multiregional scRNA-seq data set generated from 7 HCC and CCA patient tumors and their adjacent normal liver tissues.[30] *HKDC1* expression was detected mostly in cholangiocytes in the tumor-adjacent liver, malignant tumor cells, and some T cells in the tumor tissues (Figure 2C). In contrast, *HK1* and *HK2* both showed a wide range of expression across nearly all cell types within the HCC and CCA TME (Figure 2C). *HK1* levels were particularly high in tumor-associated macrophages and cancer-associated fibroblasts (CAFs) (Figure 2C). In nontumor tissues, HK1 was mainly expressed in the cholangiocytes rather than hepatocytes, while HK2 levels were very low in both cell types (Figure 2C).

We validated the scRNA-seq analysis results using RNAscope on normal human liver and tissues collected from the tumor margin of patients with HCC and CCA. The results were consistent with the scRNA-seq analysis (Figure 2D). All 3 HKs were weakly positive in cholangiocytes in the normal human liver, with *HKDC1* showing relatively stronger signals (Figure 2D, d, i, n). In HCC, upregulation of *HKDC1* was evident in the tumor cells but not that of *HK1* or *HK2* (Figure 2D, e, j, o). In CCA, both *HKDC1* and *HK1* showed significant upregulation, with *HKDC1* being highly specific to tumor cells and *HK1* being widespread (Figure 2D, g and l). In tumor-adjacent liver tissues of HCC and CCA, *HKDC1* expression remained in cholangiocytes in the portal triads, but *HK1* was primarily found in immune cells accumulated in this region (Figure 2D, f, h, k, m). *HK2* upregulation was minimal in the HCC and CCA tumors examined, consistent with the scRNA-seq results (Figure 2D, o–r). Since RNA expression may not always correlate with the protein level, we performed immunohistochemistry of HKDC1, HK1, and HK2 on liver cancer patient tumors and found their protein localization patterns consistent with the RNA results (Supplemental Figure S3, http://links.lww.com/HEP/I661). Overall, our data show that HKDC1 is the most tumor cell–specific HK in patients with HCC and CCA.

HKDC1, HK1, and HK2 are involved in different metabolic activities in HCC cells

Upon the identification of their differential distribution in liver cancer patient tumors, we compared the metabolic activities of HKDC1, HK1, and HK2 in liver cancer cells. Due to the lack of commercial resources for human CCA cell lines, we focused on 3 HCC cell lines, Huh7, PLC/PRF/5, and Hep3B. We found that Huh7 cells showed the lowest endogenous levels of all 3 HKs (Figure 3A), making this cell line a good model for comparing metabolic changes associated with their ectopic expression. We overexpressed FLAG-tagged *HK1*, *HK2*, and *HKDC1* individually in Huh7 cells and confirmed their comparable protein levels (Figure 3B). While their individual overexpression led to minor fluctuations in the endogenous levels of the other HKs, these changes were neglectable compared to the targeted ectopic expression (Supplemental Figure S4, http://links.lww.com/HEP/I661). All 3 HKs promoted Huh7 cell growth in vitro (Figure 3C). We then performed a global metabolomic analysis of the HK-overexpressing (OE) Huh7 cells, which revealed a distinct metabolic profile in HKDC1-OE cells compared to both the parental control, HK1-OE and HK2-OE cells (Figure 3D and Supplemental Table S1, http://links.lww.com/HEP/I662). HK2-OE caused the least metabolic changes among the 3 HKs under our experimental conditions. For HKDC1 and HK1, despite their structural similarity, metabolic alterations associated with their overexpression were markedly different (Figure 3E). Metabolic pathway enrichment analysis identified a significant impact of HKDC1-OE on the tricarboxylic acid (TCA) cycle (Figure 3F). In contrast, HK1-OE substantially upregulated metabolites involved in purine and pyrimidine metabolism pathways (Figure 3G). Consistently, intermediate metabolites in the TCA cycle, such as malate, were significantly upregulated in HKDC1-OE cells, whereas key metabolites of purine and pyrimidine biosynthesis pathways, including ribulose 5-phosphate, inosine, and cytidine monophosphate, were specifically elevated in HK1-OE cells (Figure 3H). Comparing HK2-OE Huh7 cells to the control cells, purine metabolism showed up as one of the top elevated pathways (Supplemental Figure S5, http://links.lww.com/HEP/I661). However, since we have shown that

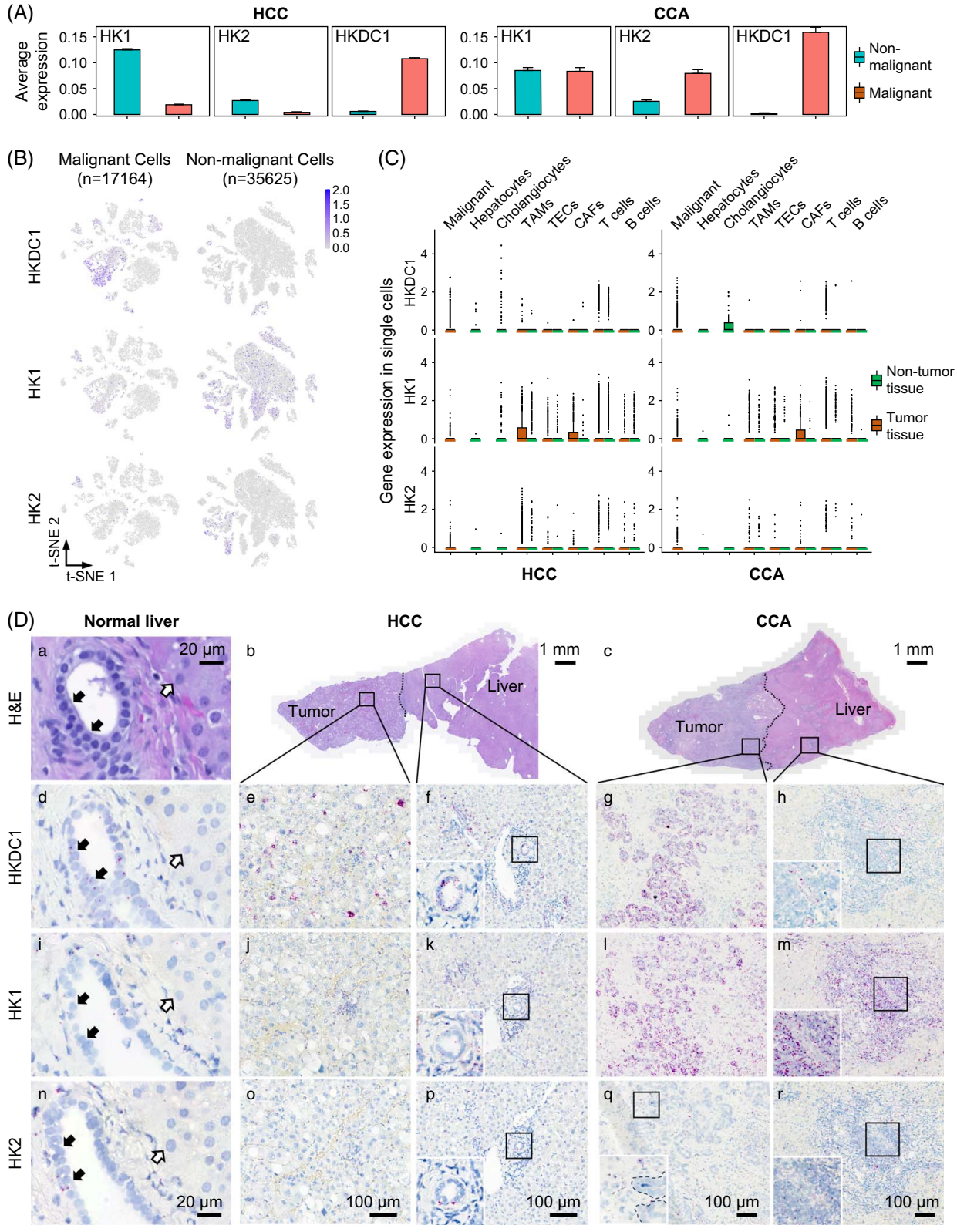

FIGURE 2 HKDC1 upregulation is highly specific to malignant cells in patients with HCC and CCA. (A) Mean expression of *HK1*, *HK2*, and *HKDC1* in nonmalignant cells and malignant cells from 46 published liver cancer patient samples, including 25 patients with HCC (left panel) and 12 patients with CCA (right panel).[29] (B) tSNE plots of *HKDC1*, *HK1*, and *HK2* expression in the malignant and nonmalignant cells in the samples in (A). (C) *HKDC1*, *HK1*, and *HK2* expression in individual cell types using the published scRNA-seq data from 7 patients with liver cancer.[30] Hepatocytes and cholangiocytes were derived from tumor-adjacent normal liver tissues of patients with HCC and CCA. (D) H&E and RNAscope staining of *HKDC1*, *HK1*, and *HK2* on the serial sections from the indicated normal human liver and HCC and CCA patient tumors. Solid arrows: normal cholangiocytes; open arrows: normal hepatocytes. Inserts: boxed area in the same image. RNAscope images on the same column share the same scale bar. Abbreviations: CAFs, cancer-associated fibroblasts; CCA, cholangiocarcinoma; H&E, hematoxylin and eosin; HKDC1, hexokinase domain containing 1; scRNA-seq, single-cell RNA sequencing; TAMs, tumor-associated macrophages; TECs, tumor-associated endothelial cells.

HK2 is mostly expressed in the nonmalignant compartment in liver cancer, our data generated in Huh7 cells unlikely reflect its metabolic functions in liver cancer. Overall, our findings demonstrate that HKDC1 is functionally nonredundant with HK1 and HK2 and is likely involved in the TCA cycle to promote tumor cell growth.

HKDC1 promotes HCC metastasis

Since Hkdc1 was a top upregulated gene in metastatic tumors and organoids in our PPTR and PNR models, we examined its prometastatic functions in the human HCC cell lines. Because of the low levels of endogenous HKDC1 in Huh7 cells (Figure 3A), we used this cell line to overexpress HKDC1 using an HKDC1-IRES-tdTomato construct (Figure 4A). Significantly accelerated cell growth was observed in HKDC1-OE Huh7 cells (Figure 4B). The protumorigenic function of HKDC1 in liver cancer has been studied in the past, however, only in subcutaneous models.[31] Considering the physiological impact of the host liver microenvironment on primary liver cancer, we established orthotopic cell line–derived xenografts by injecting the control and HKDC1-OE Huh7 cells into the liver of NOD scid gamma mice (n = 5/group). All mice were allowed to reach their humane endpoint to assess their survival. To our surprise, we found no differences in animal survival between the control and OE groups when using either 1×10^4 cells/mouse or 1×10^5 cells/mouse (Figure 4C). No liver or lung metastases were detected in any groups either. Upon further examination, we noticed that Huh7 control cells, despite their undetectable *HKDC1* expression in vitro, showed high levels of *HKDC1* in the tumors they generated in vivo (Figure 4D). This suggests that *HKDC1* expression can be induced in vivo, which may explain the limited differences observed in the tumor development between the control and OE Huh7 cells.

To exclude the potential interference of *HKDC1* upregulation in vivo, we generated *HKDC1* knockout (KO) through CRISPR/Cas9 in PLC/PRF/5 cells. We subsequently generated *HKDC1*-rescued cells by re-expressing *HKDC1* in the KO cells (KO/Res) (Figure 4E). As expected, *HKDC1*-KO PLC/PRF/5 cells showed slowed cell growth in vitro compared to the control cells, and the re-expression of *HKDC1* in the KO cells accelerated their growth (Figure 4F). However, no statistical differences in survival were observed among the mice orthotopically transplanted with the control, KO, and KO/Res PLC/PRF/5 cells (n = 5/group) (Figure 4G). No lung metastasis was found in any of the groups either. We then tested whether the addition of CAFs, which are known to promote cancer cell migration,[32,33] could help tumor cells reach the lung where HKDC1 would promote metastatic growth. We used LX2 cells, a human HSC line, and HSCs are known to be the main source of liver CAFs.[34] We cultured HKDC1-KO and HKDC1-KO/Res cells with conditioned medium of LX2 cells, followed by a transwell migration assay. We found that both HKDC1-KO and HKDC1-KO/Res cells cultured with LX2 conditioned medium showed increased migration (Supplemental Figure S6, http://links.lww.com/HEP/I661). We then tested the coinjection LX2 cells with the KO or KO/Res PLC/PRF/5 cells. Mice orthotopically injected with LX2 and KO/Res cells showed significantly shorter survival than those injected with LX2 and KO cells (n = 5/group) (Figure 4G). Moreover, 3 out of the 5 mice injected with LX2 and KO/Res cells developed distant lung metastases, which did not occur in mice injected with LX2 and KO cells (Figure 4H). Finally, we tested *HKDC1*-manipulated PLC/PRF/5 cells in a tail veil injection lung metastasis model (1×10^6 cells/mouse, n = 3/group). Unlike the limited differences we observed in the orthotopic cell line–derived xenografts, the development of lung metastasis was drastically accelerated in mice injected with KO/Res cells compared to KO cells, with the former having significantly more and larger metastases in all mice when examined 3 months after tail veil injection (Figures 4I, J). Based on these results, we conclude that HKDC1 is prometastatic predominantly through its ability to promote metastatic growth after tumor cells arrive at a premetastatic niche such as the lung.

HKDC1 supports HCC cell growth under hypoxia

Next, we compared their transcriptomes of HKDC1-manipulated PLC/PRF/5 cells through RNA-seq. Gene set enrichment analysis found hypoxia as one of the top enriched pathways when comparing KO versus control cells and KO/Res cells versus KO cells (Figure 5A). This was not unexpected as cells often undergo metabolic adaption toward enhanced glycolysis under hypoxia.[35] Consistently, we also detected a significant and positive correlation between the expression of *HKDC1* and *HIF1A* (Figure 5B), a master regulator of hypoxia,[36] in HCC and CCA patient tumor RNA-seq profiles available in The Cancer Genome Atlas Program. This correlation was also detected in the PPTR tumors and organoids (Figure 5C). RNAscope staining confirmed the colocalization of these 2 genes in HCC patient tumors and PPTR tumors (Figure 5D). Cultivation under hypoxia (1% O_2) for 24 hours induced HIF1α and HKDC1 proteins in all 3 HCC cell lines (Figure 5E). This finding promoted us to examine the impact of HKDC1 loss under hypoxia. We compared the control and 2 HKDC1-KO PLC/PRF/5 single-cell clones cultivated under normoxia (21% O_2) and hypoxia. As expected, the KO cells were able to expand, although more slowly than the control cells under normoxia. However,

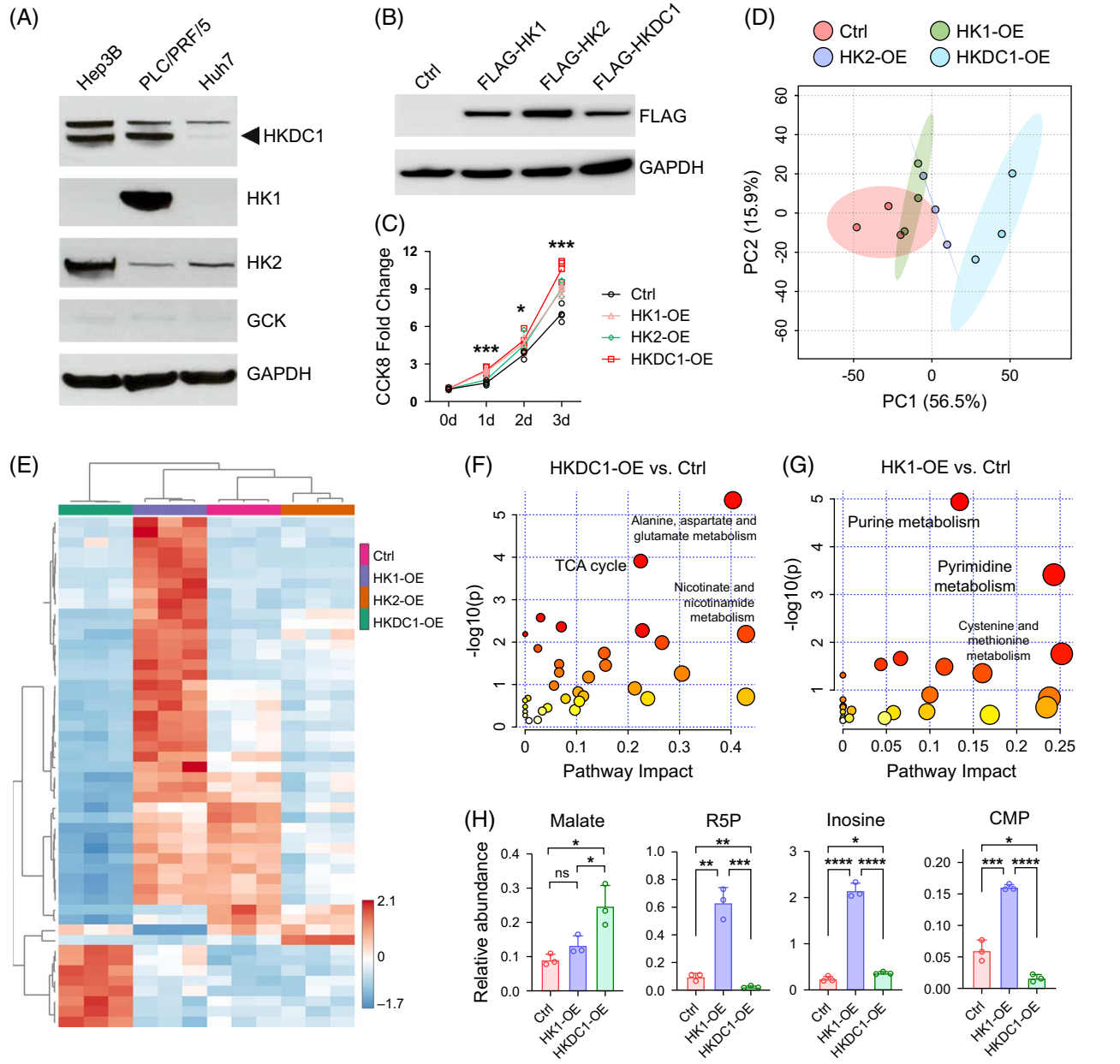

FIGURE 3 HKDC1, HK1, and HK2 are involved in different metabolic activities in HCC cells. (A) Immunoblotting of the HKs in the indicated HCC cell lines. (B) Immunoblotting of the FLAG-tagged HKs in Huh7 cells. (C) Cell proliferation assay of the HK-OE Huh7 cells. Student *t* test. *p* values: * < 0.05; *** < 0.001. (D) PCA of metabolomics from Huh7 isogenic cell lines with ectopic overexpression of control, HK1, HK2, and HKDC1, respectively. (E) Heatmap of the significantly altered metabolites among the 4 isogenic cell lines selected through one-way ANOVA test with *p* values ≤ 0.05. (F) Pathway enrichment analysis of the metabolites differentially upregulated by HKDC1-OE. (G) Pathway enrichment analysis of the metabolites differentially upregulated by HK1-OE. (H) Comparison of the relative abundances of selected metabolites determined by metabolomics in the indicated cell lines. *p* values: * < 0.05; ** < 0.01; *** < 0.001; **** < 0.0001. Abbreviations: HK, hexokinases; HKDC1, hexokinase domain containing 1; PCA, principal component analysis.

under hypoxia, we noticed a complete growth halt of the KO cells during the first 24 hours, while the control cells were able to expand similarly to the normoxic condition. The KO cells resumed the growth after 24 hours and then expanded at a similar rate as the KO cells under normoxia (Figure 5F). These results suggest that HKDC1 can be induced by hypoxia to provide growth support to HCC cells under acute hypoxic stress.

HKDC1 binds to GSK3β to stabilize β-catenin and promote liver cancer stemness

The association between HKDC1 and metastasis, organoids, and hypoxia led us to investigate its potential role in stemness properties, given that organoids are known to enrich stem cell population,[37,38] and that hypoxia induces stemness and metastasis in various cancers.[39,40] We observed colocalization of *HKDC1* expression with liver

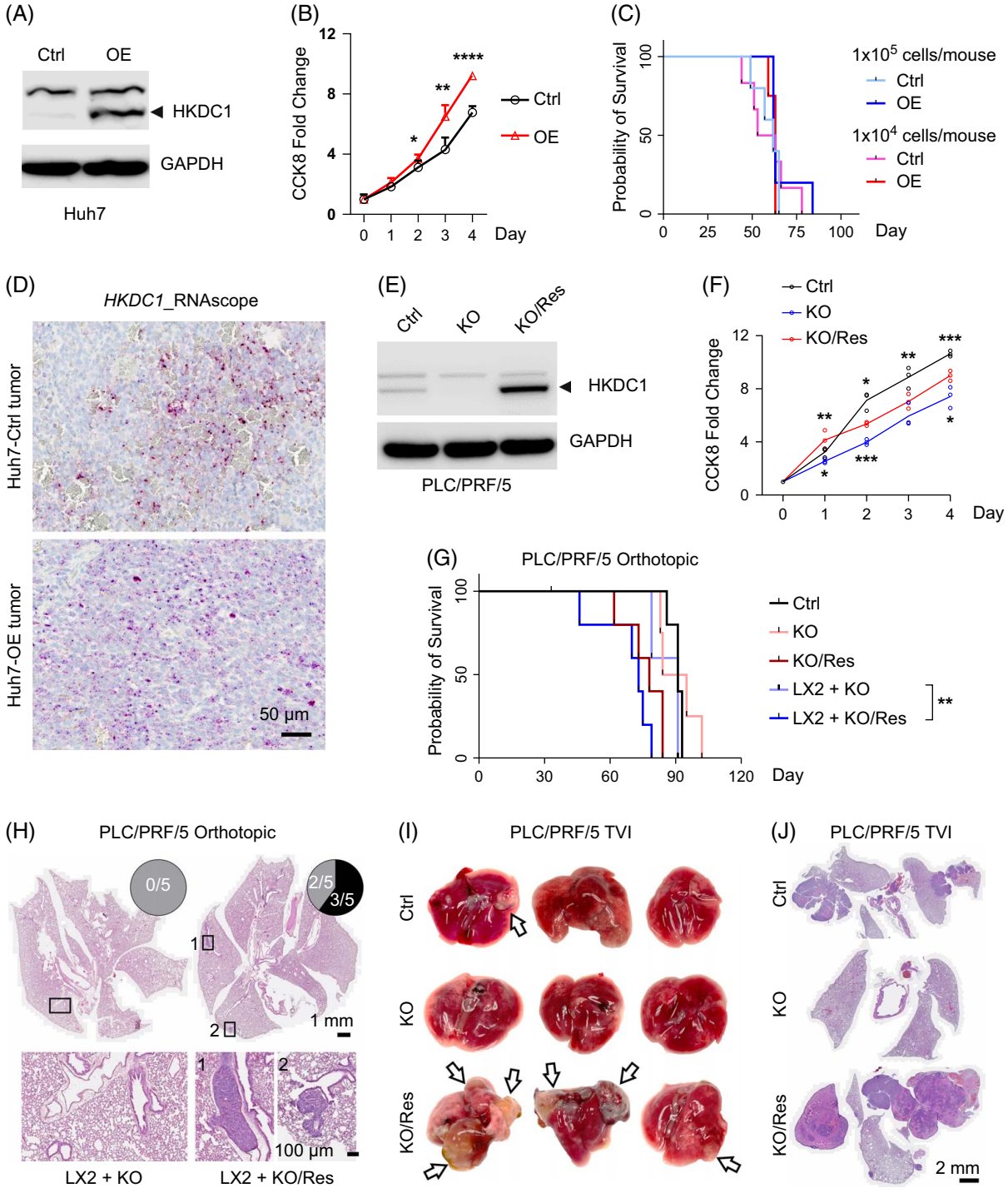

FIGURE 4 HKDC1 promotes HCC metastasis. (A) Immunoblotting of HKDC1 in the control and HKDC1-OE Huh7 cells. (B) Cell proliferation assay of the control and HKDC1-OE Huh7 cells. (C) Kaplan-Meier survival curves of mice injected with the indicated Huh7 cells. (D) *HKDC1* RNAscope on the liver tumors generated by the control and HKDC1-OE Huh7 cells. (E) Immunoblotting of HKDC1 in the control, HKDC1-OK, and -OK/Res PLC/PRF/5 cells. (F) Cell proliferation assay of the control, HKDC1-OK, and -OK/Res PLC/PRF/5 cells. *p* values on the top of the curves: KO/Res versus KO; *p* values on the top of the curves: KO versus control. (G) Kaplan-Meier survival curves of mice injected with the indicated PLC/PRF/5 cells. (H) Top: whole-lung H&E images of mice injected with LX2 mixed with the HKDC1-OK and -OK/Res PLC/PRF/5 cells, respectively; bottom: high-magnification images of the boxed areas in the top image. Pie chart: The number of mice with lung metastasis (black) and with no lung metastasis (gray). Images on the same row share the same scale bar. (I) Gross lung images of the TVI mice injected with the indicated PLC/PRF/5 cells. Arrows: lung metastasis. (J) H&E images of the lung tissues in (J). Images share the same scale bar. Statistics in (B, C, F): Student *t* test. *p* values: * < 0.05; ** < 0.01; *** < 0.001; **** < 0.0001. Statistics in (C) and (G): Log-rank (Mantel-Cox) test. *p* values: ** < 0.01. Abbreviations: H&E, hematoxylin and eosin; HKDC1, hexokinase domain containing 1; KO, knockout; TVI, tail veil injection.

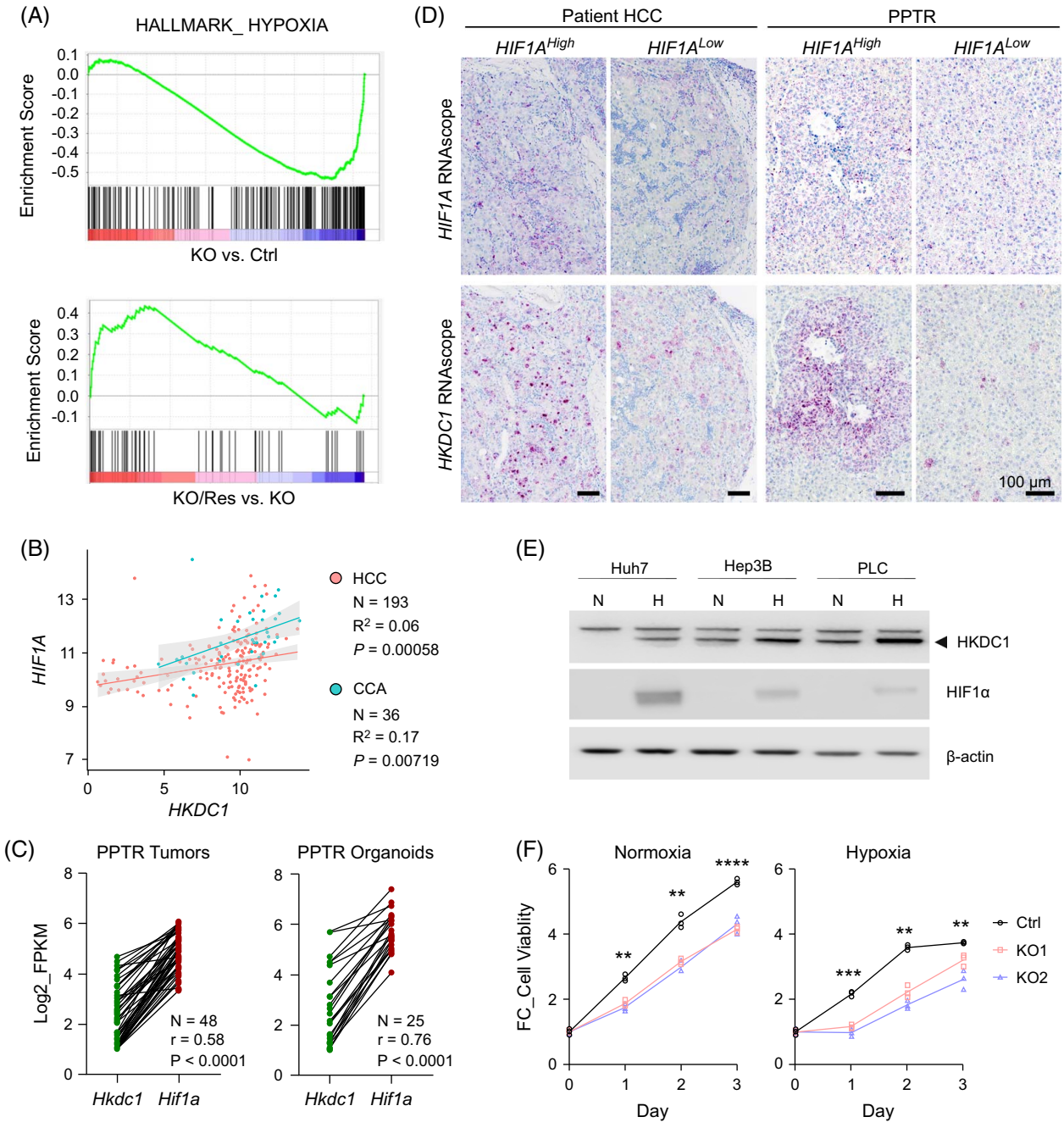

FIGURE 5 HKDC1 is induced by hypoxia to support HCC cell growth under hypoxia. (A) GSEA analysis found the enrichment of HALLMARK_HYPOXIA gene signature in HKDC1-KO versus control and KO/Res versus KO PLC/PRF/5 cells. (B) Spearman correlation analysis of *HKDC1* and *HIF1A* gene expression using RNA-seq data on HCC and CCA patient tumors in TCGA. (C) Spearman correlation analysis of *Hkdc1* and *Hif1a* gene expression in PPTR tumors and organoids. (D) *Hkdc1* and *Hif1a* RNAscope staining on HCC and PPTR tumor serial sections. Images on the same column share the same scale bar. (E) Immunoblotting of HKDC1 in the indicated cells cultivated under normoxia (N, 21% O_2) and hypoxia (H, 1% O_2) for 24 hours. (F) Cell proliferation assay of the control and HKDC1-KO PLC/PRF/5 cells under normoxia and hypoxia. *p* values on the top of the curves: control cells, hypoxia versus normoxia; *p* values on the top of the curves: KO cells, hypoxia versus normoxia. Statistics in (F) Student *t* test of the control versus KO1 cells. *p* value, ** < 0.01; *** < 0.001; **** < 0.0001. Abbreviations: CCA, cholangiocarcinoma; GSEA, gene set enrichment analysis; HKDC1, hexokinase domain containing 1; PPTR, Prom1^{CreERT2}; Pten$^{flx/flx}$; Tp53$^{flx/flx}$; Rosa-ZsGreen; TCGA, The Cancer Genome Atlas.

stem cell markers CK19,[41] EpCAM,[42] and β-catenin[43,44] in PPTR genetic tumors, HCC, and CCA patient tumors (Figure 6A). Molecularly, we studied the potential interaction between HKDC1 and β-catenin for the critical role of the β-catenin/WNT signaling pathway in liver cancer stem cells.[18,19] However, no positive association was found between the RNA levels of *Hkdc1* and *Ctnnb1* (the gene encoding β-catenin) in the PPTR and PNR organoids or HCC and CCA patient tumors (Supplemental Figure S7, http://links.lww.com/HEP/I661).

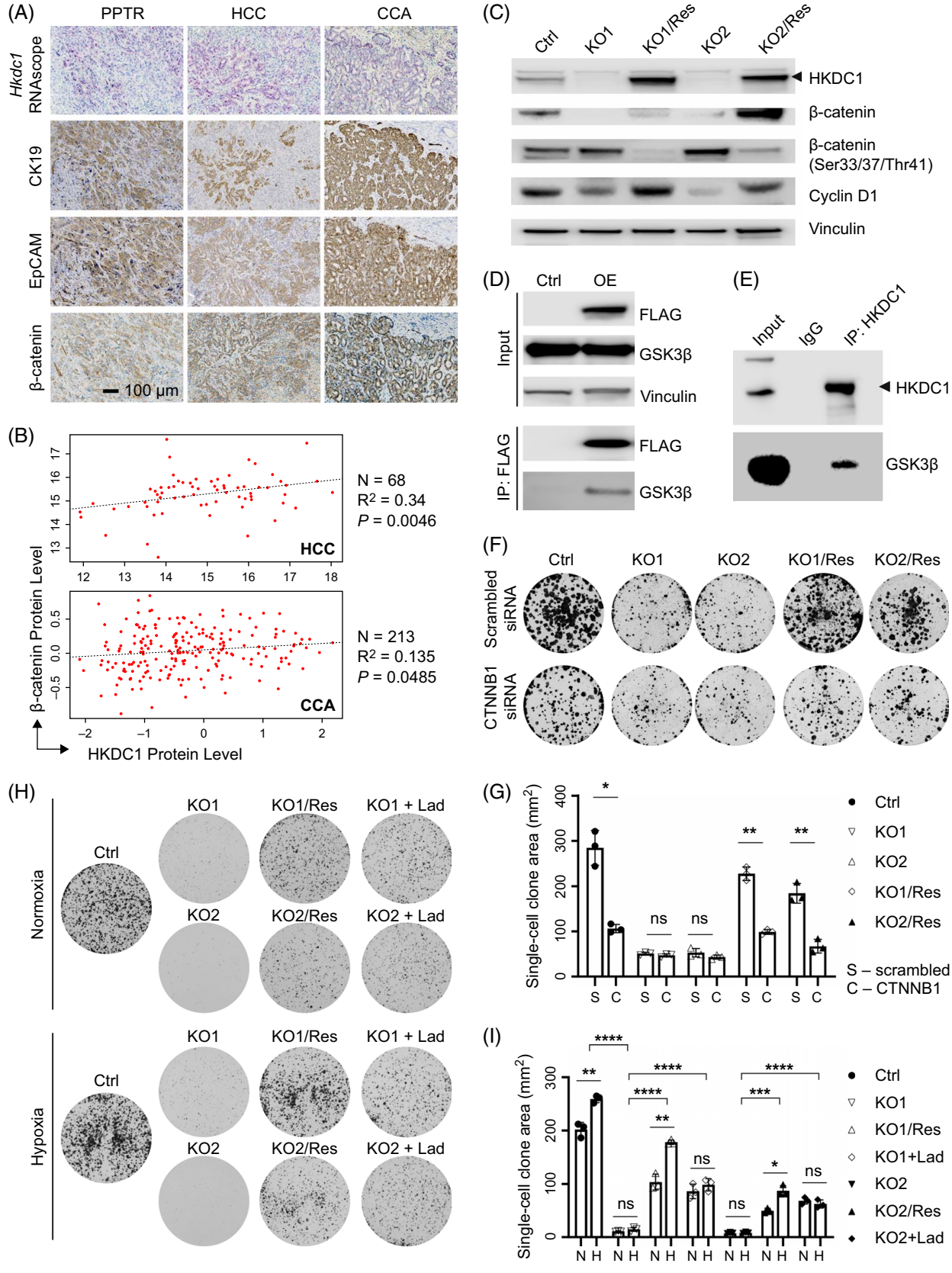

FIGURE 6 HKDC1 promotes HCC stemness by binding to GSK3β and stabilizing β-catenin. (A) RNAscope staining of *Hkdc1* and IHC staining of CK19, EpCAM, and β-catenin on serial sections of tumors from the PPTR genetic model and patients with HCC and CCA. All images share the same scale bar. (B) Spearman correlation analysis of HKDC1 and β-catenin protein levels in HCC and CCA patient tumors. (C) Immunoblotting of the indicated proteins in the control, HKDC1-OK, and -OK/Res PLC/PRF/5 cells. (D) GSK3β IP from HKDC1-FLAG-OE Huh7 cells using FLAG

antibody. (E) GSK3β IP from PLC/PRF/5 cells using HKDC1 antibody. (F) Colony formation assay of the PCL/PFR/5 cells transfected with scrambled or *CTNNB1* siRNA. (G) Image-based quantification of the total colony area in (F). (H) Colony formation assay of the indicated PCL/PFR/5 cells cultured under normoxia and hypoxia. (I) Image-based quantification of the total colony area in (H). Statistics in (A) and (G): Student *t* test. *p* values: * < 0.05; ** < 0.01; *** < 0.001; **** < 0.0001. Abbreviations: CCA, cholangiocarcinoma; HKDC1, hexokinase domain containing 1; IHC, immunohistochemistry; PPTR, Prom1^{CreERT2}; Pten$^{flx/flx}$; Tp53$^{flx/flx}$; Rosa-ZsGreen.

However, published proteomics data on patient HCC[45] and CCA[46] showed that the protein levels of HKDC1 and β-catenin were positively correlated (Figure 6B), suggesting a potential regulation of β-catenin by HKDC1 at the protein level. Indeed, in the HKDC1-manipulated PLC/PRF/5 single-cell clones, higher levels of total β-catenin protein were detected in the KO/Res cells while the KO cells showed higher levels of the phosphorylated β-catenin proteins (Ser33/37/Thr41) (Figure 6C) which is known to be associated with β-catenin protein degradation.[47] Consistently, elevated levels of cyclin D1, a downstream effector of β-catenin,[48] were found in the KO/Res cells than the KO cells (Figure 6C). Phosphorylation of β-catenin is a key mechanism mediating its ubiquitination and subsequent proteasomal degradation, a process mediated mostly by glycogen synthase kinase 3β (GSK3β).[49] Therefore, we examined the potential interaction between GSK3β and HKDC1. In Huh7 cells overexpressing FLAG-tagged HKDC1, we pulled down GSK3β with the FLAG antibody through immunoprecipitation (Figure 6D). Similarly, in PLC/PRF/5 control cells, endogenous HKDC1 was coimmunoprecipitated with GSK3β protein (Figure 6E). To test whether the observed promotion of tumor cell growth by HKDC1 is at least partially mediated by β-catenin, we performed a clonal growth assay of the control, HKDC1-KO, and HKDC1-KO/Res PCL/PRF/5 cells transfected with either scrambled siRNA or *CTNNB1* siRNA (Figure 6F). In the control group transfected with the scrambled siRNA, KO/Res cells grew significantly better than the KO cells as expected. In the *CTNNB1* siRNA knockdown group, the clonal growth of HKDC1-KO cells was not affected, while that of the HKDC1-KO/Res cells dropped significantly (Figures 6F, G). Consistently, treatment with a GSK3β inhibitor, laduviglusib (also known as CHIR99021),[50] restored the clonality of the KO cells to levels similar to the KO/Res cells under both the normoxic and hypoxic culture conditions (Figures 6H, I). Overall, these results suggest that HKDC1 can bind to GSK3β to stabilize β-catenin and promote HCC growth.

DISCUSSION

In this study, we identified HKDC1 as a top upregulated gene in the metastatic tumors and organoids from 2 liver cancer mouse models previously established by our group. HKDC1 is also upregulated in HCC and CCA patient tumors and other cancers.[13,31,46,51,–54] Compared to the other 2 HKs commonly upregulated in liver cancer, HK1 and HK2, we found HKDC1 is most specifically expressed in the malignant tumor cells in patient tumors, while HK1 and HK2 are prevalent in nonmalignant TME lineages. Our unbiased metabolomic profiling suggests that these 3 HKs are involved in distinct metabolic activities. Under the experimental conditions we used, HKDC1 is uniquely involved in regulating the TCA cycle in HCC cells. Using HCC orthotopic models, we showed that HKDC1 promoted lung metastasis, although this effect was moderate. However, for HCC cells already arriving in the lung, HKDC1 is a strong driver of their metastatic growth. Finally, we showed that HKDC1 responds to hypoxia and can promote HCC stemness by binding to GSK3β and stabilizing β-catenin protein.

HKDC1 is a recently identified HK family member. Our study confirmed its upregulation and protumorigenic roles that have been reported in various cancers, including liver cancer.[13,14,31,46,51–53] However, this study is the first to demonstrate its prometastatic function using orthotopic HCC transplantation models. This is also the first study demonstrating the cell-type specificity of HKDC1 in the normal and malignant liver in comparison to HK1 and HK2, which are also commonly upregulated in liver cancer. In the normal liver, we found that all 3 HKs are lowly expressed in cholangiocytes but not in hepatocytes. However, in HCC and CCA patient tumors, HKDC1 expression is specifically upregulated in malignant tumor cells, while HK1 and HK2 upregulation is seen across many TME lineages. We believe this finding may provide an explanation to the seemingly contradictory observations published previously that the higher HKDC1 level is associated with poorer prognosis of patients with HCC[14] but better survival of patients with CCA.[46] CCA is well known for its low tumor cellularity due to the abundant TME components.[55] More advanced CCA have a greater nonmalignant TME compartment which can result in lower tumor cell content and lower HKDC1 levels detected through bulk tumor-based analyses. Indeed, in all CCA patient samples we examined, high levels of HKDC1 expression were found in the tumor cells but not in their non-malignant TME lineages. This highlights the importance of dissecting the cellular specificity when it comes to studying molecular drivers of tumors with complex TME, such as liver cancer.

Our metabolomic profiling of HK-OE Huh7 cells suggests that these 3 HKs are involved in distinct metabolic activities. Specifically, HKDC1 demonstrates a pronounced impact on the TCA cycle, while HK1-OE predominantly affects nucleotide metabolism, and HK2-OE causes the least metabolic changes in Huh7 cells.

This involvement of HKDC1 in the TCA cycle is consistent with 2 recent studies which have revealed that HKDC1 interacts with mitochondria, the organelle where the TCA cycle occurs, to support liver cancer progression[31] and prevent cellular senescence.[56] Our identification of the regulation of β-catenin by HKDC1 at the protein level provided new insights to other potential routes through which HKDC1 modulates the TCA cycle besides its HK function in glycolysis. β-catenin is essential to the maintenance of hepatic mitochondrial homeostasis.[57] It regulates many metabolic pathways that are involved in the TCA cycle, such as nucleoside biosynthesis,[58,59] glutamate uptake and metabolism,[60] and lipogenesis,[61,62] which converts fatty acids and sugars into acetyl-CoA to feed into the TCA cycle.[63] In addition, since mitochondria play an important role in metastasis by modulating metabolic and genetic responses to dynamic TME cues,[64] it supports our observation that HKDC1 responds to hypoxia and needs assistance from CAFs to promote HCC metastasis. CAFs, one of the most critical TME components of liver cancer, are widely known for their roles in creating hypoxic TME and promoting tumor cell dissemination.[54,65] As HKDC1 is known to have a low HK activity and low expression in the normal liver,[9,10] it is conceivable that HKDC1 is not a primary nor classical HK in tumor cells but rather a stress responder, such as under acute hypoxia. It likely collaborates with mitochondria and possibly other organelles and signaling pathways, such as β-catenin/WNT pathway, as our study has shown, to maintain energy production in tumor cells under stress. This provides an interesting future direction for exploring the mechanistic link between HKDC1 and liver cancer progression from the perspective of tumor-TME interaction. Despite its low expression in normal tissues, the specific upregulation of HKDC1 in tumor cells and metastases makes it a promising therapeutic target in patients with advanced liver cancer, particularly those who have developed distant metastases. Further investigation into the precise roles and regulatory mechanisms of HKDC1 in liver tumorigenesis and metastasis is needed to fully exploit its therapeutic potential.

DATA AVAILABILITY STATEMENT

The RNA-seq datasets generated during this study are available in the NCBI GEO repository (GSE278074). The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials, or available from the corresponding author on reasonable request.

AUTHOR CONTRIBUTIONS

Li Fan and Cheng Tian conducted most of the biological experiments. The order of the co-first authors was assigned based on their intellectual contributions. Xiaoli Liu and Haiyan Tan conducted experiments. Yogesh Dhungana, Wentao Yang, Lichun Ma, and Min Ni conducted the computational analyses. Evan S. Glazer, Junmin Peng, and Jiyang Yu provided technical and intellectual support. Liqin Zhu conceived and oversaw the research. All authors contributed to the writing of the manuscript.

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CONFLICTS OF INTEREST

The authors have no conflicts to report.

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