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TITLE: AA Novel Fifth Hexokinase, HKDC1, Mediates NASH-Induced Liver Cancer Through Modulating Mitochondrial Function

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Cancer cells dynamically undergo metabolic reprogramming to enhance glucose metabolism which is essential for both energy production and providing building blocks. The role of the mitochondria has independently emerged as a key regulator of metabolic reprogramming due to their role in sensing and controlling nutrient flux and metabolism. The effect of hexokinase (HK) interaction with the mitochondria has been documented in cancer; however, the exact mechanisms are not well established. Recently a 5th HK, hexokinase domain containing 1 (HKDC1) has been shown to be significantly overexpressed in hepatocellular carcinoma (HCC) to a greater degree than other HKs. Non-alcoholic steatohepatitis (NASH) is an independent risk factor for the development of HCC particularly due to its prevalence in developing countries. The goal of this proposal is to mechanistically investigate how HKDC1 could be a link between the progression of NASH to HCC via its role at the mitochondria. Our published work (via knockout and overexpression) shows that 1) HKDC1 has a role in HCC development and progression and 2) its interaction with the mitochondria (via N-terminal) is essential for its role in HCC progression.

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1. **Introduction:** Nonalcoholic fatty liver disease (NAFLD) is a condition in which excess fat in the body is stored in the liver and it is the leading cause of chronic liver disease and the 3rd cause of liver transplantation. The disease ranges from non-alcoholic fatty liver (fat build up in liver known as steatosis) to serious disease conditions such as non-alcoholic steatohepatitis (NASH) to life-threatening liver cancer (90% of which is hepatocellular carcinoma, HCC). Screening studies have shown that at least 30- 40% of the United States adult population today has NAFLD, a significant number of which develop the more serious form of liver damage known as NASH. To makes things worse, NAFLD is more common in people who have some form of other metabolic disease such as obesity and type 2 diabetes both of which have reached epidemic proportions. To complicate things further, there are no noninvasive procedures that can reliably differentiate NAFLD from NASH, making it impossible to know the true incidence and prevalence of this disorder and of the health care related costs, considerable effort is being placed on the design of more effective screening and therapeutic strategies to prevent and reverse NAFLD and stop its progression to HCC. Therefore, therapies that target novel protein and pathways involved in metabolism have the potential to treat and control HCC. We discovered hexokinase domain containing 1 (HKDC1) a to be significantly over-expressed in many different forms of cancer and more so in HCC. We have preliminary data that shows that this novel protein (HKDC1) is over-expressed in NASH & HCC, and we hypothesize that it is a novel link between these two disease conditions (NASH and HCC). Further we have sufficient proof that HKDC1 interacts with the mitochondria, and we believe that this interaction is essential for HKDC1's role in the development of NASH and HCC. To improve pre-existing therapies and to further develop novel strategies to treat NASH and HCC, we need to understand the exact molecular mechanisms by which HKDC1 promotes the progression of these diseased conditions. The focus of this proposal is to evaluate the role and therapeutic potential of targeting HKDC1 to inhibit HCC proliferation and therefore is highly innovative and significant as it will lead to avenues that target novel oncogenic pathways independent of those targeted by existing drugs.
2. **Keywords:** Liver cancer, HKDC1, mitochondria, metabolism, hexokinase
3. **Accomplishments:** During my 2$^{nd}$ year of this grant, I was able to accomplish these goals:

**<u>Major Task 2</u>**

Subtask1: Perform experiment focusing on mitochondrial interaction of HKDC1 on cellular metabolism

**FINDINGS:**

**A) HKDC1 expression is essential for LC growth and proliferation.**

Since HKDC1 has negligible expression in the adult liver (1, 2), and its expression is upregulated in LC (3, 4), we hypothesized enhanced HKDC1 expression promotes LC proliferation. To explore this, we developed a HKDC1 knockout (KO) cell line using Crispr-Cas9 in HepG2 cells (referred as HKDC1-KO from hereon). With these cells, HKDC1 ablation results in diminished proliferation and survival (Fig 1A-B) as shown by reduced expression of proliferation marker, Ki67 (Fig 1C), bromodeoxyuridine (BrdU) incorporation (Fig 1D) and proliferating cell nuclear antigen (PCNA; Fig 1E). Next, by cell-cycle analysis, HKDC1-KO cells have a significant

increase of cells in synthesis (S) phase and a decrease in G2 phase (Fig 1F), which may be due to failure in DNA synthesis machinery or an intra-S phase arrest to progress through this phase. Cyclins and cyclin-dependent kinases (CDKs) are major effectors of the cell cycle with a key role in the cancer (5, 6) where our data shows downregulation of key cell cycle proteins such as cyclin D1 and Geminin (Fig 2E) and mRNA levels of *CCNE1, CCNF, CDK1, CDK2* and *CDK5* (Fig 1G).

Cell migration abilities are essential for cancer metastasis (7). Therefore, we used the classical trans-well assay to assess the effect of HKDC1 ablation on cell invasive properties observing that HKDC1-KO cells lost the ability to migrate and invade (Fig 1H). As cell migration depends on the synthesis of proteoglycans (8, 9), we searched for genes involved in proteoglycan synthesis in our RNA-seq data and found that they were significantly downregulated with HKDC1 ablation in HepG2 cells, which we then confirmed by qPCR (Fig 1I). In an *in vivo* xenograft model (Nu/J mice) we injected EV and KO cells sub-cutaneously and observed that all (100%) mice injected with EV cells developed tumors and no mice injected with HKDC1-KO cells developed tumors (Fig 1J-K). Next, in another model of LC, we used the classical chemical-based (diethylnitrosamine; DEN) induction of LC with our liver-specific HKDC1 knockout mouse (HKDC1-LKO) model by crossing HKDC1 floxed (HKDC1$^{f/f}$) mice (2, 10) with Albumin-Cre mice. DEN was injected at 14 days and the animals were observed for 40 weeks. As a proof of concept, we re-expressed full-length human HKDC1 (using AAVs) in a group of mice from both HKDC1$^{f/f}$ and HKDC1-LKO groups. At the terminal time point (40 weeks), we found that the HKDC1$^{f/f}$ (controls) had larger livers and more tumors than HKDC1-LKO mice (Fig 2A-B). The mice from both control and HKDC1-LKO groups where HKDC1 was overexpressed had larger livers and more tumors (Fig 2A-B). Further, histological analysis revealed that livers from HKDC1-LKO mice with AAV treatment had the highest number of BrdU and Ki67 positive hepatocytes compared to all groups while HKDC1-LKO had the least number of positive cells compared to any other group (Fig 2C-D). These data from both *in vitro* and *in vivo* LC models indicates that HKDC1 regulates several important cellular processes and establishes its role in the LC proliferation.

**B) HKDC1 ablation disrupts glucose metabolism**

Since HKDC1 is a putative HK gene, we next assessed the effect of HKDC1 ablation on other HKs, including their expression, cellular localization and cellular HK activity. We show here that HKDC1 ablation had no effect on the protein or mRNA levels of the other HKs (Supplementary Fig 4A-B). Further, total HK activity in the HKDC1-KO cells was not significantly different from EV cells (Fig 3A). Since HK2 is also expressed in LC (11), we used siRNAs against either HK2 or HKDC1 to determine the relative impacts of these HKs on HK activity in HepG2 cells. Our data shows that when HK2 was knocked down (KD), there was ~ 40% reduction in cellular HK activity (Fig 3B) compared to no change when HKDC1 was KD (Fig 3B). Then, we measured extracellular acidification rate (ECAR) and our data shows that there is no change in glycolysis and glycolytic capacity in HepG2 and SNU-475 cells with HKDC1 knockdown while there was an increase in glycolysis in Hep3B2 cells. Overall, these data are consistent with our earlier studies which show that HKDC1 is a poorly functioning HK (with low Km), as compared HK2 (2) and HK2 has a direct role in enhancing glycolysis (11).

Exploring other aspects of glucose metabolism, we observed that HKDC1 ablation significantly increases glucose uptake and consumption (Fig 3D-E). Intriguingly levels of GLUT4, which is activated by the pro-survival PI3K-Akt pathway (12), were significantly upregulated (Supplementary Fig 4E-F). Next, using steady-state

metabolomics, focused on glucose metabolism, glucose-6-phosphate levels were increased in HKDC1-KO cells (Fig 3F) without a similar increase in other glycolytic or TCA cycle metabolites; however, an increase in the levels of metabolites involved in the PPP and HBP shunts of the glycolysis occurred (Fig 3F).

Going further using targeted metabolomics (U-$^{13}C_6$ labeled glucose), the HKDC1-KO cells, showed that labeled carbons from glucose are converted to glucose-6-phosphate (Fig 3G; upper panel) but did not accumulate in the glycolytic intermediates, and rather cause an increase in PPP and HBP metabolites (Fig 3G; upper panel). Moreover, there is a significant decrease in labeled glucose carbons entering the TCA cycle (Fig 3G; lower panel). Since anaplerosis feeds metabolites to the TCA cycle (13) in addition to glucose, we performed a U-$^{13}C_5$ labeled glutamine targeted metabolomics observing that HKDC1 ablation has a significant decrease in labelled TCA cycle metabolites upon HKDC1-KO (Fig 3H; all panels). As summarized in Fig 3I, cells lacking HKDC1 have enhanced glucose consumption fueling the PPP and HBP pathways but decreased TCA cycle flux.

**Major Task 3**

Subtask 1: Investigate the effect of HKDC1 modulation on mitochondrial metabolism

**FINDINGS: HKDC1 is essential for mitochondrial function**

We have previously shown that HKDC1 localizes to the mitochondria and binds with mitochondrial proteins like VDAC (2), to further confirm this we performed subcellular fractionation with modulated HepG2 cells, to show that HKDC1 was predominantly in the membrane fraction with a lesser amount in the cytosol (Fig 4A). In contrast, HK2, which has been shown to bind to the mitochondria in some cancer cell lines (14, 15), was found to be in the cytosol and membrane fractions (Fig 4A). Interestingly, in HKDC1-KO cells, we did not see more HK2 in the mitochondrial fraction, suggesting that HKDC1 may not compete with HK2 for mitochondrial binding at these cells (Fig 4A). We confirmed this observation by examining two other LC cell lines (Fig 4B). Furthermore, we performed co-immunoprecipitation studies showing that HKDC1 and VDAC colocalize in EV cells, whereas this interaction is absent in HKDC1-KO cells (Fig 4C). Lastingly, in liver sections from DEN treated HKDC1$^{f/f}$ and HKDC1-LKO mice by immunohistochemistry, we observed that HKDC1 and VDAC colocalize in HKDC1$^{f/f}$ liver (Fig 4D) but not in HKDC1-LKO.

Mitochondrial function is essential for carcinogenesis thus, HKDC1 might be required for optimal mitochondrial function in LC. To investigate this, we performed Seahorse analysis three LC cells lines observing that both basal and maximal respiration was significantly reduced in HKDC1-KO/KD cells. These data suggest that HKDC1 is important for mitochondrial function (Fig 4E). Next, we measured activities of mitochondrial complex I, SDH and III observing that HKDC1-KO significantly reduces the activities of all three complexes (Fig 4F). Since energy in the form of ATP mainly comes from mitochondrial activity and enhanced glucose consumption observed in HKDC1-KO cells is not sufficient to maintain ATP levels as indicated by significantly reduced ATP levels (Fig 4G).

Calcium ($Ca^{2+}$) influx from the ER is essential for mitochondrial metabolism therefore, we assessed mitochondrial $Ca^{2+}$ and found that HKDC1-KO/KD cells had significantly higher levels of $Ca^{2+}$ (Fig 4H). High mitochondrial $Ca^{2+}$ also leads to decreased membrane potential (MMP) and induces reactive oxygen species

(ROS) production, we assessed both and found that HKDC1-KO cells had decreased MMP (Fig 5I) and enhanced ROS production (Fig 4J) (16-19). Investigating further, we used transmission electron microscopy to examine ER-mitochondrial appositions and structure in HKDC1-KO cells. We observed that HKDC1-KO cells displayed a markedly higher degree of ER-mitochondria contact sites and lower hepatic mitochondrial number (Fig 4K). We also observed that mitochondria from HKDC1-KO cells were rounder and swollen compared to the tubular mitochondria observed in EV cells (Fig 5K); however, a three-dimensional reconstruction is needed to confirm these observations. These data suggest that higher mitochondrial $Ca^{2+}$ is associated with mitochondrial dysfunction in HKDC1-KO cells. We further confirmed these findings using qPCR for ER stress markers including ER chaperone protein BiP (binding-immunoglobulin protein aka GRP-78), activating transcription factor 4 (ATF4) and C/EBP-homologous protein (CHOP) which are the effectors of PKR-like ER kinase (PERK) (Fig 4M) (20), and inositol requiring enzyme 1α/β (IRE1) (20) (Fig 4M). Therefore, we show here that when HKDC1 is not bound to the mitochondria (HKDC1-KO), there is increased mitochondria-ER interaction, ER stress and mitochondrial dysfunction which may result in altered metabolism and cell death.

**4. Impact:**

Here we show that HKDC1 deletion in human liver cancer cells inhibits proliferation and tumorigenesis both *in vitro* and *in vivo* and hepatocarcinogenesis in mice. While HKDC1 ablation does not change cellular HK activity, it induces glucose uptake with enhanced flux through the glycolysis shunt pathways PPP and HBP, and reduced TCA cycle metabolites. Additionally, we show an increased expression of GLUT4, which seems contradictory as GLUT4 is under the control of the PI3K-Akt pathway. While this pathway is considered pro-tumorigenic, Akt also has pro-survival functions (21, 22) therefore, increased GLUT4 expression could be a pro-survival mechanism in HKDC1-KO cells.

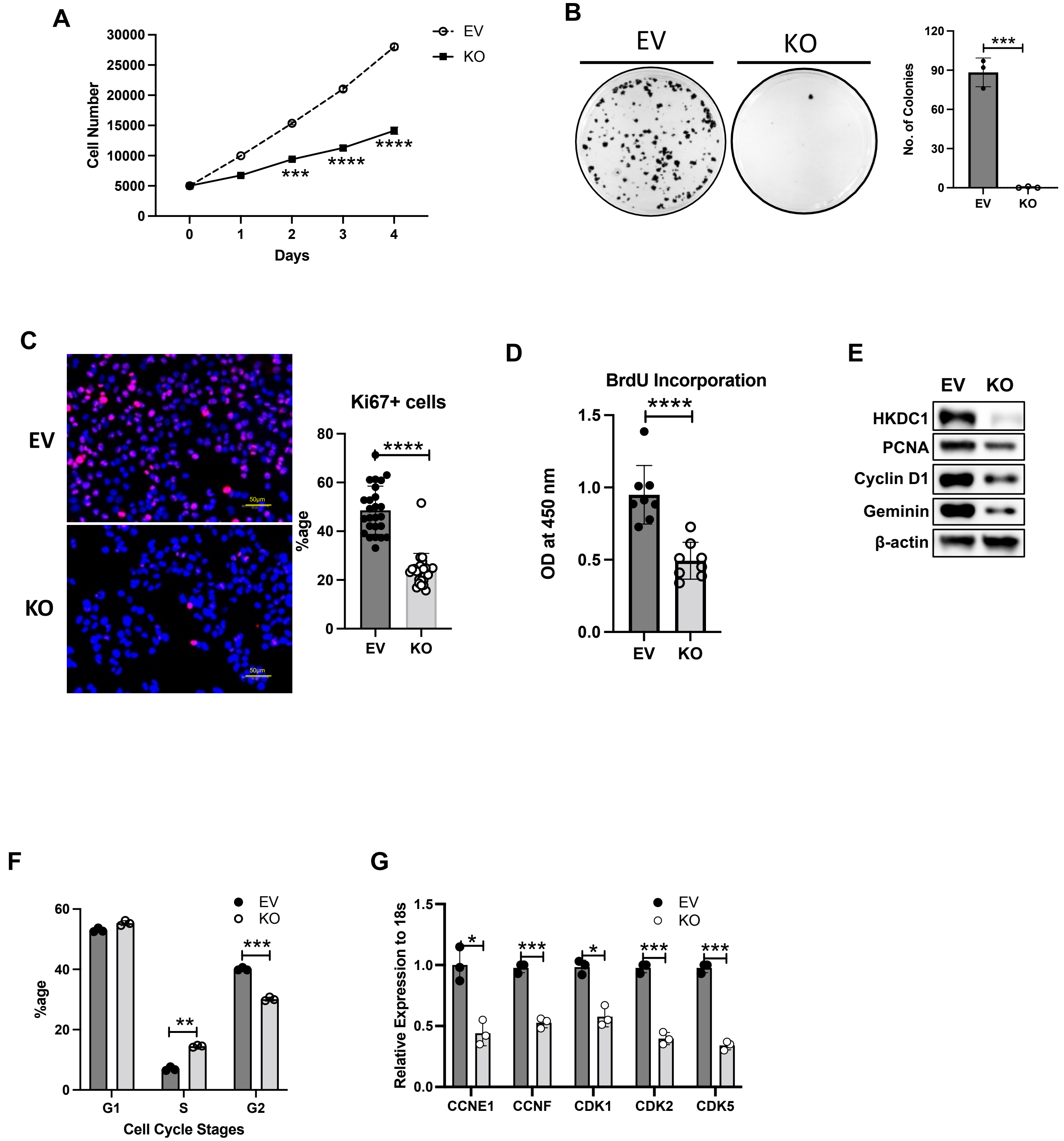
Further mitochondrial binding of hexokinases has been suggested to be necessary for cancer progression (23, 24). We provide evidence here for the first time that HKDC1 interaction with the mitochondria is essential, and its deletion induces mitochondrial dysfunction in LC. We further prove this by using a mitochondrial binding deficient HKDC1 (HKDC1-TR) which when re-expressed in HKDC1-KO cells cannot restore mitochondrial function. As cells make most of their energy in the form of ATP from mitochondrial function, HKDC1-KO cells had significantly less ATP than control cells. This decrease in cellular ATP might result in a metabolic stress where cells then resort to taking in more glucose to meet the energetic demands of the cell. However, since HKDC1-KO cells have impaired mitochondrial function, enhanced glucose flux resulting from increased uptake does not fuel the TCA cycle but is moved to glucose shunt pathways. As cancer cells in the synthetic (S) phase of the cell cycle need ATP to prepare the cells for division, a lack of ATP might result in cell cycle arrest as observed in HKDC1-KO cells, however this needs further mechanistic investigation.

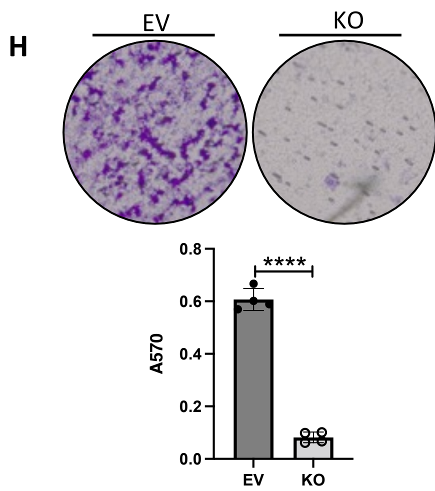
To summarize, mitochondrial bound HKDC1 regulates metabolism, proliferation, and survival of LC and HKDC1-KO significantly affects glucose flux, energy metabolism and mitochondrial function leading to less ATP thereby impacting cell cycle progression and ER stress induction. Since HKDC1 has nominal expression in normal hepatocytes, but is highly upregulated in LC, novel small molecule and peptide-based inhibitors could be designed to target HKDC1, specifically its mitochondrial interaction in LC.

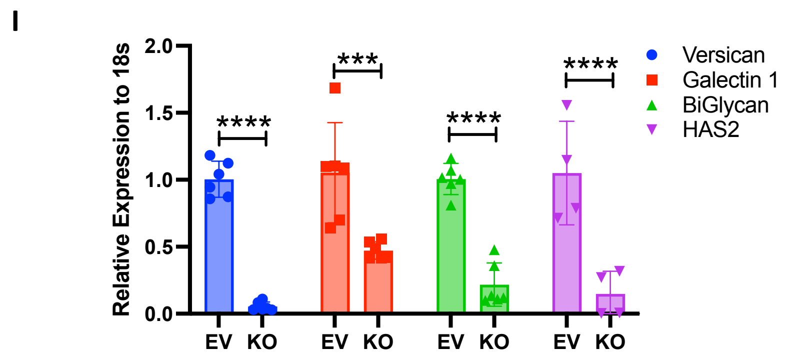
**Changes/Problems:** The main problem that I have faced since the start of this award is that research work was totally stopped due to Covid-19 related closures from March 2020 till Dec 2020. From the start of 2021 till Apr-2021 we were only allowed to work in staggered work schedules which has greatly impacted the progress of this project. We are still facing a lot of supply chain issues for very basic things. I would therefore request grant management to consider this when granting an extension so that this work can be completed.

5. **Products:** Not applicable
6. **Participants & Other Collaborating Organizations:** Not applicable
7. **Special Reporting Requirements:** Not applicable
8. **Appendices:** Not applicable

Fig 1




H
EV
KO
****
A570
0.8
0.6
0.4
0.2
0.0
EV
KO


I
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2.0
1.5
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0.5
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Galectin 1
BiGlycan
HAS2
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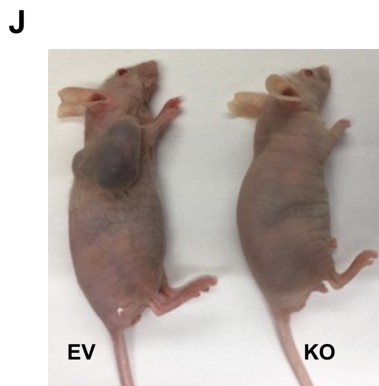
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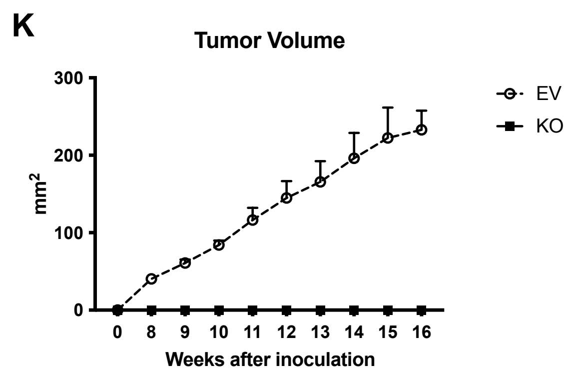
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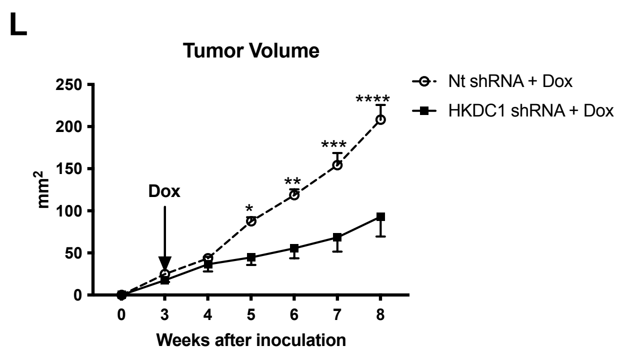
Weeks after inoculation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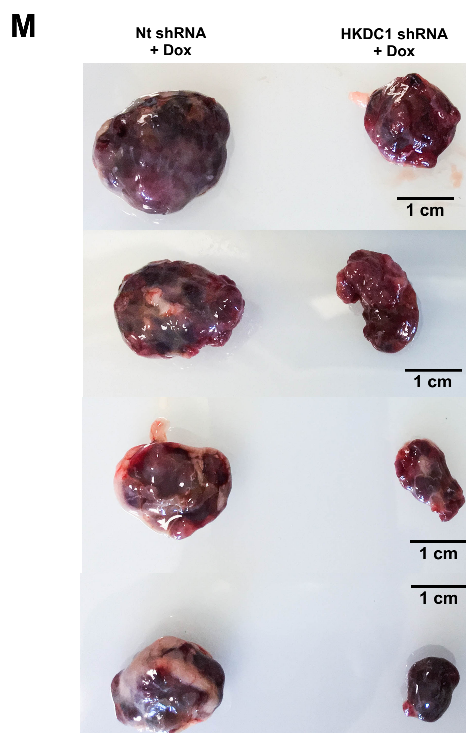
M
Nt shRNA
+ Dox
HKDC1 shRNA
+ Dox
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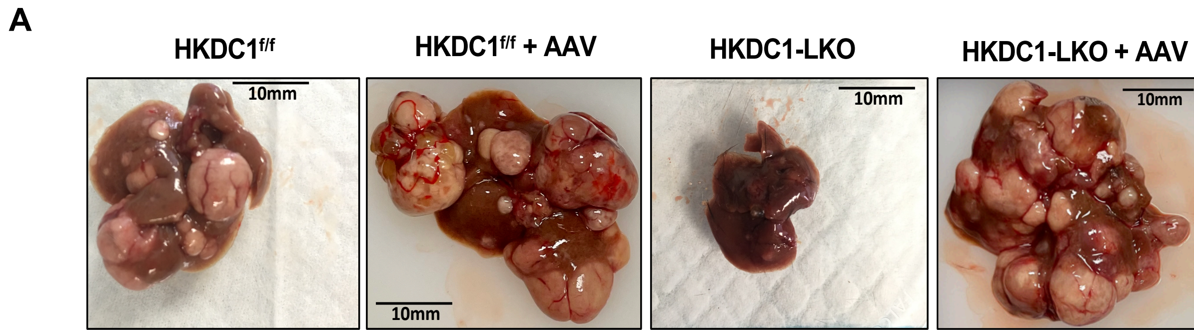
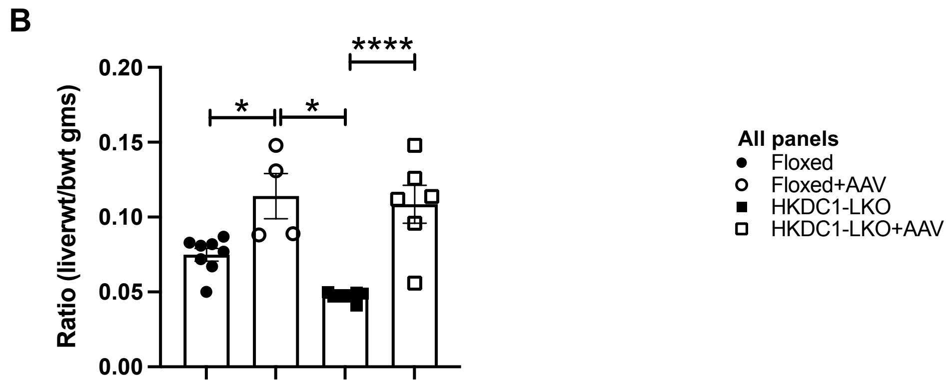
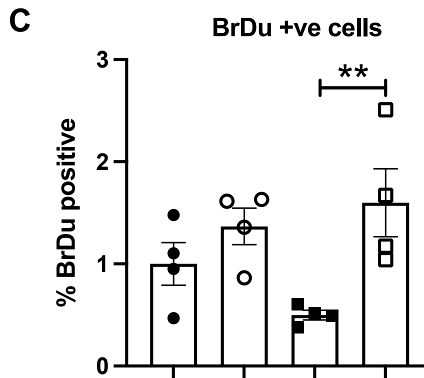
1 cm
1 cm
1 cm

Fig 2



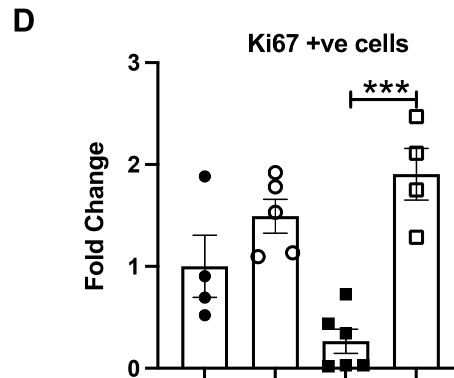
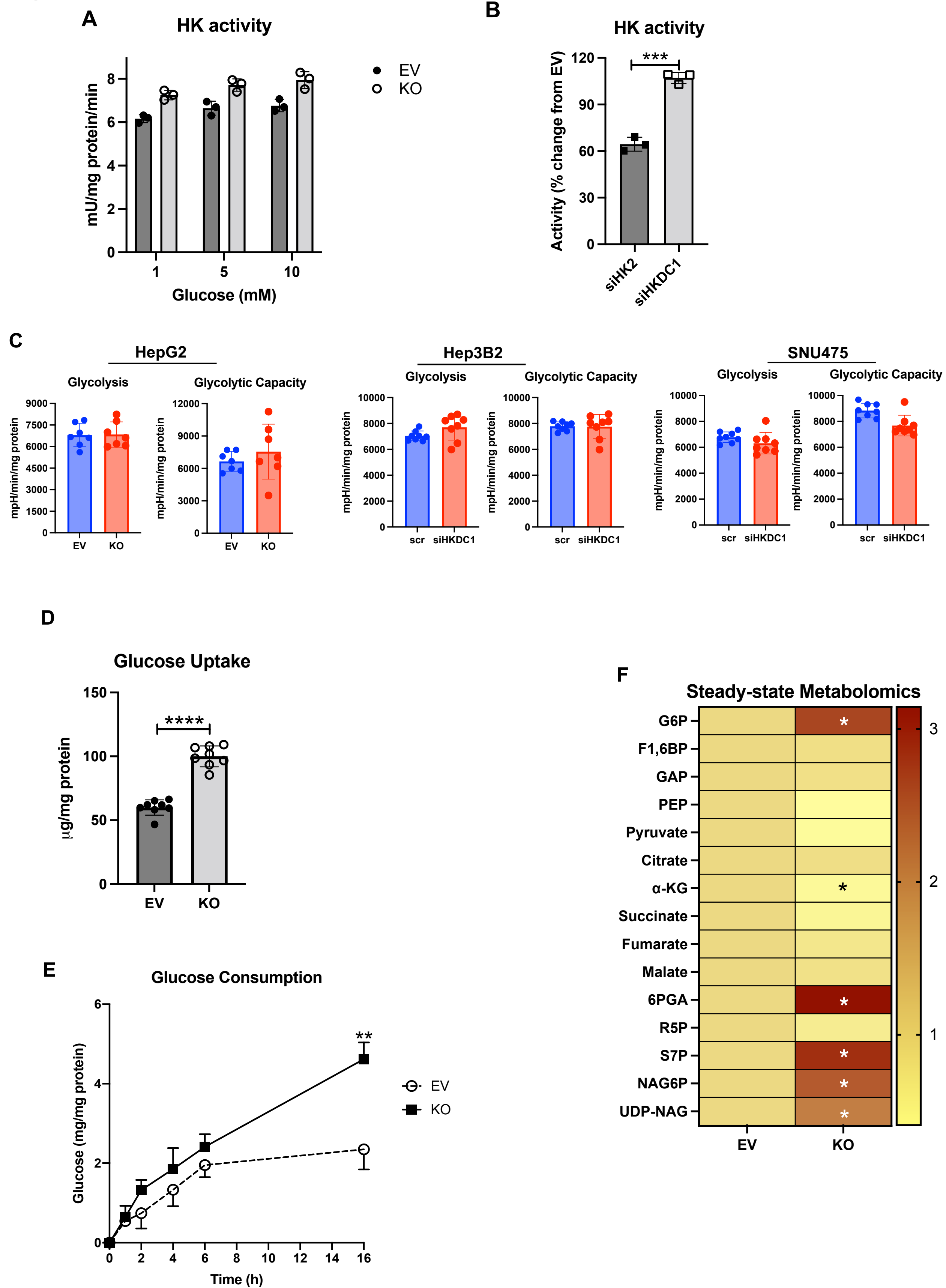



Fig 3



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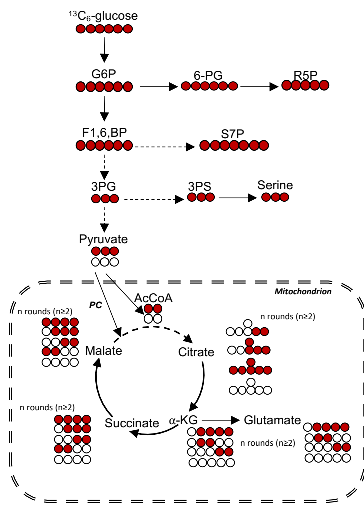


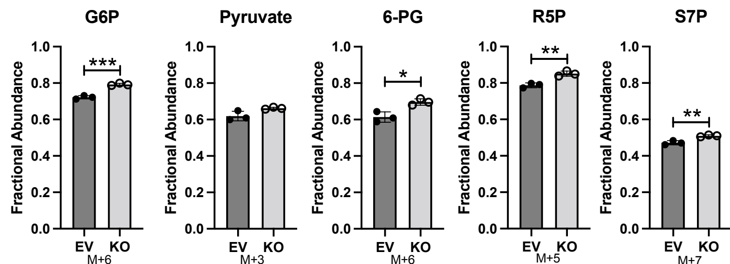


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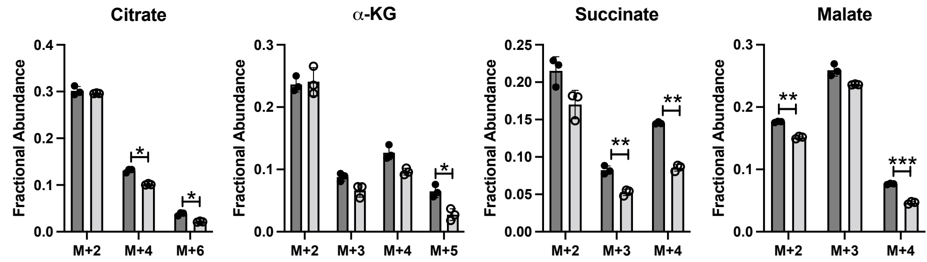
○ KO

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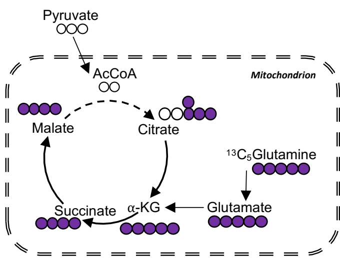


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EV
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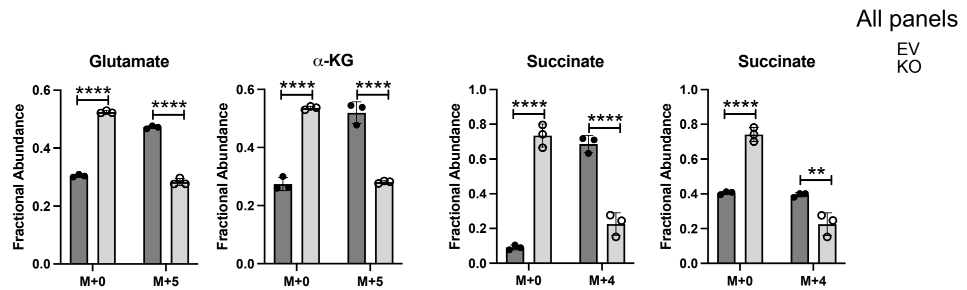


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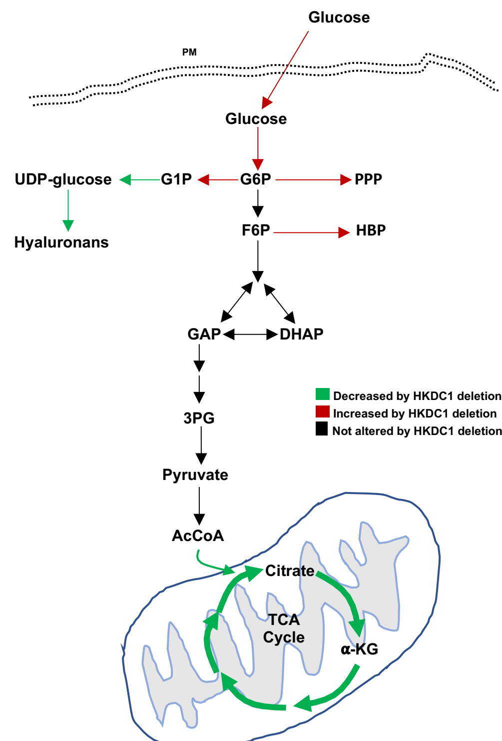


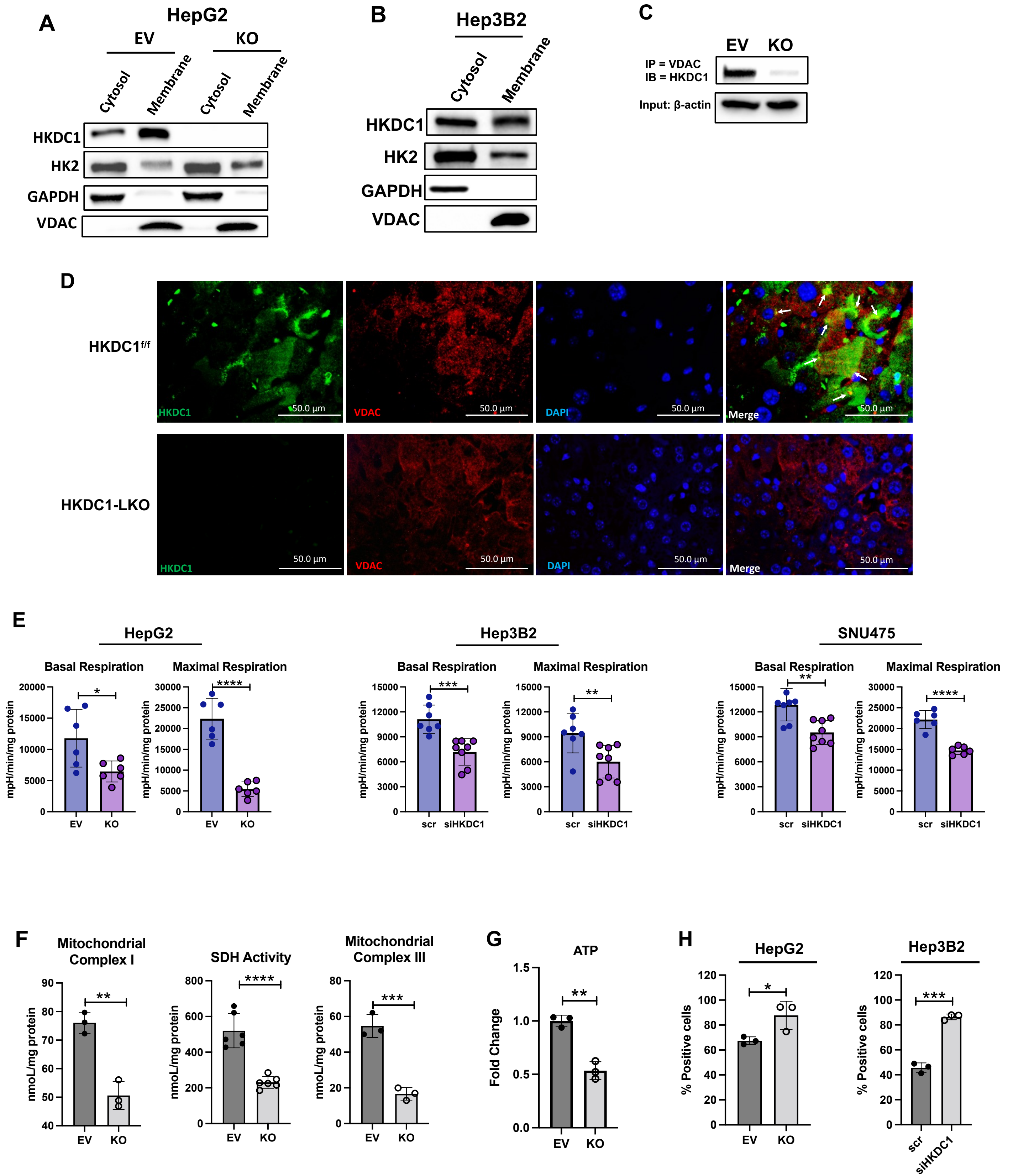
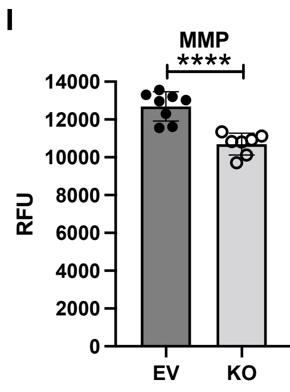


Fig 4
A
HepG2
EV
KO
Cytosol
Membrane
Cytosol
Membrane
HKDC1
HK2
GAPDH
VDAC
B
Hep3B2
Cytosol
Membrane
HKDC1
HK2
GAPDH
VDAC
C
EV
KO
IP = VDAC
IB = HKDC1
Input: β-actin
D
HKDC1f/f
HKDC1-LKO
HKDC1
VDAC
DAPI
Merge
50.0 μm
E
HepG2
Hep3B2
SNU475
Basal Respiration
Maximal Respiration
mpH/min/mg protein
EV
KO
scr
siHKDC1
F
Mitochondrial Complex I
SDH Activity
Mitochondrial Complex III
nmoL/mg protein
G
ATP
Fold Change
H
HepG2
Hep3B2
% Positive cells

I

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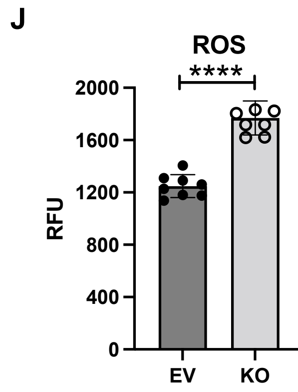


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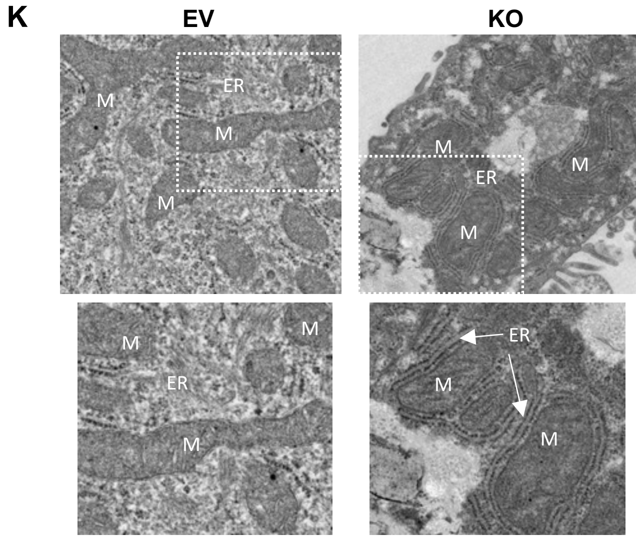


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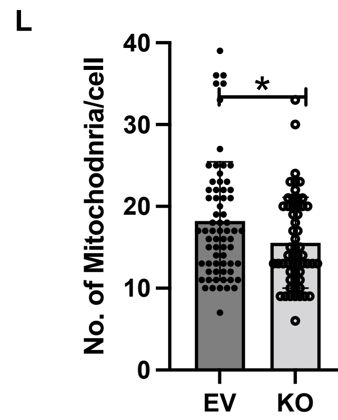


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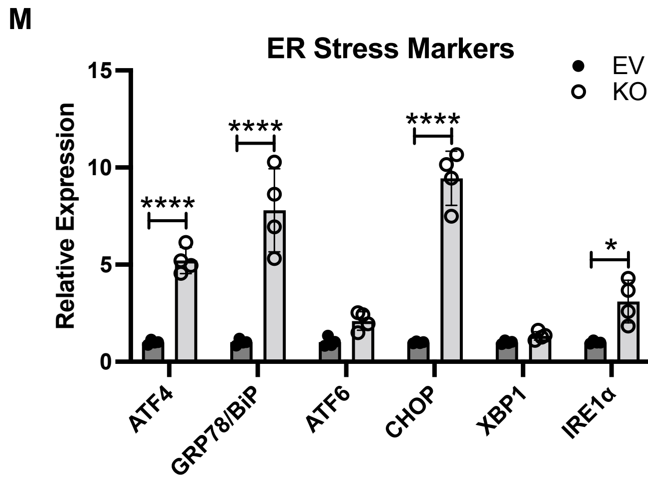

**Figure Legends:**

**Fig 1. HKDC1 is essential for LC progression and survival.** EV and HKDC1-KO cells (KO) were used for **A)** cell proliferation assay, **B)** colony forming assay (left panel images representative of 3 images per group and right panel number of colonies were counted using Image J), **C)** immunostaining for Ki67 proliferative marker (pink), nuclei are stained with DAPI (blue), **D)** Cell proliferation was measured using the BrdU assay, **E)** Western blot analysis was done in EV and KO cells for markers of cell proliferation and cell cycle (representative of 2 independent blots). **F)** Cell cycle analysis with propidium iodide, **G)** mRNA expression by qPCR for cyclins and cyclin dependent kinases vs KO cells, **H)** In invasion assay, EV and KO cell suspensions ($2 \times 10^5$ cells/well) were added to the upper chambers and allowed to invade for 72 h. Invasive cells were stained with 0.1% crystal violet (upper panel) and were measured by enzyme-linked immunosorbent assay reader using 570 nm as test wavelength (lower panel) and **I)** mRNA expression by qPCR for proteoglycan synthesis genes. **J)** *in vivo* tumor growth was assessed where $1X10^6$ EV or KO cells were inoculated into 4-6 weeks old male Nu/J mice (n=5), images were taken at endpoint (16 weeks post inoculation). **K)** Tumor size was measured weekly till 16 weeks after appearance of tumor with a vernier caliper tumor weight till the end of the study. **L)** Hep3B2 cells were transfected with shHKDC1 or (non-target) ntshRNA and transfected cells were selected with appropriate antibiotics, $1X10^6$ cells were inoculated into mice (n=4). When tumors were visible, mice were given doxycycline (in diet) for 7 days to activate shRNAs. Tumor growth was measured weekly till 8 weeks after appearance of tumor with a vernier caliper. **M)** Images of tumors at endpoint. All cell line experiments (**A-G**) were performed 2-3 times with 3-5 replicates per experiment. Values are mean ± SD; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 by Student's t-test, or 2-way ANOVA (for 2K).

**Fig 2. HKDC1 ablation impairs LC progression in an *in vivo* LC model**. Two-week-old HKDC1$^{f/f}$ and HKDC1-LKO male mice were injected with DEN (25 mg/kg). When mice were 8 weeks old, both groups were further divided into groups where one group received AAV expressing human HKDC1 (HKDC1$^{f/f}$+AAV and HKDC1-LKO+AAV) and the AAV expressing null vector was used as the control with the two other groups (HKDC1$^{f/f}$ and HKDC1-LKO), with N=3-7 per group. Ten months after DEN injection, mice were sacrificed and **A)** images of the livers are shown. **B)** Livers were weighed and ratio to body weight was calculated. Liver sections were fixed, and immunohistochemistry was performed to show **C)** BrdU and **D)** Ki67 positive hepatocytes. Regions of tumor in the histology slides were identified and omitted from analysis. Values are mean ± SD; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 by 2-way ANOVA.

**Fig 3. HKDC1-KO impairs glucose metabolism. A)** Hexokinase activity in EV and KO HepG2 cells. **B)** HepG2 cells were treated with siRNA against either HK2 or HKDC1 for 24h, cells were lysed, and hexokinase activity was assayed. **C)** Seahorse metabolic analysis (ECAR) of EV and HKDC1-KO cells (left panel) and siRNA mediated HKDC1 knockdown (siHKDC1) in Hep3B2 and SNU475 cells (center and right panels). **D)** 2-NBDG (2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose) fluorescent analog of glucose was used to assess glucose uptake in EV and KO HepG2 cells. **E)** In EV and KO HepG2 cells, glucose consumption was

assessed by measuring glucose concentration in media aliquots taken at designated time periods which was subtracted from initial glucose concentration of media, obtaining glucose being consumed by the cells. **F)** Steady-state metabolomics analysis of glycolytic and TCA cycle metabolites in cells cultured under standard growth condition (n=3, independent biological replicates). **G)** Mass isotopomer analysis of glycolytic and TCA cycle metabolites in cells cultured with 5.5 mM of [U-$^{13}C_6$] glucose and unlabeled glutamine (n=3, independent biological replicates) for 4h. **H)** Mass isotopomer analysis of TCA cycle metabolites in cells cultured with 2 mM of [U-$^{13}C_5$] glutamine and unlabeled glucose (n=3, independent biological replicates) for 4h. **I)** Schematic summarizing the changes in glucose flux from Table1 and 4E-F). Exp 4A-D were performed 2-3 independent times, with three replicates per individual experiment. Values are ± SD; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 by Student's t-test (for 4A-C) or 2 way ANOVA (for 4D-F).

**Fig 4. HKDC1 is essential for mitochondrial function in LC.** Cell fractionation experiment showing cytosolic, membrane and nuclear fractions in **A)** EV and KO HepG2 cells and **B)** Hep3B2 cells. **C)** EV and KO HepG2 cells were used in co-immunoprecipitation experiments where IP was done with anti-VDAC antibody and then immunoblotting (IB) was done with HKDC1 antibody. **D)** Liver sections from DEN treated HKDC1$^{f/f}$ and HKDC1-LKO mice were used to show HKDC1-VDAC interaction by immunohistochemical staining of HKDC1 and VDAC (images are representative of n=3 per group). **E)** Seahorse metabolic analysis (OCR) of EV and HKDC1-KO cells and siRNA mediated HKDC1 knockdown (siHKDC1) in Hep3B2 and SNU475 cells (center and right panels). **F)** Activity assay for mitochondrial complex I (left panel), SDH (center panel) and complex III (right panel) of EV and HKDC1-KO cells. **G)** Intracellular ATP levels relative to EV (fold change) (n=3). **H)** Mitochondrial $Ca^{2+}$ levels were assessed by Rhod-2AM fluorescence using flow cytometry of EV/HKDC1-KO cells (left panel) and Hep3B2 cells treated with siRNA against HKDC1 for 24h (right panel). 50,000 cells were assessed for each sample, and data was plotted on bar graphs with statistics (n=3) for each cell line **I)** Mitochondrial membrane potential was measured by TMRE fluorescence. **J)** Intracellular ROS levels are shown. **K)** HepG2 expressing empty vector (EV) or HKDC1 knockout (KO) cells were processed for TEM. 20-25 images were taken for each cell at different magnifications. Images shown here were taken at 4000X. Inset (shown in white) was enlarged (below) to show mitochondria and ER (M=mitochondria, ER=endoplasmic reticulum). **L)** Mitochondria were counted for each cell (20-27 cells per sample) by Image J software. All cell line experiments were performed 2-3 independent times with 3-8 replicates per experiments. **M)** qPCR analysis in EV and KO cells to assess mRNA levels of ER stress markers. Values are ± SD; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 by Student's t-test (for 5B, E-G, I) or 2 way ANOVA (for 5C,J).

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