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

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14. ABSTRACT

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 key data (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 1st year of this grant, I was able to accomplish these goals:

<u>Major Task 1</u>

Subtask1: Establish HKDC1 modulated cell lines (OE & KD &TR)

Subtask 2: Perform proliferation and survival experiments in modulated cell lines

As shown in Fig 1 I was able to modulate HKDC1 expression in a non-cancerous hepatocyte cell line (AML12) (**Fig 1)**. HKDC1 overexpression (OE) leads to enhanced proliferation and survival of these cell lines, showing that indeed HKDC1 enhances proliferation in cancer cells.

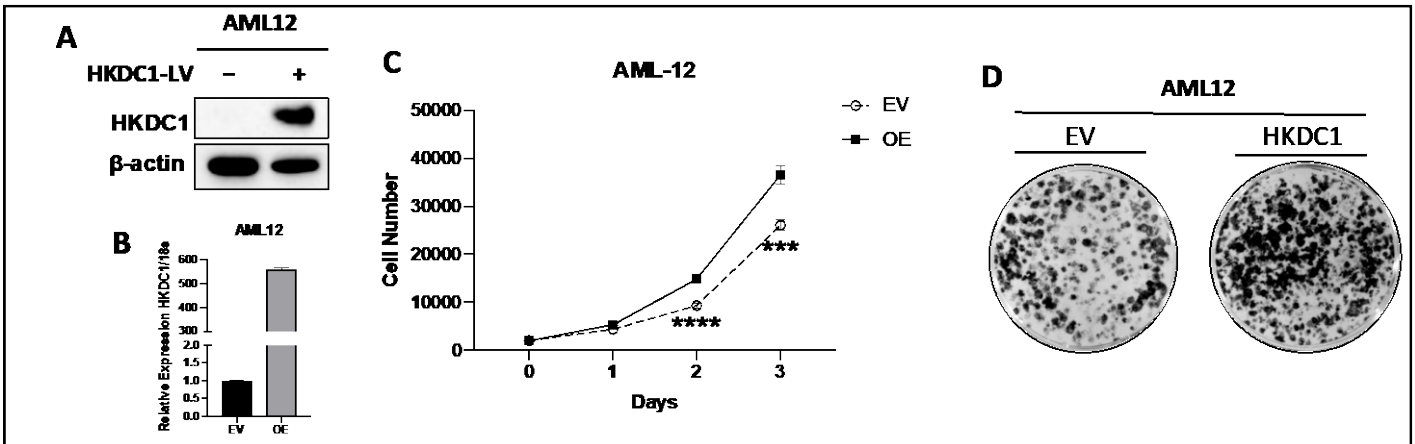

Fig 1. HKDC1 overexpression leads to proliferation. A non-cancerous hepatocyte cell line AML12 was used to overexpress either empty vector (EV) or full-length HKDC1 (OE) via lentiviral transduction to generate stable cell lines. Overexpression of human HKDC1 was confirmed by A) immunoblot and B) qPCR. These cells were then used in C) proliferation assays were cell growth was measured for 3 days after cells were plated and D) colony forming assay.

I was also able to create a HKDC1-knockout (KO) cell line in HepG2 cells (**Fig 2**) and use them to show that HKDC1-KO leads to HCC cell death and decreased survival *in vitro* (**Fig 2**). We also used these cells in an *in vivo* xenograft study where HKDC1-KO leads to inhibition of tumor formation in nude mice (**Fig 3**).

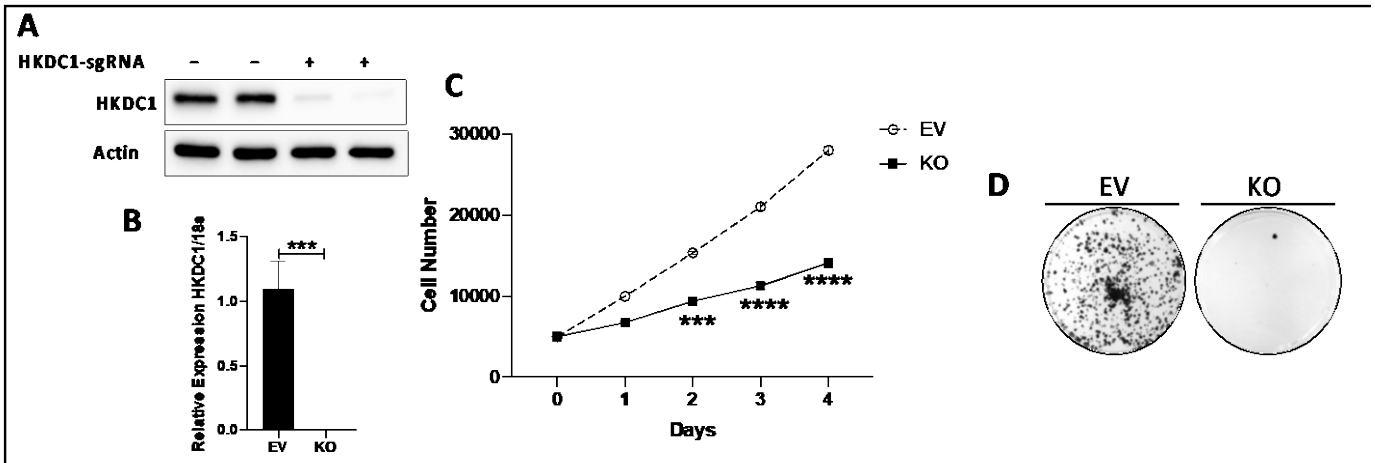

Fig 2. HKDC1 knockout leads to cell death. HepG2 cells were used to express either empty vector (EV) or sgRNA against HKDC1 lentiviral transduction to generate HKDC1 knockout cell lines (KO) stable cell lines. KO of human HKDC1 was confirmed by A) immunoblot and B) qPCR. These cells were then used in C) proliferation assays were cell growth was measured for 4 days after cells were plated and D) colony forming assay.

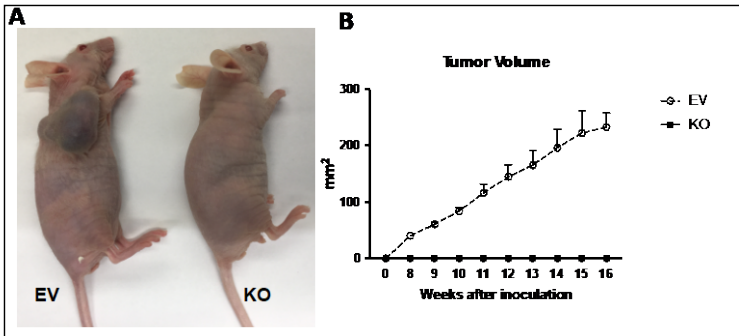

Fig 3. HKDC1 knockout inhibits tumor formation. 1X10⁹ modulated HepG2 cells (EV or KO) were injected into the flanks of Nu/J mice. The mice were monitored for 16 weeks after inoculation when animals in EV group reached end-point criteria A) images of animals bearing tumors B) tumor volume was calculated each weeks after tumors were visible.

Taking as slightly different approach we used another HCC cell line (Hep3B2) were we used shRNAs to knockdown (KD) HDKC1 in these cells. To have more control on the KD we used shRNAs under the control of doxycycline inducible promoter. We used these cells in a xenograft experiment were we show that once cells are KD of HKDC1, there is a significant inhibition of tumor growth in vivo (**Fig 4**).

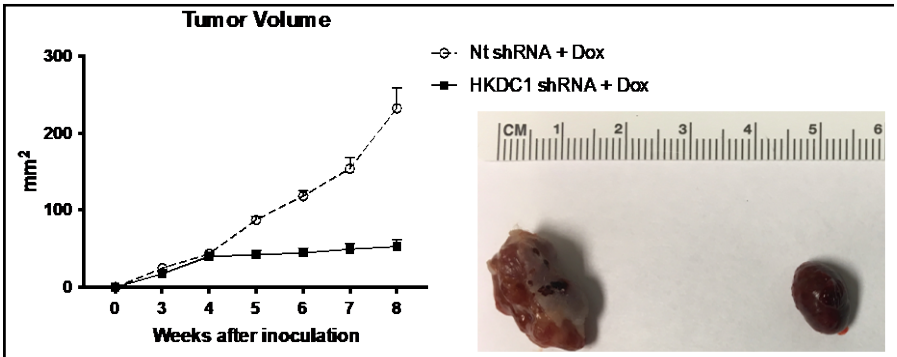

Fig 4. HKDC1 knockdown inhibits tumor formation. shRNA against HKDC1 or scramble (Nt) was introduced in Hep3B2 cells via transfection to create stable cells. $1X10^9$ modulated Hep3B2 cells were injected into the flanks of Nu/J mice. After mice had visible tumors, they were fed doxycycline in diet for 1 week to activate the shRNA. The mice were monitored for 16 weeks after inoculation when animals in EV group reached end-point criteria A) tumor volume was calculated each weeks after tumors were visible and B) images of tumors.

Lastly, we were able to reintroduce full-length (FL) and truncated (TR) version of HKDC1 (which lacks first 20 amino acids at N-terminal) in our HepG2-KO cells which do not have any endogenous HKDC1. Using these cell lines, we were able to show that 1) indeed N-terminal is essential for HKDC1's interaction with the mitochondria (**Fig 5**) and 2) that re-expressing HKDC1 in KO cells rescues the inhibition in cell proliferation but the truncated version is unable to do so (**Fig 6**) which shows that HKDC1-mitochodnria interaction for its pro-proliferative role in HCC.

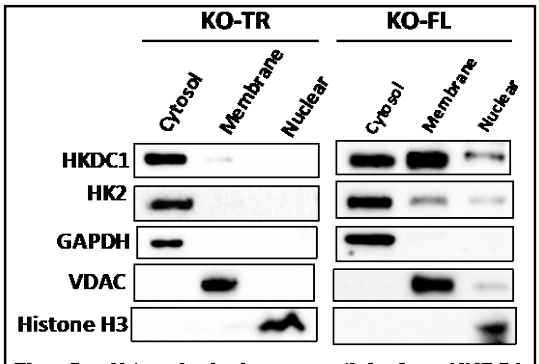

Fig 5. N-terminal is essential for HKDC1 localization to mitochondria. In HepG2 cells HKDC1 was first deleted with Crispr-Cas9 and then HKDC1 full length (KO-FL) or truncated (KO-TR) was overexpressed in these cells with lentiviral approach. Cellular fractionation to isolate cytosolic, mitochondrial and nuclear fractions was followed by immunoblot to assess levels of HK2 and HKDC1 protein in those fractions.

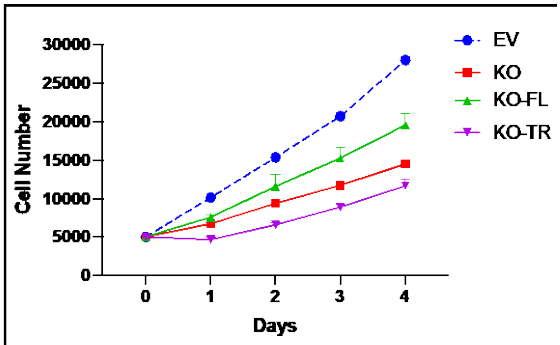

Fig 6. HKDC1 localization to mitochondria is essential for HCC proliferation. In HepG2 cells HKDC1 was first deleted with Crispr-Cas9 and then HKDC1 full length (KO-FL) or truncated (KO-TR) was overexpressed in these cells with lentiviral approach. Cell proliferation was measured by counting viable cells every day for 4 days after cell plating.

4. Impact: Based on the data collected so far:

1. HKDC1 is essential for HCC proliferation both *in vitro* and *in vivo*

2. Mitochondrial interaction of HKDC1 is essential for its role in HCC. This could be used in future studies where simply knocking of HKDC1 from the mitochondria in cancer cells (as normal hepatocytes have very low HKDC1) by using inhibitors or cell penetrating peptides could be a way to inhibit HCC growth.

5. 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. Only since the last 2-3 months I was able to work with unlimited capacity. I would therefore request grant management to consider this when granting an extension so that this work can be completed.

6. Products: Not Applicable

7. Participants & Other Collaborating Organizations: Not Applicable

8. Special Reporting Requirements: Not Applicable

9. Appendices: Not Applicable