**Elevated lipogenesis and a single-point mutation in acetyl-CoA carboxylase accelerates hepatocarcinogenesis**

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**Summary**

**The incidence of hepatocellular carcinoma (HCC) is rapidly increasing due to the prevalence of obesity and non-alcoholic fatty liver disease (NAFLD) but the molecular and metabolic triggers that initiate disease development are not fully understood. We find that targeted point mutations on the critical AMP-activated protein kinase (AMPK) phosphorylation site within acetyl-CoA carboxylase (ACC) upregulates liver de novo lipogenesis (DNL) and accelerates the development of hepatic lesions in mice. This ACC point-mutation also increases DNL and proliferation of cultured human liver cancer cells. Consistent with these findings, a novel liver specific ACC inhibitor (ND-654), which mimics the effects of ACC phosphorylation, prevents the development of HCC and improves survival in rats when used alone and in combination with the multi-kinase inhibitor sorafenib. These studies highlight the importance of DNL and ACC phosphorylation in accelerating the development of HCC and the potential utility of small molecule ACC inhibitors for treating the disease.**

## Introduction

Liver cancer now accounts for almost 750 000 deaths annually and hepatocellular carcinoma (HCC) is predominant.[1] Unfortunately, the global incidence of HCC is rapidly increasing, an effect which is attributed in part to the obesity epidemic and subsequent development of non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH).[2] Despite the growing prevalence of HCC, Sorafenib, a multi-kinase inhibitor which extends life in individuals with advanced HCC by approximately 2-3 months, is currently the only approved therapy.[3] Thus, identifying novel and effective therapeutic targets is of critical importance.

Commentato [LB1]: This sentence is choppy/passive. Perhaps re-word it:
Despite the growing prevalence of HCC, the only approved therapy is Sorafenib, a multi-kinase inhibitor shown to extend life in individuals with advanced HCC by 2 to 3 months.

Elevated liver de novo lipogenesis (DNL) is a key contributing factor in the development of NAFLD [4] and many cancers including HCC [5-6]. Consumption of diets that are high in refined carbohydrates, particularly fructose, accelerate DNL and consistent with this finding also accelerate the development of NAFLD [7] and HCC in mice [8] and potentially humans.[9] One of the central enzymes controlling DNL is acetyl-CoA carboxylase (ACC). ACC facilitates the conversion of acetyl-CoA to the metabolic intermediate malonyl-CoA. Malonyl-CoA is the first committed substrate for DNL and is also an inhibitor of fatty acid oxidation due to allosteric inhibition of carnitine palmitoyltransferase-1. Thus, ACC is vital for controlling the flux of carbon intermediates between carbohydrate and fatty acid metabolism.[10] Two isoforms of ACC exist and the enzymatic activity of both ACC1 and ACC2 can be inhibited by phosphorylation at serine 80 and serine 221 (serine 79 and serine 212 in mice), respectively, by the cellular energy sensor AMP-activated protein kinase (AMPK)[11]. Importantly, in mice with targeted knock-in (KI) mutations in which the serine phosphorylation sites on ACC1 (S79) and ACC2 (S212) are converted to an alanine (known herein as ACC KI mice), there is a loss of AMPK-mediated ACC inhibition, elevated hepatic malonyl-CoA, lipogenesis and early signs of NAFLD/NASH in young animals.[11] Studies have shown that the inhibition of ACC reduces cell proliferation in some cancers [12] but, the role of AMPK phosphorylation of ACC in HCC is currently unknown. This is important because ACC phosphorylation is reduced with type 2 diabetes and obesity[13] (conditions known to increase NAFLD and HCC risk) and new small molecules which mimic the effects of AMPK phosphorylation on ACC have recently been developed for the treatment of NAFLD/NASH[14] and non-small cell lung cancer (NSCLC)[15].

Commentato [LB2]: Should there be any statement on what the aims/outcomes/hypotheses for the paper?

## Results and Discussion

To examine the role of ACC phosphorylation in controlling HCC development, WT and ACC KI mice were maintained on a control chow diet with or without fructose supplemented in the drinking water to enhance rates of DNL. An elevated respiratory quotient (RQ) is indicative of high rates of whole-body DNL[16] and under standard chow fed conditions there were no differences in RQ (Figure 1A) or any other metabolic parameters between WT and ACC KI mice (Supplementary Table 1). As anticipated fructose treatment increased the RQ of WT mice during the dark (feeding cycle), an

effect that was more dramatic in ACC KI mice (Figure 1A), and was not due to differences in food and water intake or activity levels which were comparable to WT controls (Supplementary Table 1). Consistent with the increase in RQ, fructose increased the expression of the DNL proteins fatty acid synthase (FASN), ATP citrate lyase (ACLY) and ACC in the livers of WT and ACC KI mice. Fructose treatment decreased the activating phosphorylation of AMPK at Thr172 in both WT and ACC KI mice; however, the loss of ACC phosphorylation in ACC KI mice was the only detectable difference between genotypes (Figure 1B & 1C). Fructose fed ACC KI mice also increased liver lipogenesis in vivo (Figure 1D) indicating that AMPK phosphorylation of ACC is vital to suppress DNL.

When mice were injected with the HCC initiator diethylnitrosamine (DEN) which promotes aspects of the human disease[17], both WT and ACC KI mice had indications of hepatocarcinogenesis, including the presence of altered hepatocyte foci, hyperplastic nodules and HCC (Figure 1E). Importantly, despite similar sized lesions (Figure 1F); ACC KI mice had twice as many lesions per liver as WT controls (Figure 1G) indicating that ACC phosphorylation is vital to restrain hepatocarcinogenesis. This increase in the number of lesions was independent of alterations in factors known to accelerate tumorigenesis including adiposity, liver triglyceride, insulin resistance, inflammatory cytokines and markers of liver fibrosis all of which were comparable between genotypes (Supplementary Figure 1A-H). These data indicate that AMPK phosphorylation of ACC is vital for restraining the development of hepatocarcinogenesis.

Recently, the discovery of a new class of potent, highly specific, isozyme-nonselective, allosteric, protein-protein interaction ACC inhibitors has been reported[14]. These compounds interact within the phosphopeptide-acceptor and subunit dimerization site of the biotin carboxylase domain of both ACC1 and ACC2 to prevent dimerization and inhibit enzymatic activity. The first of these drugs, ND-630, was shown to reduce hepatic steatosis in rats with diet-induced obesity[14] and is now under investigation in clinical trials of NASH (NCT02856555). The second, ND-646, was recently shown to inhibit the growth of NSCLC[15]. To further examine the role of ACC in hepatocarcinogenesis, we utilized a third compound in this series, ND-654, for the following studies.

As compared to ND-630 and ND-646, ND-654 has been modified to allow for enhanced uptake by hepatocytes. To confirm hepatoselective delivery of ND-654, rats were first treated with a single oral dose of 10 mg/kg ND-654 and the concentration of ND-654 was measured after 1 hour in the liver, muscle and plasma (Figure 2A). Under these conditions, the liver concentration of ND-654 was ~2700 fold higher than that of muscle and ~100 fold higher than that of plasma. Next, rats were treated with a single oral dose of 10 mg/kg ND-654 and the concentration of ND-654 was measured over 8 hours in the liver and plasma (Figure 2B). Concentrations in the plasma were in the nM range and slowly decreased over time, while liver concentrations declined over time but still remained above 1 µM even after 8 hours. To assess the effects of ND-654 on ACC activity, rats were treated with a single oral dose of different concentrations of ND-654 (0.3, 3, and 30 mg/kg) and the presence of malonyl CoA was determined 1 hour later as a measure of ACC inhibition (Figure 2C,D). While there was no change in malonyl CoA

concentration in the muscle, malonyl CoA levels in the liver were reduced by 80%. These data illustrate that ND-654 is a potent ACC inhibitor which selectively targets the liver.

We next tested whether ND-654 would inhibit hepatocarcinogenesis in a rat model of sequential cirrhosis and HCC that we have previously shown to resemble aspects of the human disease at the biochemical, histological, and molecular level[17]. Repeated, weekly intraperitoneal administration of 50 mg/kg DEN in rats causes progressive inflammation and fibrosis followed by development of cirrhosis at 12 weeks and HCC around 15 weeks. In our first study, DEN-injured rats were administered 10 mg/kg ND-654 daily by oral gavage beginning at the start of week 15 when HCCs are first apparent and were sacrificed at the beginning of week 19. During this short-term administration of ND-654, we observed no significant differences in group mean body weight, although administration of DEN did significantly reduce body weight compared to PBS controls (Supplementary Figure 2A). Likewise, while liver enzymes, like alanine transaminase (ALT) and aspartate transaminase (AST), were elevated in the serum of DEN-injured rats compared to controls, ND-654 treatment did not cause further elevations (Supplementary Figure 2B,C). Consistent with the inhibition of ACC[14], serum triglyceride levels were reduced with ND-654 (Supplementary Figure 2D). Overall, these data demonstrate that ND-654 was effectively delivered to the liver and was well-tolerated in cirrhotic rats.

After 4 weeks of treatment, we counted and measured surface tumors that were $\geq$ 5 mm as nodules of this size have previously been shown to be predominantly HCCs in this model[17-19]. ND-654 reduced tumor burden in DEN-injured rats by 55% as the number of tumor nodules decreased from 15.8 ± 4.7 in DEN animals to 7.1 ± 3.7 in DEN animals treated with ND-654 (Figure 2E,F). Liver weight as a percentage of body weight decreased from 6.9 ± 1.5% in DEN animals to 5.1 ± 1.9% in those that received ND-654 (Figure 2G). ND-654 binds to the BC domain of ACC and prevents phosphorylation by AMPK, and therefore phospho-ACC levels have been proposed as a useful biomarker to assess effective drug targeting[15]. Consistently, we observed decreased ~~p-~~ACC phosphorylation in both the surrounding cirrhotic liver and tumor nodules from drug-treated animals (Supplementary Figure 2E,F). However, proliferation, as assessed by proliferating cell nuclear antigen (PCNA) protein levels, only decreased in the tumors in the ND-654 group (Supplementary Figure 2E,F). In addition, PCNA staining decreased in tumors in the ND-654 group (Figure 2H, middle panel) and necrosis was also observed in some tumors from the ND-654 group (Figure 2H, right panel) but there was no evidence of apoptosis as assessed by cleaved caspase-3 staining (Figure 2H).

Given these promising results, we repeated the study to determine whether ND-654 could increase survival in this lethal model of cirrhosis-driven HCC. For this experiment, DEN-injured rats received: vehicle control, 10 mg/kg ND-654, or 30 mg/kg ND-654 by oral gavage daily beginning at the start of week 14. Remarkably, ND-654 significantly increased the median survival from 114 days in vehicle controls to 126 days in the 10 mg/kg group ($p = 0.02$) and 129 days in the 30 mg/kg ($p = 0.009$). There was no significant difference between the 10 and 30 mg/kg group and in fact 3 animals in the 10 mg/kg group lived the longest suggesting that higher doses of ND-654 do not increase survival in this model (Figure 2I).

**Commentato [LB3]:** In figure, can you use 2 different symbols rather than the same * stacked?

Patients assigned to the control arm of HCC clinical trials receive standard-of-care therapy which is currently sorafenib for patients with advanced-stage disease. We therefore decided to examine whether ND-654 would increase the efficacy of sorafenib in the DEN rat model. For this experiment, DEN-injured rats received: vehicle control, 10 mg/kg ND-654, 10 mg/kg sorafenib, or the combination of 10 mg/kg ND-654 and 10 mg/kg sorafenib by oral gavage daily beginning at the start of week 13. ND-654 and sorafenib had similar efficacy in reducing HCC incidence (the number of tumor nodules was $16.4 \pm 8.4$ in the control group compared to $9.7 \pm 4.7$ in the ND-654 group (a 41% decrease) and $7.0 \pm 4.6$ in the sorafenib group (a 57% decrease)) (Figure 3A-C). While both compounds caused tumor necrosis (Figure 3B), the combination of sorafenib and ND-654 was especially effective at reducing HCC incidence by 81% (the number of tumor nodules was $3.1 \pm 2.6$ in the ND-654 + sorafenib group) (Figure 3 A-C). Sorafenib led to a reduction in liver weight as a percent of body weight which was associated with a marked ductular reaction an effect which was maintained when combined with ND-654 (COMBO) despite modest reduction in body mass in this group (Supplementary Figure 3A-C). While sorafenib increased serum levels of triglycerides (Supplementary Figure 3D), the addition of ND-654 reduced their levels and also decreased phosphorylated -ACC levels in both liver and tumor tissue as observed in the first study (Figure 3D-E). Finally, the combination of sorafenib and ND-654 was also observed to effectively decrease tumor proliferation as assessed by PCNA protein levels (Figure 3E).

To establish whether AMPK phosphorylation of ACC was also important for increasing proliferative capacity in human liver cancer cells, the ACC KI mutation was generated in HepG2 cells using CRISPR-Cas9 (Supplementary Figure 4A). Sequencing analysis indicated successful insertion of the ACC1 S80A KI mutation into the ACC KI HepG2 cells but not control cells. When the HepG2 cells were stimulated with the potent AMPK activator phenformin, AMPKα Thr172 and ACC phosphorylation increased as expected in WT cells, but no ACC phosphorylation was detectable in ACC KI cells (Figure 4A), indicating the ACC KI mutation was successfully inserted and ACC2 phosphorylation was negligible in this cell line. The specificity of the mutation in ACC KI cells was evident as the total protein expression of AMPK and ACC was unaltered as was the phosphorylation of AMPK Thr172 and another AMPK substrate, ULK1[20] (Figure 4A) indicating that AMPK activity and the ability phosphorylate other substrates was maintained. Consistent with the elevated DNL and increased hepatocarcinogenesis in ACC KI mice in vivo, HepG2 cells expressing the ACC KI mutation had a 4-fold increase in lipogenesis and 2-fold increase in proliferation (Figure 4B). This increase in proliferation did not appear to be the result of alterations in the phosphorylation of other growth signaling pathways which was comparable between WT and ACC KI cell lines (Supplementary Figure 4B). We also examined the effects of ND-654 on HepG2 cells. As expected, treatment with ND-654 eliminated phospho-ACC levels (Figure 4C) and reduced the proliferation of HepG2 cells over time (Figure 4D) and alike to observations in the ACC KI cells, this was not associated with alterations in protein phosphorylation of other growth signaling pathways (Supplementary Figure 4C). These data indicate that inhibition of ACC activity through phosphorylation at Ser79 is vital for inhibiting HCC

**Commentato [LB4]:** There's inconsistency with short-form of pACC. I removed p-ACC in text above, and now it's shortened here as phospho-ACC

proliferation in a cell autonomous manner independently of alterations in other growth signaling pathways.

Since the approval of sorafenib, HCC clinical trials have largely been unsuccessful and the lack of patient selection in trial design is at least partly responsible for this failure.[21-22] Over the last decade, attempts have been made to define molecular subclasses of HCC and several reports have now shown that these subclasses due correlate with treatment response *in vitro*.[23-26] We recently described an HCC gene classification system that is highly reproducible between clinical datasets and divides HCC into three major subclasses termed S1, S2 and S3.[27-28] S1 tumors compose 28-31% and S2 tumors compose 23-24% of HCC in clinical data sets. Both S1 and S2 tumors are associated with worse prognosis compared to S3 tumors. To explore ACC pathway-related molecular aberrations in human HCC tissues, we analyzed somatic genomic DNA copy number alterations, mutations, and genome-wide transcriptome profiles of 374 human HCC tissues obtained from The Cancer Genome Atlas data portal (https://gdc.cancer.gov). Interestingly, somatic DNA amplification and mutations in the ACC1 (*ACACA*) and ACC2 (*ACACB*) genes were only observed in 17 and 12 tumors (4.5% and 3.2%), respectively, and did not associate with any of the HCC molecular subclasses. By comparison, S2 tumors, which express several traditional HCC biomarkers like AFP and GPC-3,[27-28] had high ACC1 expression suggesting that these tumors are readily identifiable and might be an attractive target for ACC inhibition (Figure 4F).

In conclusion, our studies show that genetic alterations resulting in reduced phosphorylation of ACC augment HCC development in mice and proliferation of human liver cancer cells. Importantly, we also establish that pharmacological targeting of this pathway using ND-654, a novel liver directed small molecule, is effective in reducing HCC and improving survival in rats and that this molecule has additive effects to the current standard of care sorafenib. While mutations in AMPK or ACC in HCC have not been described, it is well documented that both obesity and type 2 diabetes, two primary risk factors for developing HCC, reduce AMPK activity and the phosphorylation of ACC[13] as well as increase rates of liver DNL.[4] These data suggest that therapies aimed at mimicking the effects of AMPK phosphorylation on ACC using small molecules such as ND-654 may be valuable for treating HCC particularly those in the S2 subclass.

**Commentato [u5]:** THE FIRST (21) REFERENCE IS INCORRECT: IT SHOULD BE PAGE 408-424

THE TEXT IS ALSO INCORRECT. I READ THE PAPER I RECELLED A DIFFERENT COMMENTS ON SORAFENIB. AS MATTER OF FACT, THE AUTHOR MENTIONED DIFFERENT CAUSES OF TRIAL UNSUCCESS. THUS I WOULD CHANGE THE SENTENCE IN THIS WAY"... Since the approval of sorafenib, HCC clinical trials have largely been unsuccessful due to

**Commentato [LB6]:** Typo? Do correlate?

**Supplemental Table 1:** Activity levels, food and drink consumption, $VO_2$ and $VCO_2$ from WT and ACC KI mice maintained on a chow diet with or without fructose supplemented in the drinking water.

| | | Chow | | Chow + Fructose | | *p*-value | |
|---|---|---|---|---|---|---|---|
| | | WT | ACC KI | WT | ACC KI | effect of diet | effect of genotype |
| *Activity Level (beam breaks)* | 24 hour | 22169±2495 | 19665±2870 | 16728±2977 | 16941±1842 | 0.14 | 0.67 |
| | Light phase | 3156±475.8 | 2718±247.6 | 3042±595.6 | 3331±355.6 | 0.55 | 0.86 |
| | Dark phase | 19013±2104 | 16947±2766 | 13687±2515 | 13610±1671 | 0.09 | 0.66 |
| *Food consumption (g)* | 24 hours | 3.9±0.08 | 3.9±0.29 | 2.2±0.18 | 1.795±0.52 | < 0.01 | 0.64 |
| | Light phase | 1.1±0.14 | 1.2±0.24 | 0.8±0.12 | 0.6±0.18 | 0.02 | 0.63 |
| | Dark phase | 2.7±0.13 | 2.7±0.18 | 1.3±0.12 | 1.2±0.35 | < 0.01 | 0.78 |
| *Drink consumption (ml)* | 24 hours | 5.2±0.58 | 7.3±1.08 | 6.6±0.80 | 5.9±0.08 | 0.98 | 0.35 |
| | Light phase | 1.6±0.31 | 2.3±0.63 | 2.3±0.22 | 1.7±0.14 | 0.85 | 0.92 |
| | Dark phase | 3.6±0.27 | 4.9±0.45 | 4.2±0.64 | 4.2±0.15 | 0.92 | 0.2 |
| *$VO_2$ (ml/kg/hr)* | 24 hours | 2210±77.47 | 2348±31.51 | 2337±179.7 | 2655±89.4 | 0.05 | 0.04 |
| | Light phase | 1871±70.16 | 2014±38.54 | 2054±183 | 2303±78.57 | 0.03 | 0.07 |
| | Dark phase | 2559±87.76 | 2695±46.27 | 2634±184.1 | 3023±103.8 | 0.09 | 0.03 |
| *$VCO_2$ (ml/kg/hr)* | 24 hours | 2050±49.77 | 2179±41 | 2240±186.6 | 2633±99.22 | 0.01 | 0.03 |
| | Light phase | 1714±62.1 | 1826±41.86 | 1927±193.3 | 2145±99.93 | 0.03 | 0.16 |
| | Dark phase | 2399±41.04 | 2540±58.56 | 2557±185.4 | 3081±97.3 | < 0.01 | < 0.01 |

Data are presented as the mean the ± SEM.

## Figure Captions

**Figure 1. AMPK phosphorylation of ACC is vital for limiting hepatocarcinogenesis and cellular proliferation.**

(A) The RQ of WT and ACCKI mice during the dark cycle (7 pm- 7 am) after being maintained on either a chow diet alone or chow diet plus fructose for 4 months (n= 4 WT chow, n=5 WT fructose, n=7 ACC KI chow, n=7 ACC KI fructose). (B) and (C) Protein levels of FASN, ACLY, ACC total protein, ACC phospho-Ser79, AMPK α total protein, AMPK α phospho-Thr172 and β-actin in the liver from WT and ACC KI mice (n= 4 WT chow, n=5 WT fructose, n=6 ACC KI chow, n=6 ACC KI fructose). (D) Incorporation of [3H]–acetate into total hepatic lipid (n=4). Representative images (E), diameter (F) and number (G) of hepatic lesions in photomicrographs of livers from DEN-treated ACC KI and WT mice maintained on fructose diet for 4 months (Haematoxylin and eosin stain, bar represents 1000 µm, n= 8 WT, n=6 ACC KI).
* significantly different WT fructose vs ACC KI fructose, $p < 0.05$
** significantly different from WT, $p < 0.05$
*** main effect of diet, $p < 0.05$

**Figure 2. ND-654 selectively targets the liver and inhibits HCC proliferation.** Male Wistar rats were divided into three groups (n = 8 per group). The first group received weekly intraperitoneal (IP) injections of PBS as control for 18 weeks. The second group received weekly IP injections of DEN (50 mg/kg diluted in PBS) for 18 weeks. The third group received weekly IP injections of DEN for 18 weeks as above and were also treated with ND-654 (10 mg/kg) once daily by oral gavage beginning at 15 weeks. In the DEN model, rats develop liver fibrosis after 8 weeks which progresses to cirrhosis at 13 weeks and HCC beginning at 15 weeks. (E) Representative images of gross livers are shown. (F) Tumor nodules ≥5 mm were counted. (G) Liver weight (LW) as a percentage of body weight (BW) was measured at the end of the study. (H) Representative images of H&E, proliferating cell nuclear antigen (PCNA; proliferative marker) and cleaved caspase-3 (apoptosis marker) staining of tumor are shown (100X magnification). The left column shows a representative tumor from the DEN group, the middle column and right columns show representative tumors from the DEN + ND654 group with reduced proliferation and extensive necrosis (N), respectively. (I) Male Wistar rats were divided into three groups (n = 10 per group). The first group received weekly IP injections of DEN (50 mg/kg diluted in PBS), the second and third groups received weekly IP injections of DEN as above and were also treated with either 10 or 30 mg/kg ND-654 once daily by oral gavage beginning at 14 weeks. Survival was examined by a Kaplan-Meier analysis.
* significantly different from PBS, $p < 0.05$
** significantly different from DEN, $p < 0.05$

**Figure 3. ND-654 improves the efficacy of sorafenib in cirrhotic rats with HCC.** DEN-injured rats received: vehicle control (VEH), 10 mg/kg ND-654 (ND-654), 10 mg/kg sorafenib (SOR), or the combination of 10 mg/kg ND-654 and 10 mg/kg sorafenib (COMBO) by oral gavage for 5 weeks. (A) Representative images of whole livers are

shown. (B) Representative images of H&E stainings of tumor illustrating increased necrosis (N) in treated tumors (100X magnification). (C) Tumor nodules ≥5 mm were counted. Levels of phosphorylated ACC (pACC), total ACC, proliferating cell nuclear antigen (PCNA), and actin were measured by western blot analysis in (D) liver tissue and (E) tumors (representative blot from 3 independent experiments).
* significantly different from PBS, $p < 0.05$
** significantly different from DEN, $p < 0.05$
**** significantly different from DEN, DEN + ND-654, and DEN + SOR, $p < 0.05$

**Figure 4. AMPK phosphorylation of ACC limits HepG2 proliferation.** (A) AMPK Thr172, ACC Ser80, and ULK1 (Ser555) phosphorylation in WT and ACCKI HepG2 cells after treatment with phenformin (2 mM) for 1 hour (representative blot from 3 independent experiments). (B) De novo lipogenesis measured by incorporation of 3-H acetate into lipid over 1 hour (means of two independent experiments performed in triplicate) and (C) Proliferation in WT and ACCKI HepG2 cells over 72 hours (n=7). (D) HepG2 cells were treated with or without ND-654 (10 μM) for 72 hours and levels of p-ACC, total ACC, and actin were measured by western blot analysis (representative blot from 3 independent experiments). (E) Proliferation in HepG2 cells treated with or without ND-654 (10 μM) was measured at 1 day, 2 days, 4 days, and 6 days post treatment by an MTT assay (representative results from 3 independent experiments). (F) Genomic DNA structural alterations, AMPK pathway modulation, and expression of ACC pathway-related genes in 374 human HCC tissues are shown according to the transcriptomic HCC subtypes (S1, S2, and S3 subtypes). Gene set involved in AMPK-mediated fatty acid oxidation was suppressed in the S1 subtype and partially in the S2 subtype. Gene expression of *ACACA* (encoding ACC1) and *ACACB* (encoding ACC2) was restricted to the S2 and S3 subtypes, respectively. DNA structural alterations (i.e., somatic DNA amplification and mutations) in *ACACA* and *ACACB* genes were observed only in 17 and 12 tumors (4.5% and 3.2%), respectively, with no association with the pathway modulation and gene expression.
* significantly different from WT cells, $p < 0.05$

**Supplementary Figure 1. Metabolic parameters in DEN injected WT and ACCKI mice maintained on a chow diet supplemented with fructose in the drinking water for 4 months.** (A) Body weight (n=8 WT, n=6 ACC KI), (B) adiposity (% of body weight) (n=8 WT, n=6 ACC KI), (C) liver mass relative to body mass (n=8 WT, n=6 ACC KI) (D) triacylglycerol content (n=8 WT, n=6 ACC KI), (E) glucose tolerance test (n=7 WT, n=6 ACC KI), (F) insulin tolerance test (n=8 WT, n=6 ACC KI), (G) Hepatic RNA expression of selected proinflammatory cytokines *TNFA* and *IL6* and (n=8 WT, n= 6 ACC KI) and (H) fibrosis-related genes *TIMP1, COL1A1, COL4A1, ACTA2* (n= 6-7 WT, n=5-6 ACC KI).

**Supplementary Figure 2. ND-654 was well-tolerated in cirrhotic rats.** Male Wistar rats were divided into three groups (n = 8 per group). The first group received weekly intraperitoneal (IP) injections of PBS as control for 18 weeks. The second group received weekly IP injections of DEN (50 mg/kg diluted in PBS) for 18 weeks. The third group received weekly IP injections of DEN for 18 weeks as above and were also treated with

ND-654 (10 mg/kg) once daily by oral gavage beginning at 15 weeks. In the DEN model, rats develop liver fibrosis after 8 weeks which progresses to cirrhosis at 13 weeks and HCC beginning at 15 weeks. (A) Body weight was measured at the end of the study. Serum levels of (B) alanine transaminase (ALT), (C) aspartate transaminase (AST), and (D) triglycerides (TG) were measured.
* significantly different from PBS, $p < 0.05$

**Supplementary Figure 3. ND-654 inhibits ACC and decreases tumor proliferation.** Male Wistar rats were divided into three groups. The first group received weekly intraperitoneal (IP) injections of PBS as control for 18 weeks. The second group received weekly IP injections of DEN (50 mg/kg diluted in PBS) for 18 weeks. The third group received weekly IP injections of DEN for 18 weeks as above and were also treated with ND-654 (10 mg/kg) once daily by oral gavage beginning at 15 weeks. In the DEN model, rats develop liver fibrosis after 8 weeks which progresses to cirrhosis at 13 weeks and HCC beginning at 15 weeks. Levels of phosphorylated acetyl coA carboxylase (p-ACC), total ACC, proliferating cell nuclear antigen (PCNA), and actin were measured by western blot analysis in (A) liver tissue and (B) tumors (representative blot from 3 independent experiments).

**Supplementary Figure 4. ND-654 does not increase sorafenib toxicity in cirrhotic rats.** DEN-injured rats (n = 10 per group) received: vehicle control (VEH), 10 mg/kg ND-654 (ND-654), 10 mg/kg sorafenib (SOR), or the combination of 10 mg/kg ND-654 and 10 mg/kg sorafenib (COMBO) by oral gavage for 5 weeks. (A) Representative images of H&E stainings of non-tumoral liver tissue from each group shows a marked ductular reaction in the DEN + SOR group (40X magnification). (B) Liver weight (LW) as a percentage of body weight (BW) was measured at the end of the study. (C) Body weight was measured at the end of the study. (D) Serum levels of triglycerides (TG) were measured.
* significantly different from PBS, $p < 0.05$
*** significantly different from DEN and DEN + ND-654, $p < 0.05$
**** significantly different from DEN, DEN + ND-654, and DEN + SOR, $p < 0.05$

**Supplementary Figure 5. Gene targeting strategy and phospho-kinase array profile in WT and ACC KI HepG2 cells.** (A) Gene targeting sequence for generation of the WT and HepG2 ACC KI cell line. (B) Human phospho-kinase array data showing phosphorylation status of selected kinase targets performed on cell lysates from WT and ACCKI HepG2 cells (n=2). (C) Human phospho-kinase array data showing phosphorylation status of selected kinase targets performed on cell lysates from HepG2 cells treated with or without ND-654 (10 μM) for 6 days (n=2).

**Methods (online only)**

**Animals**

ACCKI mice have been described previously.[11] ACCKI and wildtype male mice were housed in specific pathogen free micro isolator cages maintained under a 12 hour light/dark cycle at a constant temperature of 23°C. All animals were maintained on a normal chow diet (Teklad 22/5 rodent diet). Fructose treated animals were given water supplemented with fructose (30%) as has been performed by others to accelerate HCC development[29] starting at 8 weeks continuously for 16 weeks. Mice were allowed access to food and water ad libitum. To induce HCC animals were injected with the chemical carcinogen diethylnitrosamine (DEN, Sigma) at a concentration of 25 mg/Kg body weight at 2 weeks of age as previously described.[30] With the exception of a single WT animal all mice had developed signs of hepatocarcinogenesis after 6 months. Indirect calorimetry experiments were performed using the Oxymax Comprehensive Lab Animal Monitoring System (Columbus Instruments) as we have described previously.[31] Glucose and insulin tolerance tests were performed as we have described previously.[11] In vivo hepatic lipogenesis was measured by assessing 3-[H] acetate incorporation into the total hepatic lipid fraction as we have described previously.[11] At the end of the experiment, mice were anesthetized using ketamine/xylazine, tissues were removed and a portion was fixed in formalin or frozen in liquid nitrogen for histological and molecular/biochemical analysis. All of the experiments performed in this study were approved by the Animal Research Ethics Board at McMaster University.

Male Wistar rats (Charles River Laboratories, Wilmington, MA) were housed in accordance with the guidelines of the Massachusetts General Hospital Institutional Animal Care and Use committee and received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" of the National Academy of Sciences. Rats were subjected to either control PBS or low-dose 50 mg/kg DEN (Sigma, St. Louis, MO) injected IP once per week over the course of 18 weeks. In the first study, DEN-injured rats began treatment with either vehicle control or ND-654 10 mg/kg (n = 8 per group) by oral gavage at 15 weeks and were sacrificed at the end of 18 weeks. In the second study, DEN-injured rats began treatment with either vehicle control, ND-654 10 mg/kg, or ND-654 30 mg/kg (n = 10 per group) by oral gavage at 14 weeks and continued treatment until death. In the third study, DEN-injured rats began treatment with either vehicle control, ND-654 10 mg/kg, sorafenib 10 mg/kg, or ND-654 10 mg/kg + sorafenib 10 mg/kg (n = 10 per group) by oral gavage at 13 weeks and were sacrificed at the end of 18 weeks. At the time of sacrifice rats were anesthetized and sedated. A terminal blood collection was performed by cardiac puncture and livers were removed for measurement of weight, snap frozen for further analysis or fixed in formalin for histology. Serum levels of several biochemical markers including alanine transaminase (ALT), aspartate transaminase (AST), albumin (Alb), glucose (Glu), and triglycerides (TG) were measured as previously described.[17-18]

## Histology

The major lobes of the liver were removed and fixed in 10% neutral formalin, embedded in paraffin, sectioned and stained with H&E as we have described previously.[11] The number of tumor nodules per liver cross section were quantified from scanned images by researchers who were blinded to the treatment groups using NIS-Elements software (Nikon).

For rat studies, formalin-fixed samples were embedded in paraffin, cut into 5 µm-thick sections and stained with hematoxylin-eosin (H-E) according to standard procedures. Additional sections were stained with an antibodies specific for proliferating cell nuclear antigen (PCNA) and cleaved caspase-3 (both from Cell Signaling Technologies). All slides were reviewed blindly by the same liver pathologist.

## Cell line experiments

The ACC1 KI mutation was introduced into HepG2 (ATCC® HB-8065™) human liver cancer cells via transfection with gRNA/Cas9 all in one vector (custom made from GenScript) with (KI) and without (WT, sham transfected) donor ACC1 (Acetyl-CoA carboxylase 1) Ser80Ala knock in plasmid. Puromycin selection medium was added to transfected cells for 48 hrs. Pooled cells were propagated and analyzed by PCR and SURVEYOR mutation detection analysis. Clones that harboured the mutation were further selected, analyzed and purified by single cell cloning and the knock-in mutation was confirmed by sequencing. For experimental procedures cells were maintain in MEM supplemented with 10% fetal bovine serum (Gibco). Lipogenesis and proliferation rates in HepG2 cells were assessed as we have described previously.[32] Phospho-kinase array data in HepG2 cells was performed using a Human Phospho-Kinase Array Kit from R&D Systems Inc. and was analyzed as recommended by the manufacturer.

To assess the effects of ND-654 on HepG2 cells, cells were plated in triplicate at a density of $5 \times 10^4$/mL in a 24-well plate. After 24 h, medium containing vehicle control or ND-654 was added. After 72 hours of drug exposure cell number was estimated by colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT; Sigma). The absorbance at 562 nm was measured with a spectrophotometric plate reader (Emax, Molecular Devices) and the experiment was repeated twice to ensure reproducibility.

## Western Blotting

Protein abundance was determined using Western blot analysis as we have described previously[11] using the following commercially available antibodies: ACC (#3676), ACC pSer79/221 (#3661), AMPK pan □ (#2532), AMPK pThr172 (#2532), FASN (#3189), ACLY (#4332), β-Actin (#4967), GAPDH (#5174), ULK1 (#8054) ULK1 Ser555 (#5869) all from Cell Signaling Technologies.

**Transcriptomic Analysis**

Somatic genomic DNA copy number alterations, mutations, and genome-wide transcriptome profiles of 374 human HCC tissues were obtained from The Cancer Genome Atlas data portal (https://gdc.cancer.gov). Transcriptomic Molecular HCC subtypes determined by our previous transcriptome meta-analysis[27] were determined by using Nearest Template Prediction (NTP) algorithm.[33] Molecular pathway modulation in each individual sample was determined by modified gene set enrichment analysis.[34-35]

**Statistics**

Data are shown as means with error bars representing the SEM. Significant differences between means were determined using Student's t-test or 2-way ANOVA with Bonferroni's *post hoc* test where appropriate. A $p$ value of $\leq 0.05$ was considered significant.

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