Disrupting Mitochondrial Pyruvate Uptake Directs Glutamine into the TCA Cycle away from Glutathione Synthesis and Impairs Hepatocellular Tumorigenesis

Graphical Abstract

Highlights
- The MPC is retained in HCC, supporting TCA cycle pyruvate metabolism
- MPC disruption directs glutamine to the TCA cycle away from glutathione synthesis
- Glutathione synthesis is diminished, impairing hepatocellular tumorigenesis

Authors
Sean C. Tompkins, Ryan D. Sheldon, Adam J. Rauckhorst, ..., Adam J. Dupuy, Douglas R. Spitz, Eric B. Taylor

Correspondence
eric-taylor@uiowa.edu

In Brief
Tompkins et al. utilize stable glutamine isotope tracers in vivo and ex vivo to demonstrate hepatocyte MPC disruption increases TCA cycle glutamine utilization at the expense of glutathione synthesis and decreases hepatocellular tumorigenesis.
Disrupting Mitochondrial Pyruvate Uptake Directs Glutamine into the TCA Cycle away from Glutathione Synthesis and Impairs Hepatocellular Tumorigenesis

Sean C. Tompkins,1 Ryan D. Sheldon,1,9 Adam J. Rauckhorst,1,9 Maria F. Noterman,1 Shane R. Solst,2 Jane L. Buchanan,1 Kranti A. Mapuskar,2 Alvin D. Pewa,1,9 Lawrence R. Gray,1 Lalita Oonthonpan,1 Arpit Sharma,1 Diego A. Scerbo,1 Adam J. Dupuy,3,4 Douglas R. Spitz,2,4 and Eric B. Taylor1,4,5,6,7,8,10,*

1Department of Biochemistry, University of Iowa Carver College of Medicine, Iowa City, IA 52240, USA
2Free Radical and Radiation Biology Program, Department of Radiation Oncology, University of Iowa Carver College of Medicine, Iowa City, IA 52240, USA
3Department of Anatomy and Cell Biology, University of Iowa Carver College of Medicine, Iowa City, IA 52240, USA
4Holden Comprehensive Cancer Center, University of Iowa Carver College of Medicine, Iowa City, IA 52240, USA
5Fraternal Order of Eagles Diabetes Research Center (FOEDRC), University of Iowa Carver College of Medicine, Iowa City, IA 52240, USA
6Abboud Cardiovascular Research Center, University of Iowa Carver College of Medicine, Iowa City, IA 52240, USA
7Pappajohn Biomedical Institute, University of Iowa Carver College of Medicine, Iowa City, IA 52240, USA
8FOEDRC Metabolomics Core Research Facility, University of Iowa Carver College of Medicine, Iowa City, IA 52240, USA
9These authors contributed equally
10Lead Contact
Correspondence: eric-taylor@uiowa.edu
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SUMMARY

Hepatocellular carcinoma (HCC) is a devastating cancer increasingly caused by non-alcoholic fatty liver disease (NAFLD). Disrupting the liver Mitochondrial Pyruvate Carrier (MPC) in mice attenuates NAFLD. Thus, we considered whether liver MPC disruption also prevents HCC. Here, we use the N-nitrosodiethylamine plus carbon tetrachloride model of HCC development to test how liver-specific MPC knockout affects hepatocellular tumorigenesis. Our data show that liver MPC ablation markedly decreases tumorigenesis and that MPC-deficient tumors transcriptomically downregulate glutathione metabolism. We observe that MPC disruption and glutathione depletion in cultured hepatomas are synthetically lethal. Stable isotope tracing shows that hepatocyte MPC disruption reroutes glutamine from glutathione synthesis into the tricarboxylic acid (TCA) cycle. These results support a model where inducing metabolic competition for glutamine by MPC disruption impairs hepatocellular tumorigenesis by limiting glutathione synthesis. These findings raise the possibility that combining MPC disruption and glutathione stress may be therapeutically useful in HCC and additional cancers.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a devastating global health problem as the fifth most common malignancy and second greatest cause of cancer mortality worldwide (Degasperi and Colombo, 2016). HCC is often and increasingly caused by non-alcoholic fatty liver disease (NAFLD) (Dyson et al., 2014; Estes et al., 2018; Margini and Dufour, 2016; Younossi et al., 2015). Additionally, the two primary risk factors for NAFLD and NAFLD-mediated HCC are obesity and diabetes. (Margini and Dufour, 2016). Although the mechanisms underlying NAFLD-mediated HCC are not fully understood, factors arising from the deranged metabolism induced by chronic overnutrition are agreed to be causal factors. These include aberrant insulin and insulin growth factor 1 (IGF-1) signaling, chronic inflammation, and increased oxidative damage (Kutlu et al., 2018). As the obesity, diabetes, and NAFLD epidemics grow, new therapies are urgently needed to curb consequently increasing NAFLD-mediated HCC incidence (Baffy et al., 2012).

The recently identified mitochondrial pyruvate carrier (MPC) has emerged as a potential diabetes and NAFLD therapeutic target (Bricker et al., 2012; Colca et al., 2018; Gray et al., 2015; Herzig et al., 2012; McCommis et al., 2015, 2017; Rauckhorst et al., 2017). The MPC resides in the inner mitochondrial membrane and transports pyruvate from the cytosol into the mitochondrial matrix (Bricker et al., 2012; Herzig et al., 2012). Thus, the MPC occupies a critical metabolic node by linking glycolysis with mitochondrial metabolism. We and others demonstrated that liver-specific MPC disruption in diabetic mice decreases hyperglycemia (Gray et al., 2015; McCommis et al., 2015). Subsequent studies showed that chemical and genetic MPC disruption attenuates NAFLD by decreasing tricarboxylic acid (TCA) cycle flux and metabolite pool sizes, inflammation, and fibrosis (McCommis et al., 2017; Rauckhorst et al., 2017). Thus, MPC disruption could be expected to prevent HCC.

Conversely, hepatocyte MPC disruption decreases gluconeogenesis, thereby phenocopying a defining and consistent HCC feature (Facciorusso et al., 2016; Hirata et al., 2016; Khan 2016).
et al., 2015; Likhitrattanapisal et al., 2016; Ma et al., 2013; Shang et al., 2016). Moreover, in several cancers MPC deficiency promotes the Warburg effect, stemness, and proliferation (Flores et al., 2017; Gray et al., 2015; Li et al., 2017a, 2017b; McCommis et al., 2015; Sandoval et al., 2017; Schell et al., 2017). Thus, a critical question is whether the anti-NAFLD or pro-HCC metabolism effects of MPC disruption dominate to decrease or increase HCC development. Furthermore, we considered that parsing this pro- and anti-cancer tension could isolate metabolic variables fundamentally underlying cancer development. Because chemical MPC inhibition is now being tested in clinical trials to treat NAFLD, this problem is also timely and clinically important (Colca et al., 2018). Here, we implemented the well-established diethylnitrosamine (DEN) plus carbon tetrachloride (CCL₄) chronic oxidative stress mouse model of HCC development, which recapitulates human HCC genetic heterogeneity (Chappell et al., 2016; Marrone et al., 2016; Pound and McGuire, 1978; Uehara et al., 2014). Remarkably, compared to wild-type (WT) mice, liver-specific MPC knockout (MPC LivKO) mice developed two-thirds fewer liver tumors with increased tumor apoptosis. Using a combination of unbiased transcriptomic profiling, biochemical and cell-based assays, and stable-isotope metabolomic tracing, we discovered that MPC disruption reroutes glutamine away from glutathione synthesis into the TCA cycle as a mechanism for impaired hepatocellular tumorigenesis. Elevated mitochondrial glutamine anaplerosis is recognized as an important cancer mechanism for sustaining TCA cycle flux (Altman et al., 2016; Vander Heiden and DeBerardinis, 2017; Zhang et al., 2017). We show here, surprisingly, that mitochondrial glutamine utilization can be anti-cancer by competitively limiting glutathione synthesis, a key component of tumor initiation, promotion, and progression (Bansal and Simon, 2018; Harris et al., 2015; Huang et al., 2013; Lam et al., 2017).

**RESULTS**

**MPC1 and MPC2 Are Highly Expressed in Human HCC**

The MPC is a heterodimer comprising two obligate protein subunits, namely, MPC1 and MPC2. Loss of either destabilizes the other and eliminates the MPC complex (Bricker et al., 2012; Herzig et al., 2012; Oonthonpan et al., 2019; Tavoulari et al., 2019). To understand the potential importance of MPC function in human HCC, we analyzed MPC1 and MPC2 expression in The Cancer Genome Database (TCGA), a comprehensive multi-dimensional map of key genomic changes in 33 types of cancer (Cerami et al., 2012). Within the TCGA, HCC was the second highest median MPC1-expressing and the highest median MPC2-expressing tumor type (Figures 1A and 1B). Retention and expression of MPC genes is striking because HCC universally downregulates the MPC-mediated process of gluconeogenesis (Gray et al., 2015; Ma et al., 2013; McCommis et al., 2015; Shang et al., 2016). Thus, the retained expression of MPC1 and MPC2 in TCGA HCCs suggested the MPC may have a yet-to-be-discovered pro-tumorigenic function.

**Liver-Specific MPC Disruption Decreases Hepatocellular Tumorigenesis**

We previously observed that MPC LivKO mice are resistant to high-fat-diet-induced hyperglycemia, liver inflammation, and liver fibrosis (Gray et al., 2015; Rauckhorst et al., 2017). Here, we aimed to test whether this protective effect would extend to HCC prevention. We induced HCC in WT (Mpc1fl/fl) and MPC LivKO (Mpc1fl/fl+AlbCre) mice by using a low-dose version of the well-established N-nitrosodiethylamine (DEN) plus carbon tetrachloride (CCL₄) model (Uehara et al., 2014). DEN randomly mutates the genome, and CCL₄ produces hepatic oxidative stress and inflammation, accelerating HCC development. In contrast to oncogene-induced HCC, the DEN/CCL₄ model recapitulates the genetic heterogeneity of human HCC (Chappell et al., 2016; Marrone et al., 2016). WT and MPC LivKO mice were injected with DEN (1 mg/kg) on postnatal day 15. This was followed by twice-weekly (0.2 mL/kg) CCL₄ injections with body weight measurements starting at 8 weeks of age. At 24 weeks of age, after 16 weeks of CCL₄ injections, mice were euthanized.
Figure 2. Hepatic Mpc1 Deletion Decreases Hepatocellular Carcinoma Development

(A) Body weight of WT and MPC LivKO mice over course of CCl4 treatment; n = 24–29 biological replicates.

(B) Liver tumor burden at euthanasia, determined by grossly dissecting each liver lobe and examining for and counting visible tumors; please see STAR Methods section for additional detail; n = 24–29 biological replicates.

(C) Liver mass at euthanasia, determined by removing and weighing whole livers; n = 24–29 biological replicates.

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16 weeks of CCl₄ injections, mice were euthanized and gross tumor burden was visually determined by two individuals blinded to liver genotype. Data from two separate cohorts generated 1 year apart are presented combined. Results from individual cohorts are shown in Figure S1. Animal weights did not differ throughout the study (Figure 2A). Compared to WT mice, MPC LivKO mice had significantly fewer tumors per liver, smaller average tumor size, smaller liver weights, and a smaller liver-to-body weight ratio (Figures 2B–2E). qPCR analysis of normal adjacent and paired tumor tissue confirmed Mpc1 expression loss in MPC LivKO mice. Conversely, WT tumors compared to normal-adjacent tissue significantly increased Mpc1 expression (Figure 2F). Western blots confirmed loss of both Mpc1 and Mpc2 protein in MPC LivKO livers (Gray et al., 2015; McCombs et al., 2015) (Figures 2G and S2A). These results indicate that MPC LivKO livers are less susceptible to chronic metabolic-injury-induced HCC. Representative images show decreased MPC LivKO tumor numbers (Figure 2H).

We considered whether genotypic differences in DEN-induced DNA damage could explain decreased MPC LivKO liver tumorigenesis. However, analyzing livers of 15-day-old mice 24 h after DEN injection for the DNA damage marker 8-OHdG revealed a 10-fold DNA damage increase compared to vehicle controls but no differences between genotypes (Figure 2). Genotyping and western blots of these developing livers showed Cre to be present in MPC LivKO livers but that Mpc1 protein loss was not yet complete (Figures S2B and S2C). Furthermore, dihydroethidium (DHE) fluorescence of fresh-frozen liver slices from normal chow-fed, untreated mice was not different between genotypes, consistent with no difference in basal reactive oxygen species production (Figure S3). Measuring the plasma liver damage markers aspartate aminotransferase (AST) and alanine aminotransferase (ALT) after 9 weeks of CCl₄ treatment and at time of sacrifice (16 weeks of CCl₄ treatment) also demonstrated no difference between genotypes (Figure 2J). These measurements suggest that attenuated MPC LivKO mouse HCC development cannot be explained by diminished CCl₄ liver injury susceptibility.

**MPC LivKO Tumors Exhibit Increased Apoptosis**

Because MPC LivKO tumors were smaller than WT tumors, we questioned if they had decreased proliferation or increased apoptosis. We performed immunohistochemistry on liver tumor sections for a cell proliferation marker, Ki67, and an apoptosis marker, cleaved-caspase 3. WT and MPC LivKO livers showed no difference in the Ki67-positive nuclei fraction, consistent with no proliferation rate difference (Figure 2K). Compared to WT, MPC LivKO livers showed significantly increased cleaved-caspase-3-positive nuclei, indicating a higher apoptosis rate (Figure 2L).

**RNA Sequencing Reveals Genotype Divergent Changes in Glutathione Metabolism Genes**

To understand how MPC loss resulted in markedly fewer and slightly smaller tumors with a higher apoptosis rate, we sought to identify signatures of genetic selection. We performed RNA sequencing to compare WT and MPC LivKO tumor and paired normal-adjacent tissue transcript levels. Unsupervised clustering analysis identified six gene expression change groups: (1) increased in both WT and LivKO tumors; (2) increased in WT tumors; (3) increased in MPC LivKO tumors; (4) decreased in both WT and LivKO tumors; (5) decreased in WT tumors; and (6) decreased in MPC LivKO tumors (Figures 3A and 3B; Table S1). The two clinically utilized human HCC markers Alpha-fetoprotein (Afp) and Glypican-3 (Gpc3) were upregulated in both WT and MPC LivKO tumors, providing evidence of similarity to human HCC (Masuzaki et al., 2012). Indeed, Ingenuity Pathway Analysis (IPA) identified the HCC pathway as common to both MPC LivKO and WT tumors (Figure 3C).

Interestingly, RNA sequencing data revealed genotype-divergent expression of glutathione-consuming genes. In the cluster of 14 genes upregulated in WT tumors, two, Gsta1 and Gstp2, are both glutathione-S-transferases (GSTs). GSTs detoxify oxidized lipid aldehyde derivatives and electrophilic xenobiotics by glutathione conjugation, decreasing toxicity and facilitating elimination. Conversely, three GSTs, Gsta2, Gsta3, and Mgst1 were in the cluster of 108 genes downregulated in MPC LivKO tumors. The same cluster contained glutathione peroxidase (Gpx1), another glutathione consuming enzyme. Thus, WT tumors increased but MPC LivKO tumors decreased expression of glutathione metabolizing genes. Strikingly, IPA identified the pathway “Glutathione Depletion in Liver” as specific to MPC LivKO tumor samples (Figure 3C). These RNA sequencing and IPA results led us to consider whether glutathione depletion due to MPC disruption could be a mechanism for impaired HCC development.

**The MPC Mediates Increased Tumor Glutathione Content**

Glutathione, an anti-oxidant tripeptide, is critical for tumor initiation, and glutathione levels are elevated in HCC and correlate...
with cell growth rate (Harris et al., 2015; Huang et al., 2013; Lam et al., 2017). Therefore, we measured glutathione content in tumor and normal adjacent tissue. WT normal tissue and MPC LivKO normal tissue samples did not have different mean total glutathione levels. However, compared to adjacent normal tissue, WT tumors, but not LivKO tumors, increased total glutathione (Figure 3D). Similarly, when measured as net change in glutathione from each normal-adjacent sample to its paired tumor sample, WT tumors increased total glutathione levels significantly more than MPC LivKO tumors (Figure 3E). The reduced glutathione (GSH) to oxidized glutathione (GSSG) ratio was not different between tumor and adjacent normal tissue and was unaffected by genotype, suggesting unchanged overall thiol redox balance under the basal conditions of tissue harvest (Figure 3F).

We first tested how the specific MPC inhibitor UK5099 affected Hepa-1-6 mouse hepatoma cell viability. To induce glutathione stress, proliferating Hepa-1-6 cells were treated with the glutathione-depleting agent buthionine sulfoximine (BSO) and either UK5099 or vehicle for 48 h. Control experiments confirmed that 48-h BSO treatment depleted Hepa-1-6 glutathione (Figure S4A). BSO treatment dose-dependently decreased cell viability measured by resazurin reduction, and this effect was amplified by combined UK5099 treatment (Figure 4A). This demonstrated a synthetic effect of MPC inhibition and glutathione depletion. Notably, UK5099 treatment did not affect cell viability compared to vehicle (Figure 4B). We next questioned whether viability could be rescued by the thiol antioxidant and partial glutathione precursor N-acetylcysteine (NAC). Indeed, BSO + UK5099-treated Hepa-1-6 cell viability was partially rescued by NAC supplementation when measured by resazurin reduction (Figure 4B) or crystal violet staining (Figure S4B).
To test the effect of glutathione depletion and MPC disruption on clonogenic potential, we treated Hepa1-6 cells with BSO, UK5099, and NAC combinations for 72 h and then plated cells for colony-forming assays. BSO + UK5099-treated cells generated about two-thirds fewer colonies than BSO-treated cells (Figure 4C). This demonstrates that glutathione depletion and MPC disruption synthetically lethality extend to clonogenic potential, the preferred culture model for tumorigenic potential. We observed MPC disruption decrease glutathione levels in primary hepatocytes, 5 mM glucose (E), or 5 mM glucose supplemented with 5 mM glutamine (F), treated with DMSO or 5 μM UK5099; n = 4–6 biological replicates. Data are presented as mean ± SEM, compared by t test unless otherwise noted (*p < 0.05, **p < 0.01, ***p < 0.001). See also Figure S4.

Stable Isotope Tracing Reveals MPC Disruption Reroutes Glutamine Carbon into the TCA Cycle and away from Glutathione

We observed MPC disruption decrease glutathione levels in MPC LivKO tumors, Hepa1-6 cells, and glutathione-stressed primary hepatocytes. We considered that glutamine is utilized as both a TCA cycle fuel and precursor for glutathione synthesis and, thus, could competitively link these fundamental cellular processes. We aimed to test the hypothesis that MPC-disrupted hepatocytes re-route glutamine toward the TCA cycle, away from glutathione synthesis. We performed experiments in vivo with whole liver and ex vivo with primary hepatocytes to survey glutamine carbon partitioning between the TCA cycle and glutathione. To most acutely capture these effects, we administered [U-13C]glutamine tracer intraperitoneally to mice that were treated with MPC LivKO or WT for 24 h and immediately freeze-clamped at 30 min after the first injection.
Hepatic extracts were analyzed by gas chromatography-mass spectroscopy (GC-MS) metabolomic profiling and stable isotope tracing. Principal-component analysis (PCA) confirmed the WT and MPC LivKO liver metabolomes to be systematically different (Figure S5A). Mass isotopologue analysis revealed the M+5 glutamine, M+5 glutamate, M+5 α-ketoglutarate, M+4 succinate, M+4 fumarate, M+4 malate, and M+4 citrate isotopologue fractions to be greater in MPC LivKO mice (Figure 5A; Table S2). This demonstrates greater enrichment of M+5 glutamine into the TCA cycle of MPC LivKO livers. As an additional measure of mitochondrial glutamine utilization, we examined the ratio of M+5 α-ketoglutarate to M+5 glutamine. MPC LivKO livers manifested a nearly 3-fold increase in this ratio, consistent with a 3-fold increase in TCA cycle glutamine entry (Figure 5B).

To understand how glutamine and α-ketoglutarate total concentration differences could contribute to 13C-enrichment differences, we measured metabolite pool sizes (Table S3). Metabolite profiling revealed WT and MPC LivKO liver glutamine and glutamate levels to be identical (Figures 5C and 5D). Notably, MPC LivKO liver α-ketoglutarate trended greater (p = 0.058), whereas other TCA cycle intermediates were decreased or not different (Figures 5E and S5B). The unchanged glutamine but increased α-ketoglutarate pool size corroborate ratiometric comparisons of M+5 glutamine and M+5 α-ketoglutarate isotopologue fractions. This is consistent with increased glutamine channeling into the TCA cycle through α-ketoglutarate to compensate for lost MPC LivKO mitochondrial pyruvate uptake. Notably, total metabolite profiling also revealed MPC LivKO livers to have more than two-fold greater 2-hydroxybutyrate.

Figure 5. In Vivo (U)13C Glutamine Tracing Demonstrates Increased TCA Cycle Glutamine Utilization in MPC LivKO Livers
(A) Traced isotopologue distribution in liver tissue after WT (white) and MPC LivKO (red) mice were intravenously injected with 400 mg/kg (U)13C glutamine. M+n corresponds to the number, n, of 13C carbons being incorporated into the metabolite; n = 6–7 biological replicates.
(B) Mean normalized ratio of the M+5 α-ketoglutarate signal to the M+5 glutamine signal for WT and MPC LivKO; n = 6–7 biological replicates.
(C–F) Relative abundance of glutamine (C), glutamate (D), α-ketoglutarate (E), and 2-hydroxybutyrate (F) in WT and MPC LivKO livers; n = 6–7 biological replicates. Data are presented as mean ± SEM, compared by t test performed unless otherwise noted (*p < 0.05, **p < 0.01, ***p < 0.001). See also Figure S5 and Tables S2 and S3.
2-hydroxybutyrate is a specific marker of glutathione stress and production by the transsulfuration pathway (Lord and Bralley, 2008). Thus, increased 2-hydroxybutyrate in MPC LivKO livers is consistent with adaptive glutathione synthesis (Figure 5F).

To mechanistically test for glutathione synthesis differences arising from MPC disruption, we moved from an in vivo glutathione tracer model to a more malleable primary hepatocyte tracer model. Importantly, a goal of this experiment was to reasonably recapitulate how MPC disruption affects glutamine metabolism during the high glutathione stress and genetic selection during hepatocyte transformation, which cannot be measured in established tumors. To examine glutamine flux into glutathione synthesis during acute glutathione stress, primary hepatocyte glutathione was depleted by mBCI chemical conjugation. This was followed by incubating primary hepatocytes in media containing (U)\(^{13}\)C-labeled glutamine, with either vehicle or UK5099 treatment (Figure 6A). (U)\(^{13}\)C-labeled glutamine can generate either M+3 or M+5 glutathione, depending on whether it is first oxidatively routed through the TCA cycle before incorporation into glutathione (Figure 6B). Primary hepatocyte total glutathione levels were similarly depleted by mBCI treatment (Figure 6C). Compared to vehicle, hepatocytes treated with the MPC inhibitor UK5099 had significantly less total glutathione (Figure 6D).

Next, liquid chromatography-mass spectrometry (LC-MS), which, in contrast to GC-MS, enables detection of intact glutathione, mass isotopologue analysis revealed that the M+5 glutathione fraction of total glutathione was significantly less in UK5099-treated hepatocytes (Figure 6E). Thus, MPC-inhibited hepatocytes had decreased direct flux of (U)\(^{13}\)C-glutamine into glutathione synthesis. The concentration of newly synthesized M+5 glutathione was then calculated by multiplying the M+5 isotopologue fraction by total glutathione concentration. UK5099-treated hepatocytes had a significantly decreased M+5 glutathione isotopologue total concentration (Figure 6F). Importantly, MPC inhibition did not change the GSH to GSSG ratio, which is consistent with impairment of glutathione synthesis versus modulation of thiol redox state (Figure 6G).

We repeated this experiment with WT and MPC LivKO primary hepatocytes. mBCI treatment similarly depleted WT and MPC LivKO hepatocyte total glutathione levels (Figure 6H). Compared to WT, MPC LivKO hepatocytes re-synthesized less total glutathione and had a decreased M+5 glutathione total concentration, demonstrating impaired glutathione synthesis (Figures 6J and 6K). MPC deletion did not change the GSH to GSSG ratio (Figure 6L). To enable ratiometric comparison of M+5 glutathione enrichment to M+5 glutamine loading, we modified our mass spectrometry analytical workflow from that used for the immediately preceding experiments using UK5099 to disrupt MPC activity to also detect intact glutamine. As expected, compared to WT hepatocytes, MPC LivKO hepatocytes exhibited a significantly decreased M+5 glutathione to M+5 glutamine ratio, further demonstrating that MPC disruption decreases glutamine flux into glutathione (Figure S6). Together, these data demonstrate that MPC-disrupted hepatocytes increase glutaminolysis to maintain the TCA cycle, re-synthesize glutathione at a slower rate, and utilize less glutamine for glutathione synthesis.

**DISCUSSION**

Type 2 diabetes and NAFLD are now the fastest growing HCC risk factors due to the availability of hepatitis B vaccines and hepatitis C treatments (Estes et al., 2018; Facciourusso et al., 2016; Ferlay et al., 2010; Kutlu et al., 2018; Margini and Dufour, 2016; Schütte et al., 2016). Thus, the changing etiology of HCC requires new prevention strategies. Disrupting liver MPC activity has recently been shown to attenuate diabetes and NAFLD (Gray et al., 2015; McCommis et al., 2015, 2017; Rauckhorst et al., 2017). This led us to question if MPC disruption could attenuate HCC development. However, given that MPC disruption induces a canonical, HCC-like metabolism by decreasing gluconeogenesis, an expected result was not necessarily clear (Gray et al., 2015; McCommis et al., 2015). We found that MPC disruption markedly decreased HCC development and discovered a previously unrecognized relationship among mitochondrial anaplerosis, glutamine metabolism, and tumorigenesis.

To understand the contribution of the MPC to HCC development, we utilized liver-specific MPC knockout (MPC LivKO) mice and a spontaneous, chronic metabolic-injury-driven HCC model. Strikingly, compared to WT mice, MPC LivKO mice developed two-thirds fewer tumors. Moreover, MPC LivKO tumors were smaller with increased apoptosis. In previous investigations, increased pyruvate anaplerosis in NAFLD increased TCA cycle flux, oxidative stress, and inflammation (Satapati et al., 2012, 2015). In two similar studies, liver MPC disruption prevented NAFLD-mediated TCA cycle metabolite pool expansion and decreased markers of inflammation and fibrosis (McCommis et al., 2017; Rauckhorst et al., 2017). Here, we demonstrate that MPC disruption markedly decreased chronic metabolic-injury-driven tumorigenesis. This result contributes to the growing body of literature illuminating how decreasing hepatic mitochondrial pyruvate uptake protects from progressive liver disease and demonstrates that decreasing gluconeogenesis is not necessarily oncogenic.

Our results reveal that metabolic competition for glutamine may be exploited to impair tumorigenesis. Through biochemical and stable isotope tracing assays, we found that loss of hepatocyte MPC activity limits glutathione synthesis. MPC disruption has previously been shown to increase mitochondrial glutamine utilization in cancer models, primary cortical neurons, and skeletal muscle and, conversely, to decrease glutamine utilization in incubated mouse retinas (Bader et al., 2018; Divakaruni et al., 2017; Grenell et al., 2019; Sharma et al., 2019; Vacanti et al., 2014; Yang et al., 2014). Our work here advances the understanding of cancer glutamine metabolism by demonstrating, first, that glutamine partitioning between TCA cycle oxidation and glutathione synthesis can be competitive and, second, that the MPC controls hepatocellular tumorigenesis, likely by modulating glutamine partitioning between these two fates.

Glutathione is critical for tumor initiation, a time of high reactive oxygen species (ROS), across multiple tumor types (Carter et al., 2014; Yang et al., 2014). In numerous tumor types, glutathione levels positively correlate with resistance to treatment, invasiveness, and metastasis (Bansal and Simon, 2018; Hatem et al., 2017). Glutathione may also be consumed by GSTs. GSTs conjugate glutathione to xenobiotic and
Figure 6. Ex Vivo (U)13C Glutamine Tracing Demonstrates MPC Disruption Impairs Hepatocyte Glutathione Synthesis

(A) Schematic illustration of the time course for glutathione depletion and (U)13C glutamine tracing in primary hepatocyte experiments.

(C–G) Primary hepatocytes were isolated from 12-week-old WT mice.

(H–L) Primary hepatocytes isolated from 17- to 20-week-old WT and MPC LivKO mice.

(B) Schematic demonstrating metabolic path U-13C glutamine incorporation into glutathione or the TCA cycle. Green denotes 13C label, and gray denotes 12C non-label.

(C) Total glutathione determined enzymatically in primary hepatocytes following glutathione depletion induced by 40 min of treatment with 50 μM monochlorobimane; n = 3 biological replicates.

(D) Total glutathione determined after 1 h of (U)13C glutamine chase following glutathione depletion as in (A). During the chase period, primary hepatocytes were incubated with 5 mM (U)13C glutamine, 5 mM glucose, and DMSO (Veh) or 5 μM UK5099 (UK); n = 3 biological replicates.

(E) Glutathione mass isotopologue distribution after chase period described in (A); n = 3.

(F) Concentration of M+3 and M+5 glutathione calculated by multiplying total glutathione shown in (C) with the isotopologue fractional enrichment shown in (D); n = 3 biological replicates.

(G) GSH to GSSG ratios in DMSO (Veh)- and UK5099 (UK)-treated hepatocytes. Ratios are calculated from metabolite mass spectrometry signal intensity and normalized to vehicle.

(H) Total glutathione determined enzymatically in WT and MPC LivKO primary hepatocytes following glutathione depletion as in (A); n = 4 biological replicates.

(I) Total glutathione determined in WT and MPC LivKO primary hepatocytes after 1 h of (U)13C glutamine chase following glutathione depletion as in (A). Primary hepatocytes were incubated with 5 mM (U)13C glutamine, 5 mM glucose; n = 3 biological replicates.

(J) Glutathione mass isotopologue distribution after chase period described in (A); n = 4.

(K) Concentration of M+3 and M+5 glutathione calculated by multiplying total glutathione shown in (I) with the isotopologue fractional enrichment shown in (J); n = 3 biological replicates.

(L) GSH to GSSG ratios in WT and MPC LivKO hepatocytes. Ratios are calculated from metabolite mass spectrometry signal intensity and normalized to WT; n = 4 biological replicates.

Data are presented as mean ± SEM, compared by t test performed unless otherwise noted (*p < 0.05, **p < 0.01, ***p < 0.001). See also Figure S6.
endogenous waste molecules, facilitating excretion. GSTs of the alpha class (GSTsαs) are predominately expressed in the liver and comprise the majority of differentially expressed isoforms in our RNA sequencing dataset. The WT tumor GSTsα upregulation and MPC LivKO tumor GSTsα downregulation are consistent with glutathione scarcity limiting MPC LivKO HCC development and an adaptive glutathione sparing response. Hepatocytes require extraordinarily high glutathione synthesis rates for conjugating chemical waste during detoxification metabolism (Geenen et al., 2013; Pajares and Pérez-Sala, 2018). Thus, MPC disruption may exploit a metabolic vulnerability to which developing HCCs are inherently sensitive, leading to lethal glutathione depletion during the stress of HCC initiation and early progression.

Notably, here, hepatoma experiments differ from in vivo tumorigenesis experiments by two critical parameters. First, MPC inhibition in hepatomas was acute, and second, in vivo tumorigenesis selected for MPC LivKO HCCs more resistant to glutathione stress. In contrast to the high cell death observed in hepatoma clonogenic assays, the elevated but still minimal apoptosis observed in MPC LivKO versus WT tumors does not detect apoptosis at the critical threshold of tumor initiation, which was likely higher. Thus, the greater glutathione depletion observed in acutely MPC-inhibited hepatomas may better recapitulate focal glutathione depletion in pre-neoplastic lesions and mitotic catastrophe of transformed cells, before selection and progression to HCC.

Lastly, we note limitations of this work and potential future directions. Although the CCl4 tumorigenesis protocol used here is a more extreme oxidative stress than encountered by human livers during obesity and diabetes, we expect that the low-dose CCl4 model we utilized increased translational fidelity compared to higher dose CCl4 or single oncogene HCC models. To determine how well results here translate to obesity and NASH-mediated HCC, it will be important to test the effects of MPC disruption in chronic overnutrition models of HCC development. Furthermore, although the combination of in vivo and ex vivo culture approaches used here address how chronic MPC disruption changes hepatocellular tumorigenesis and how acute MPC disruption and glutathione depletion stress changes hepatoma survival, we did not test how acute MPC disruption affects HCC growth and survival nor how acute or chronic MPC disruption affects HCC invasiveness and metastatic potential. Thus, important future directions include determining how hepatocyte MPC disruption changes pre-neoplastic lesion glutathione metabolism and survival and how acute and chronic MPC disruption change clinically relevant advanced HCC outcomes.

In conclusion, we report that MPC disruption in hepatocytes decreased metabolic-injury-driven hepatocellular tumorigenesis. We demonstrate that adaptive mitochondrial glutamine utilization resulting from MPC disruption limits glutamine availability for glutathione synthesis. Taken together, our work reveals a previously unrecognized link between pyruvate metabolism and glutathione content and identifies the MPC as a plausible HCC prevention target. Our findings raise the possibility that combining MPC disruption and glutathione stress may be therapeutically useful in HCC and additional cancers.

STAR METHODS
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SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2019.07.098.

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AUTHOR CONTRIBUTIONS
S.C.T., R.D.S., A.J.R., M.F.N., and E.B.T. wrote and edited the manuscript. All authors read and commented on a draft manuscript. S.C.T., R.D.S., A.J.R., M.F.N., S.R.S., D.R.S., and E.B.T. designed experiments. S.C.T., R.D.S., A.J.R., M.F.N., S.R.S., J.L.B., K.A.M., D.A.S., L.R.G., A.D.P., L.O., A.S., and A.J.D. performed experiments.

DECLARATION OF INTERESTS
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### STAR★METHODS

#### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Mpc1                | Cell Signaling | Cat#14462; RRID: AB_2773729 |
| Mpc2                | Cell Signaling | Cat#46141; RRID: AB_2799295 |
| VDAC                | Cell Signaling | Cat#4664; RRID: AB_10557420 |
| Cleaved Caspase-3   | Cell Signaling | Cat#9661; RRID: AB_2341188 |
| Ki67                | Abcam     | ab#16667; RRID: AB_302459 |
| Goat anti-Rabbit DyLight 800 | ThermoFisher | SAS-35571; RRID: AB_2556775 |
| Goat anti-Mouse DyLight 800 | ThermoFisher | SAS-10176; RRID: AB_255675 |
| DAKO EnVision + Dual Link System-HRP (DAB+) | Dako | K4065 |
| **Bacterial and Virus Strains** |        |            |
| AAV8.TBG.Pi.Cre.rBG | UPenn Viral Vector Core | AV-8-PV1090 |
| AAV8.TBG.Pi.Null.bGH | UPenn Viral Vector Core | AV-8-PV0148 |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| Penicillin-Streptomycin | ThermoFisher | 15140-122 |
| Insulin             | Sigma    | I9278      |
| Dexamethasone       | Sigma    | D4902      |
| Capillary blood tubes | Sarstedt | 16.444.100 |
| Fetal Bovine Serum  | Atlanta Biologicals | S11150 |
| Glutamax            | Gibco    | 35050-06   |
| Protease Arrest     | G Biosciences | 786-437 |
| Igepal CA-630       | Sigma    | I8896      |
| N-nitrosodiethylamine (DEN) | Sigma | N0258 |
| Trizol              | Life Technologies | 15596018 |
| High-Capacity cDNA Reverse Transcription Kit | Applied Biosystems | 4368814 |
| SYBR Green ER SuperMix | Life Technologies | 11760-100 |
| DNeasy Kit          | QIAGEN   | #69504     |
| Benzonase           | Sigma    | E1014      |
| Alkaline Phosphatase | ThermoFisher | EF0654 |
| miRNeasy            | QIAGEN   | #217004    |
| Sulfosalicylic acid | Sigma    | 90275      |
| Pierce BCA Assay Kit | ThermoFisher | 23225 |
| 2-vinylpyridine     | Sigma    | 132292     |
| Resazurin           | Sigma    | R7017      |
| UK5099              | Tocris   | 4186       |
| L-Buthionine-sulfoximine | Sigma | B2515 |
| N-acetyl cysteine   | Sigma    | A9165      |
| Monochlorobimane    | Sigma    | 69899      |
| U-13C Glutamine     | Cambridge Isotopes | CLM-1822 |
| Methoxymamine hydrochloride (MOC) | Sigma | 226904 |
| N,O-Bis(trimethylsilyl)trifluoroacetamide (TMS) | Sigma | 155195 |
| Carbon Tetrachloride (CCl4) | Sigma | 289116 |
| Dihydroethidium     | ThermoFisher | D1168 |
| Williams E Media    | ThermoFisher | 12551-032 |

(Continued on next page)
LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Eric Taylor (eric-taylor@uiowa.edu). This study did not generate new unique reagents.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mouse Models
Animal work was performed in accordance with the University of Iowa Animal Care and Use Committee (IACUC). Mice were group housed up to 5 mice/cage and maintained on Harlan Scientific 2920i diet. Littermate paired mice were used in all experiments. Constitutive, hepatocyte-specific Mpc1 knockout and wild-type mice were generated as previously reported (Gray et al., 2015). Briefly, Mpc1<sup>fl/fl</sup> mice were crossed with Mpc1<sup>fl/fl</sup>Alb<sup>Cre/Cre</sup> producing Mpc1<sup>fl/fl</sup>Alb<sup>Cre/Cre</sup> (LivKO) and Mpc1<sup>fl/fl</sup> (WT) mice. Acute,
hepatocyte-specific Mpc1 knockout (LivKO) and wild-type (WT) mice were generated by retro-orbitally injecting Mpc1fl/fl mice with AAV8.TBG.PI.Cre.rBG (AAV-Cre, LivKO) or AAV8.TBG.PI.Null.bGH (AAV-EV, WT) using 1x10^{11} genome copies per mouse.

For the tumorigenesis studies: at 15 days of age mice were treated with 1 mg/kg N-nitrosodiethylamine (DEN) intraperitoneally. A 100 mg/ml stock solution of DEN was prepared in PBS and filtered. On injection day, 1 mg (10 μL of 100 mg/ml stock solution) DEN was added to 15 mL of filtered PBS to make a DEN injection solution of 0.067 μg/μl. Mice were then injected with 1 mg/kg. Depending on body weight, injection volume ranged from approximately 75 μL to 125 μL. A 40 μl/ml stock solution of carbon tetrachloride (CCl4) was prepared by mixing 400 μL CCl4 to 9.6 mL of filtered corn oil. Mice were injected with 0.2 ml/kg CCl4 twice weekly from age 8 to 24 weeks.

**Cell Lines**
Hepa1-6 cells were cultured in High Glucose DMEM supplemented with 10% Fetal Bovine Serum, GlutaMAX, and 1% Penicillin-Streptomycin. Cells were cultured at 37°C with 20% oxygen and 5% carbon dioxide.

**METHOD DETAILS**

**Primary Hepatocyte Isolation**
Mpc1fl/fl mice treated with AAV-Cre (LivKO) or AAV-EV (WT) were used to harvest primary hepatocytes as described previously (Gray et al., 2015). Briefly, livers were first perfused with a buffered collagenase and trypsin inhibitor. Hepatocytes were liberated from the liver capsule. This initial cell suspension was centrifuged at 50 x g for 6 min, and the supernatant, enriched with Kupffer and other non-hepatocyte (non-parenchymal) cell types was discarded. The hepatocyte-enriched pellet was washed by gently resuspending the cell pellet followed by centrifuging for 3 minutes at 50 x g to further remove debris, dead cells, and non-parenchymal cells. This washing step was repeated for a total of 2 washes. Hepatocytes were plated at a density of 166,666 cells/cm² and allowed to attach for 4 hours in Williams E media, supplemented with 5% FBS, 1% Penicillin-Streptomycin, 10 nM insulin, and 10 nM dexamethasone.

**Tissue harvest and tumor analysis**
Tumor burden for each liver was determined separately by two individuals blinded to genotype. Mice were euthanized in the ad-lib fed state between 0900 and 1200 and livers were immediately harvested and weighed. Next, each liver lobe was grossly dissected and examined for visible tumors, which were counted and measured in diameter using calipers. A section of the left lateral lobe was fixed in 10% neutral-buffered formalin for paraffin-embedding. The remaining liver was divided into normal adjacent and paired tumor tissue, snap-frozen in liquid nitrogen and stored at −80°C until further biochemical analysis.

**Plasma analysis**
Tail vein blood was collected using capillary tubes. Tubes were centrifuged at 3,000 x g for 15 minutes and the supernatant was collected. Prior to analysis, this plasma was diluted to 1:3 with 0.9% saline. Samples were analyzed for Aspartate aminotransferase (AST) activity and Alanine aminotransferase (ALT) activity following the manufacturers’ protocols.

**Immunohistochemistry**
Formalin-fixed tumor samples were processed and embedded in paraffin. Tissue sections (4 μm) were deparaffinized and stained with Ki67 and Cleaved Caspase-3. Immunohistochemistry was visualized using DAKO EnVision + Dual Link System-HRP (DAB+) for 5 minutes. Tumor slides were imaged using an Olympus BX-61 light microscope while blinded to genotype. Three separate high-power field images/slide/mouse were imaged and analyzed using ImageJ. The positive nuclei fraction was determined using: threshold particle analysis. Threshold particle analysis identifies the number of both total nuclei and IHC-reactive nuclei by colorimetric threshold. The ratio of IHC-reactive/total nuclei was defined as % positive.

**Western Blots**
Snap-frozen liver tissue was homogenized in a buffer containing 40 mM HEPES, 120 mM NaCl, 50 mM NaF, 5 mM Sodium Pyrophosphate decahydrate, 5 mM β-glycerolphosphate, 1 mM EDTA, 1 mM EGTA, 10% Glycerol (v/v), 1% Igepal CA-630 (v/v), with 1X Protease Arrest protease inhibitor and 1 mM DTT. Homogenates were incubated at 4°C for 30 minutes and centrifugation at 21,000 x g before the supernatants were collected. Proteins were separated by 10% Tricine-SDS-PAGE gel, transferred to 0.22 μM nitrocellulose membranes, and blocked with TBST (50 mM Tris, 150 mM NaCl, and 0.05% Tween20) supplemented with 5% nonfat dry milk. Blocked membranes were incubated with primary antibodies at 4°C overnight and the following day fluorescent secondary antibodies for 1 hour at RT. Finally, immuno detected proteins were visualized using the Li-Cor Odyssey CLx system.

**Quantitative PCR (qPCR)**
Total RNA from liver tissue was extracted using TRIzol method. cDNA synthesis of equal amounts of RNA from each sample was achieved using the High-Capacity cDNA Reverse Transcription Kit. SYBR Green ER SuperMix was used during qPCR. Relative abundance of mRNA was normalized to the abundance of u36B4 mRNA.
8-hydroxy-2-deoxyguanosine (8-OHdG) ELISA Assay
15 day old constitutive, hepatocyte-specific Mpc1 knockout (MPC LivKO) and wild-type (WT) mice were injected with either 1 mg/kg of DEN or PBS. 24 hours after injection mice were euthanized, and their livers were flash frozen in liquid nitrogen. DNA was isolated from frozen liver tissue using a QIAGEN DNeasy Kit. DNA was digested by the endonuclease Benzonase for 1 hour at 37°C followed by a 30 minute treatment with alkaline phosphatase at 37°C. Samples were diluted to a DNA concentration of 100 ng/μl. From there, 5 μg of DNA was added per well and DNA/RNA Oxidative Damage ELISA Kit instructions were followed.

Transcriptomic sequencing
Total RNA was collected from tumor and paired normal-adjacent liver samples using the QIAGEN miRNeasy kit. RNA from four samples each of wild-type tumor (WT-Tumor), paired wild-type normal-adjacent (WT normal adjacent), MPC LivKO tumor (MPC LivKO-Tumor), and paired MPC LivKO normal-adjacent (MPC LivKO normal adjacent) tissue was isolated. Each tumor and its paired normal-adjacent tissue were analyzed in a paired manner. Library preparation and sequencing were performed using the Illumina mRNA-Seq workflow. Initial mapping was done using the HISAT2 software program. The resulting BAM files were analyzed using the RNaseq pipeline in the Partek Genomics Suite software package. For data normalization, the raw number of reads for each transcript was converted to Fragments Per Kibobase of transcript per Million mapped reads (FPKM). FPKM values were log transformed and unsupervised clustering was performed on samples based on normalized expression of genes with variation in Euclidean distance among samples of at least 2.5 standard deviations using Cluster 3 software. Heatmaps were generated using Java TreeView software. Pathway analysis was conducted using the QIAGEN Ingenuity Pathway Analysis software program.

DHE Assay
10 mm sections of OCT-frozen liver tissue were cut and placed on the same slide. Tissue sections were stained with 10 mM DHE for 15 minutes in PBS and imaged using an Olympus FLUOVIEW FV1000 confocal microscope. Sections were co-treated with 10 mM antimycin A during DHE staining as positive control. Mean fluorescence intensity of at least 200 cell nuclei was determined for each image and normalized to nuclei from untreated normal tissue.

Glutathione Assay
Tumor and normal adjacent tissue samples were homogenized in 5% SSA (5-Sulfosalicylic acid) buffer at the time of sacrifice. The samples were centrifuged to remove precipitated proteins. Supernatants were assayed for total glutathione (GSH) content according to the Sigma Glutathione Assay Kit method. Glutathione disulfide (GSSG) was measured after reacting the sample for 2 hours with 20 μl of a 1:1 mixture of 2-vinylpyridine and ethanol per 100 μl of sample to mask GSH. GSSG was measured GSH-depleted samples using using the Sigma Glutathione Assay Kit. Glutathione determinations were normalized to protein by solubilizing SSA precipitates in 0.1 N NaOH and measuring protein content using the BCA Assay Kit.

AlamarBlue Assay
Hepa1-6 cells were treated with BSO (L-Buthionine-sulfoximine), UK5099, and N-acetyl-cysteine (NAC) for 48 hours. Viability was assessed by resazurin cell viability assay ex/em 560nm/590nm. In short, 25,000 cells were plated per well of a standard 96-well plate. Media was replaced with 200 μL of media containing corresponding treatment conditions and allowed to grow for 48 hours. After 48 hours of growth, 20 μL of 0.15 mg/ml resazurin was added per well, incubated for 3 hours, and then fluorescence was measured.

Crystal Violet Cell Cytotoxicity Assay
Hepa 1-6 cells (7500 cells/well) were seeded on a clear, 96-well plate. After 18 hours, cells were treated with 200 μL of media containing DMSO, 5 μM UK5099, 1 mM BSO, 1 mM BSO + UK5099, 5 mM NAC, 1 mM BSO + 5 mM NAC, or 1 mM BSO + 5 mM NAC + 5 μM UK5099. Media containing treatment was changed every 24 hours until cells had grown for 48. A live/dead assay was then performed using a the Crystal Violet Cell Cytotoxicity Assay Kit. Cells were washed with 200 μL of 1x washing solution and incubated in 50 μL of crystal violet staining solution for 20 minutes. Cells were washed four times with 200 μL of 1x washing solution to remove dead cells. Cells were then incubated in 100 μL of solubilization solution for 20 minutes, and absorbance was measured at 595 nm.

Clonogenic Assay
1.5 × 10^5 Hepa-1-6 cells were plated in 60 mm dishes for 48 hours and treated in DMEM supplemented with 10% FBS, 1% sodium pyruvate, and 1% Pen Strep with 1 mM BSO, 5 μM UK5099, and 10 mM NAC for 72 hours prior to clonogenic assay. Cells were detached using 0.25% trypsin, combined with floating cells, and pelleted via centrifugation at 200 x g for 5 minutes. Pellets were resuspended in fresh complete DMEM media and the total cell population was counted using a Beckman Coulter Counter. 500–20,000 cells per dish for clonogenic survival were then plated in 60-mm dishes that had been previously plated one day prior with a feeder layer of 100,000 immortalized chinese hamster ovary cells that had been lethally irradiated with 30 Gy of 250 kVp X-rays. Clones were grown for 8 days in complete media, fixed with 70% ethanol, stained with Coomassie blue, and colonies greater than 50 cells were counted. The plating efficiencies of treatment groups for each cell line were normalized to the control groups. Feeder layer alone plates were included in every experiment to ensure that all the feeder cells had been clonogenically inactive. The survival analysis
was performed using a minimum of two cloning dishes per experimental condition, and the experiment were repeated a minimum of three times.

**Monochlorobimane Assay**

Hepatocytes were washed with PBS and DMEM media containing 5 mM glucose and 50 μM monochlorobimane or 5 mM glucose plus 5 mM glutamine and 50 μM monochlorobimane was added. Immediately after media addition cells were analyzed (ex/em 370/490) on a 90 s interval for 40 minutes.

**In-vivo stable isotope tracer measurement**

Mpc1 LivKO (AAV-Cre) and wild-type (AAV-EV) mice were retro-orbitally injected with a solution of 200 mM (U)\(^{13}\)C glutamine at a dosage of 400 mg/kg whole body mass. Two injections were administered 15 minutes apart. 30 minutes after the first injection, mice were anesthetized with isoflurane and the left lateral lobe of the liver was rapidly frozen using a freeze-clamp apparatus. Frozen lobules of liver were lyophilized overnight. Lyophilized tissue was disrupted using a bead mill homogenizer in ice cold 4:5:4.5:1 methanol/acetonitrile/H\(_2\)O to extract metabolites. Homogenate was incubated at –20°C for 1 hour followed by a 10 minute centrifugation at maximum speed. Supernatants were transferred to fresh tubes and evaporated using a speed-vac. The resulting dried metabolite extracts were derivatized using methoxyamine hydrochloride (MOC) and N,O-Bis(trimethylsilyl)trifluoroacetamide (TMS). Briefly, dried extracts were reconstituted in 60 μL of 11.4 mg/mL MOC in anhydrous pyridine, vortexed for 5 min, and heated for 1 hour at 60°C. Next, 40 μL TMS was added to each sample, and samples were vortexed for 1 minute before heating for 30 minutes at 60°C. Samples were then immediately analyzed using GC/MS. GC chromatographic separation was conducted on a Thermo Trace 1300 GC with a TraceGold TG-SSMS column. 1 μL of derivatized sample was injected into the GC operating in split mode (split ratio: 20:1; split flow: 24 μL/min, purge flow: 5 mL/min, Carrier mode: Constant Flow, Carrier flow rate: 1.2 mL/min). The GC oven method was as follows: 80°C for 3 minutes, ramp to 280°C at a rate of 20°C/minute to a maximum temperature of 280°C, and the oven was held at 280°C for 8 minutes. The injection syringe was washed 3 times with methanol and 3 times with pyridine between each sample. Metabolites were detected using a Thermo Q-Exactive Plus or Thermo ISQ 7000 mass spectrometers. The Q-Exactive Plus mass spectrometer was operated from 3.90 to 21.00 minutes in EI mode (–70eV) in full scan (56.7-850 m/z) at 60K resolution and the ISQ 7000 mass spectrometer was operated in SIM mode for glucose molecular ion isotopologues (m/z: M+0 = 554, M+1 = 555, M+2 = 556, M+3 = 557, M+4 = 558, M+4 = 559, M+5 = 560). XCalibur was used for istopologue peak identification and integration. Metabolite profiling data was analyzed using Tracefinder 4.1 utilizing standard verified peaks and retention times.

**Primary Hepatocyte Tracer Measurement**

Hepatocytes were washed with PBS and media was replaced with DMEM containing 5 mM glucose, 5 mM glutamine, and 50 μM monochlorobimane for 40 minutes to deplete cellular GSH levels. Hepatocytes were washed and media was replaced with DMEM containing 5 mM glucose and 5 mM (U)\(^{13}\)C glutamine and DMSO or the specific MPC-inhibitor, 5 μM UK5099, for 1 hour. Following the incubation, hepatocytes were washed 2 times with ice-cold PBS, 2 times with ice-cold distilled water, and then immediately frozen in liquid nitrogen. Frozen plates of hepatocytes were lyophilized overnight and extracted using cold (~20°C) 4.5:4.5:1 methanol/acetonitrile/H\(_2\)O by 2 cycles of vortexing, freeze-thawing, and water bath sonication. The samples were then incubated at –20°C for 1 hour followed by a 10 minutes centrifugation at maximum speed. Supernatants were transferred to fresh tubes and evaporated using a speed-vac. Samples were resuspended in 60 μL of 1:1 acetonitrile:water, sonicated for 20 minutes, vortexed for 5 minutes, incubated for 1 hour at –20°C, and centrifuged for 10 minutes at max speed to remove any protein carry over. Supernatants were collected and transferred to autosampler vials. A Phenomenex Luna aminopropyl column attached to a Thermo Vanquish Flex UHPLC separated metabolites using a HILIC mobile phase system (A:20mM NH\(_4\)-Acetate and 20mM NH\(_4\)OH; B:90:10 acetonitrile:water). LC flow was 0.2 mL/min. The gradient was as follows: 85% B, linear decrease to 0% B by 34 min, and holding at 0% B from 34-38 minutes. The mass spectrometer operated in negative mode. Targeted selected ion monitoring (tSIM) was used to isolate GSSG isotopologues (tSIM: 610.1447-622.1447 m/z; M+0 = 611.1451 m/z, M+1 = 612.1485 m/z, M+2 = 613.1518 m/z, M+3 = 614.1552 m/z, M+4 = 615.1585 m/z, M+5 = 616.1619 m/z) and GSH isotopologues (tSIM: 305.0765-313.0765 m/z; M+0 = 306.0765 m/z, M+1 = 307.0799 m/z, M+2 = 308.0832 m/z, M+3 = 309.0866 m/z, M+4 = 310.0899 m/z, M+5 = 311.0933 m/z). XCalibur was used for istopologue peak identification and integration.

**Principle Component Analysis**

Principle Component Analysis (PCA) score plot of the first and second principle components (PCs) generated from a dataset of 86 metabolites extracted from MPC LivKO and WT liver tissue. The website ClustVis (https://biit.cs.ut.ee/clustvis/) was used to generate the PCA plot. ClustVis utilizes R code (https://www.r-project.org) for generation of PCA plots.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

As indicated within figure legends the Student’s t test was used when comparing the means of two groups, and one- and two-way analysis of variance (ANOVA) with Tukey’s post hoc testing was used when comparing the means of more than two groups. A
limited number of statistical outliers were excluded after identification by the Grubbs test. P values less than 0.05 were considered statistically significant (*p < 0.05, **p < 0.01, ***p < 0.001). Data were organized and analyzed using Microsoft Excel, SigmaPlot, and GraphPad Prism software. Sample size (n) and definition of “n” are found in figure legends.

DATA AND CODE AVAILABILITY

Transcriptomic data generated during this study are available at the Gene Expression Omnibus database: GSE132728 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE132728).
Supplemental Information

Disrupting Mitochondrial Pyruvate Uptake Directs Glutamine into the TCA Cycle away from Glutathione Synthesis and Impairs Hepatocellular Tumorigenesis

Sean C. Tompkins, Ryan D. Sheldon, Adam J. Rauckhorst, Maria F. Noterman, Shane R. Solst, Jane L. Buchanan, Kranti A. Mapuskar, Alvin D. Pewa, Lawrence R. Gray, Lalita Oonthonpan, Arpit Sharma, Diego A. Scerbo, Adam J. Dupuy, Douglas R. Spitz, and Eric B. Taylor
Figure S1, related to Figure 2

Cohort 1

A

B

C

D

Figure S1, related to Figure 2

Cohort 2

E

F

G

H
Figure S1, related to Figure 2.
(A-C) Cohort 1 MPC LivKO and WT mice body weight over course of injections (A), gross liver tumor burden (B), and gross liver weight (C), n=13-15 biological replicates.
(D) Cohort 1 average tumor size as measured by calipers at time of sacrifice WT tumors n=37, LivKO tumors n=25. Note, for Cohort 1, not all animals had their tumors measured by calipers.
(E-G) Cohort 2 MPC LivKO and WT mice body weight over course of injections (E), gross liver tumor burden (F), and gross liver weight (G), n=11-16 biological replicates.
(H) Cohort 2 average tumor size as measured by calipers at time of sacrifice WT tumors n=115, LivKO tumors n=29. Note, for Cohort 2, all animals had their tumors measured by calipers.
Data are presented as mean ± SEM (*p<0.05, **p<0.01, ***p<0.001).
Figure S2, related to Figure 2

A

| Mpc1 | H15s-M1-KO | H15s-M2-WT | H15s-M3-KO | M15s-M1-WT | M15s-M2-WT | M15s-M3-WT | M15s-M4-WT | M15s-M5-WT | M15s-M6-WT | M15s-M7-WT | M15s-M8-WT | M15s-M9-WT | M15s-M16-WT |
|------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|
| Mpc2 | H15s-M1-KO | H15s-M2-WT | H15s-M3-KO | M15s-M1-WT | M15s-M2-WT | M15s-M3-WT | M15s-M4-WT | M15s-M5-WT | M15s-M6-WT | M15s-M7-WT | M15s-M8-WT | M15s-M9-WT | M15s-M16-WT |
| VDAC | H15s-M1-KO | H15s-M2-WT | H15s-M3-KO | M15s-M1-WT | M15s-M2-WT | M15s-M3-WT | M15s-M4-WT | M15s-M5-WT | M15s-M6-WT | M15s-M7-WT | M15s-M8-WT | M15s-M9-WT | M15s-M16-WT |

B

Cre

M1 M2 M3 M4 M5 M6 M7 M8 M9 M10 M11 M12 M13 M14

C

| Mpc1 | M1 | M2 | M3 | M4 | M5 | M6 | M7 | M8 | M9 |
|------|----|----|----|----|----|----|----|----|----|
| VDAC | M10 | M11 | M12 | M13 | M14 | M15 | M16 |
**Figure S2, related to Figure 2.**

(A) Western blot for Mpc1 and Mpc2 in normal-adjacent liver tissue from both hepatic carcinogenesis cohorts. VDAC control.

(B) PCR genotyping of livers from MPC LivKO and WT mice used in Figure 2I. Presence of a Cre band at 565 base pairs denotes MPC LivKO genotype. Image mode converted from RBG to greyscale.

(C) Western blot of livers mice used in Figure 2I for Mpc1. Cre is present and active in MPC LivKO mice at 16 days of age, however the deletion is only partial in the developing mouse liver. VDAC control.
Figure S3, related to Figure 2

A

WT  WT AntA  LivKO  LivKO AntA

B

DHE Signal

Normalized MFI / 300 nuclei

WT  LivKO  WT AA  LivKO AA
Figure S3, related to Figure 2.
A) 10 mM sections of WT or MPC LivKO liver were stained with 10 mM dihydroethidium (DHE) on the same slide for 30 min prior to imaging by confocal microscopy. For a positive control, tissue sections were incubated with 10 mM antimycin A during DHE staining.
(B) The mean fluorescence intensity (MFI) of 200 nuclei from six randomly selected areas quantified using ImageJ software, n=4 biological replicates.
Data are presented as mean ± SEM (*p<0.05, **p<0.01, ***p<0.001).
Figure S4, related to Figure 4

A

Total GSH

nmoles GSH eqv/mg protein

Control

BSO

***

B

Crystal Violet 48 hr

Normalized Absorbance

Veh

UK5099

BSO

UK5099

NAC

BSO + NAC

UK5099

BSO + UK5099

NAC

UK5099

BSO + NAC + UK5099
Figure S4, related to Figure 4.
(A) Total glutathione (GSH) levels in Hepa1-6 cells treated with 1 mM BSO for 48 hours, n=4 replicate wells.
(B) Hepa1-6 cells treated for 48 hours with vehicle, 1 mM buthionine sulfoximine (BSO), 5 μM UK5099 (UK), 1 mM BSO + 5 μM UK5099, 5 mM N-acetyl cysteine (NAC), 1 mM BSO + 5 mM NAC, or 1 mM BSO + 5 μM UK5099 + 5 mM NAC. Viability measured by crystal violet assay, n=8 replicate wells.
Data are presented as mean ± SEM (*p<0.05, **p<0.01, ***p<0.001).
Figure S5, related to Figure 5

A

B

In-vivo Total Metabolite Profiling

WT
LivKO

Pyruvate
Lactate

Citrate

Malate
Fumarate

Isocitrate

α-Ketoglutarate

Succinate

Glutamine

Glutamate
Figure S5, related to Figure 5.
(A) Principle Component Analysis (PCA) score plot of the first and second principle components (PCs) generated from 86 metabolites extracted from MPC LivKO and WT liver tissue. PCA demonstrates clear separation between MPC LivKO and WT. PC1 explains 23.3% of chemical variance while PC2 explains 22.9%. From the PCA data two separate groups were observed corresponding to WT and MPC LivKO mice. Each dot represents an individual animal. PCA plot created using ClustVis (https://biit.cs.ut.ee/clustvis/). n=6-7 biological replicates.
(B) Relative abundance of TCA cycle metabolites, glutamate, glutamine, pyruvate, and lactate in livers of MPC LivKO and WT mice after injection of (U)\(^{13}\)C- glutamine, n=6-7 biological replicates. Note, the glutamine, glutamate, and α-ketoglutarate panels are shown in Figure 5. Data are presented as mean ± SEM (*p<0.05, **p<0.01, ***p<0.001).
Figure S6, related to Figure 6

M+5 GSH / M+5 GLN

Ratio

WT  LivKO

*
Figure S6, related to Figure 6.
Ratio of M+5 glutathione to M+5 glutamine signal in MPC LivKO or WT primary hepatocytes, n=4 biological replicates. Data are presented as mean ± SEM (*p<0.05, **p<0.01, ***p<0.001).