mTORC2 promotes tumorigenesis via lipid synthesis

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Summary

Dysregulated mammalian TOR (mTOR) promotes cancer, but underlying mechanisms are poorly understood. We describe an mTOR-driven mouse model that displays hepatosteatosis progressing to hepatocellular carcinoma (HCC). Longitudinal proteomic, lipidomic and metabolomic analyses revealed that hepatic mTORC2 promotes *de novo* fatty acid and lipid synthesis leading to steatosis and tumor development. In particular, mTORC2 stimulated sphingolipid (glucosylceramide) and glycerophospholipid (cardiolipin) synthesis. Inhibition of fatty acid or sphingolipid synthesis prevented tumor development, indicating a causal effect in tumorigenesis. Increased levels of cardiolipin were associated with tubular mitochondria and enhanced oxidative phosphorylation. Furthermore, increased lipogenesis correlated with elevated mTORC2 activity and HCC in human patients. Thus, mTORC2 promotes cancer via formation of lipids essential for growth and energy production.

Significance

mTOR signaling is activated in most tumors, yet the molecular mechanisms by which mTOR drives tumorigenesis are poorly understood. In a mouse model that develops hepatosteatosis and liver cancer, we find that mTORC2 promotes fatty acid, sphingolipid and glycerophospholipid synthesis, and thereby tumorigenesis. Thus, our study examines in unprecedented depth the role of mTORC2 and lipogenesis in tumorigenesis. Our findings demonstrate a role for mTORC2 in lipid-mediated oncogenesis that could be exploited for targeted cancer therapies.

Introduction

Cancer is a disorder characterized by increased metabolic activity leading to enhanced cell growth and proliferation. Thus, cancer cells exhibit metabolic features that are distinct from non-cancerous cells (Hanahan and Weinberg, 2011; Pavlova and Thompson, 2016). One such feature is elevated fatty acid (FA) synthesis; non-cancerous cells rely on exogenous sources (Schulze and Harris, 2012). Various enzymes that mediate FA and lipid synthesis are transcriptionally up-regulated in tumors (Menendez and Lupu, 2007). Although clinical trials for lipogenesis inhibitors are ongoing (Beloribi-Djefaflia et al., 2016), the regulation and function of lipids in tumors remain elusive.

Liver cancer is the fifth most common cancer worldwide and has poor prognosis (Llovet et al., 2016). One risk factor for liver cancer is Non-Alcoholic Fatty Liver Disease (NAFLD). NAFLD is characterized in part by excessive accumulation of triglycerides (TG) in hepatocytes (also known as hepatosteatosis), due to enhanced hepatic *de novo* FA synthesis (Lambert et al., 2014). NAFLD may progress to Non-Alcoholic Steatohepatitis (NASH) and ultimately hepatocellular carcinoma (HCC) (Postic and Girard, 2008).

FA synthesis is induced by growth factors, through the transcription factor SREBP1c. Upon growth factor stimulation, precursor SREBP1c is proteolytically processed to yield mature Sterol Regulatory Element-Binding Protein-1c (SREBP1c) which is translocated into the nucleus to activate expression of the FA biosynthesis genes *Acaca*, *Fasn*, *Scd* (Horton et al., 2002). Tumors frequently exhibit activated SREBP1c (Li et al., 2016; Ricoult et al., 2015).

FAs are assembled from acetyl groups derived mainly from citrate. Citrate produced by the TCA cycle in mitochondria is shuttled to the cytoplasm and converted, by ATP-Citrate Lyase (ACLY), to acetyl-CoA and oxaloacetate. Oxaloacetate is converted to pyruvate, generating NADPH that provides reducing power for lipid synthesis. Acetyl-CoA is converted to malonyl-CoA by Acetyl-CoA Carboxylase (ACC). Fatty Acid Synthase (FASN) then condenses acetyl-CoA and several molecules of malonyl-CoA to produce palmitate (16 carbon unit). Stearoyl-CoA Desaturase (SCD) desaturates palmitate thereby generating monounsaturated FAs (MUFAs).

Other desaturases (FADS) generate highly polyunsaturated FAs (PUFAs). FAs can be stored in the form of TG. Alternatively, FAs are utilized for the synthesis of sphingolipids (SLs) and glycerophospholipids (GPLs) that are ultimately used as signaling molecules or membrane building blocks.

The evolutionarily conserved Ser/Thr kinase Target of Rapamycin (TOR) controls metabolic pathways that mediate cell growth. TOR forms two structurally and functionally distinct protein complexes termed TOR Complex 1 (TORC1) and TORC2 (Loewith et al., 2002). Mammalian TORC1 (mTORC1) is activated by nutrients, growth factors and cellular energy (Laplante and Sabatini, 2012; Shimobayashi and Hall, 2016). Growth factors stimulate mTORC1 via PI3K-PDK1-AKT signaling that inhibits the negative regulator and tumor suppressor TSC complex, consisting of TSC1 and TSC2 (Dibble and Cantley, 2015). The tumor suppressor PTEN negatively regulates both mTORC1 and mTORC2 signaling. mTORC1 directly or indirectly phosphorylates S6K, ribosomal protein S6, CAD, and ULK among others. Growth factors activate mTORC2 which phosphorylates several members of the AGC kinase family, including AKT at Ser473 (AKT-pSer473), to control various cellular processes (Sarbassov et al., 2006). mTORC2 physiologically controls hepatic FA synthesis via AKT and SREBP1c (Hagiwara et al., 2012; Yuan et al., 2012). mTORC2 is also tumorigenic (Guertin et al., 2009; Guri and Hall, 2016), but little is known about the underlying mechanism(s).

Results

Liver specific activation of mTOR signaling promotes FA synthesis, hepatosteatosis, and HCC.

To study the role of mTOR signaling in cancer, we generated mice lacking *Tsc1* and *Pten* specifically in the liver (termed L-dKO mice). The L-dKO *(Tsc1$^{loxP/loxP}$; Pten$^{loxP/loxP}$; Alb-Cre)* mice exhibited reduced expression of TSC1 and PTEN in the liver, and concomitant activation of mTORC1 and mTORC2 signaling, as compared to age-matched littermate control mice *(Tsc1$^{loxP/loxP}$; Pten$^{loxP/loxP}$)* (Figure 1A). The L-dKO mice displayed disproportionately increased liver weight (hepatomegaly), starting at 4 weeks

of age (Figure S1A-C). L-dKO mice also exhibited increased serum levels of liver damage markers ALT, AST and LDH (Figure S1D), and elevated hepatic expression of cancer-associated genes *Afp* and *Aldh* (Figure S1E). L-dKO mice invariably presented liver cancer, detected microscopically at ~ 12 weeks of age, and died at ~20 weeks of age at which time the liver was replete with tumors (Figure S1A). Histopathological analysis confirmed liver cancer, HCC and rarely (~10%) cholangiocarcinoma (Figures 1B, S1F). A fibrotic rim, indicative of NASH, demarcated the tumors (Figure 1B). BrdU incorporation confirmed increased hepatocytes proliferation in tumors (Figure S1G). Collectively, and consistent with previous studies (Kenerson et al., 2013), the above results indicate that dysregulated hepatic mTOR causes liver cancer.

To identify early, mTOR-dependent events that promote liver cancer development, we performed longitudinal, unbiased quantitative proteomics and phosphoproteomics on liver samples from 4, 8 and 12 week-old L-dKO mice and littermate control mice. Pathway enrichment analysis of proteins and phosphorylation events that were consistently and significantly (>2 fold) up- or down-regulated at all three ages revealed enrichment of FA and lipid synthesis pathways (Figure S1H-K). Importantly, FA and lipid synthesis pathways were up-regulated specifically in livers developing HCC, i.e., livers from L-dKO mice (Figure 1C; Tables S1 and S2). Immunoblot analyses confirmed increased expression of FA biosynthetic enzymes in liver lysates from L-dKO mice (Figure 1A). Immunoblotting also showed increased levels of mature SREBP1c (Figure 1A), while qPCR analysis revealed increased expression of the *Acaca*, *Fasn,* and *Scd* genes (Figure S1L), indicating that the increase in expression of FA synthesis enzymes was at the transcriptional level. Hepatic expression of CD36, a long-chain fatty acid transporter that contributes to hepatosteatosis (Steneberg et al., 2015), was increased in L-dKO mice (Figures 1A, S1L), suggesting enhanced FA uptake in addition to synthesis. Hepatic expression of genes involved in lipid secretion (*Apob, Mttp*) or degradation (*Atgl, Acox1, Mcad*) was unchanged (Figure S1M). ACLY phosphorylation at serine 455 was increased in livers of L-dKO mice, as determined by phosphoproteomics and confirmed by immunoblotting (Figure 1A, C). ACLY phosphorylation correlated with

lipogenesis (Das et al., 2015). Up-regulation of lipid (in addition to FA) synthesis pathways was also confirmed, as described further below. Resected HCCs from 20 week-old L-dKO mice (12 tumors, n=4 mice) displayed increased expression of FA and lipid synthesis pathways, as determined by proteomic and transcriptomic analyses and confirmed by immunoblotting and immunohistochemistry (Figure 1A-C). We note that in experiments in which we used liver samples from 20 week-old mice, the samples were excised tumors (HCCs), whereas samples from younger mice (4, 8, 12 and 16 weeks) were whole liver.

We next investigated FA accumulation (hepatosteatosis) and its correlation with tumorigenesis. Macroscopically, livers from L-dKO mice appeared fatty starting at 8 weeks of age. Lipid droplet specific Oil-red-O (ORO) staining confirmed hepatosteatosis (Figure 1D). H&E staining revealed enlarged hepatocytes, beginning at 8 weeks of age, likely due to a combination of lipid droplet accumulation and mTOR-driven cell growth (Figure S1N, S1O). Consistent with enhanced lipid droplet accumulation, hepatic TG levels were increased in L-dKO mice (Figure 1E). Hepatic TG accumulation was more pronounced in *ad-libitum* fed mice compared to mice starved overnight (Figure S1P). We note that all subsequent experiments were performed with *ad-libitum* fed mice. In addition to increased hepatic TG levels, L-dKO mice exhibited key clinical pathologies of NASH (inflamed hepatosteatosis), including hepatocyte ballooning, Mallory-Denk bodies, glycogenated nuclei, lobular inflammation and increased IL-6 (STAT3-pTyr705) and TNF signaling, starting at 8 weeks of age (Figures S1Q-S). Hepatic immune cell infiltration was further confirmed by FACS analyses (Figure S1T). The above observations indicate that hepatosteatosis and NASH precedes HCC in L-dKO mice, as observed clinically.

To investigate the effect of activated hepatic mTOR signaling and hepatosteatosis on whole-body metabolism, we performed indirect calorimetry. The respiratory exchange ratio (RER) was lower in L-dKO, compared to littermate control mice, despite little-to-no difference in feeding behavior or physical activity, suggesting that L-dKO mice are more reliant on FA oxidation for energy production (Figure S1U-W). L-dKO mice were unaltered in total fat or lean mass compared to controls, as determined by

longitudinal whole-body fat composition analyses (EchoMRI) (Figure S1X). Since L-dKO mice displayed hepatosteatosis and hepatomegaly but did not exhibit an overall increase in fat or lean mass, we investigated whether L-dKO mice exhibit cancer cachexia (wasting syndrome) that may offset increased liver mass. Cachexia is characterized by substantial tissue loss, in particular of skeletal muscle (sarcopenia) and adipose tissue. Indeed, L-dKO mice exhibited sarcopenia and reduced adipose tissue mass (Figure S1Y-Z). Thus, L-dKO mice exhibit whole-body cachexia-like effects, as observed in hepatosteatosis patients (Lee et al., 2015).

FA synthesis is required for tumor development.

To determine whether FA synthesis is required for tumor development, 6 to 8 week-old L-dKO and littermate control mice were treated with the FASN inhibitor orlistat (Sounni et al., 2014) or drug vehicle alone daily for 12 weeks. Orlistat-treated L-dKO mice displayed significantly fewer hepatic lipid droplets and liver tumors, and exhibited reduced serum liver damage markers ALT, AST and LDH, compared to L-dKO mice treated with drug vehicle alone (Figures 2A-B, S2A-C). Orlistat treatment did not reduce total liver or body weight (Figure S2D). Furthermore, orlistat treatment had no effect on AKT-pSer473 and S6-pSer235/6 (Figure S2E), indicating that drug action was not via inhibition of mTOR. Thus, FA synthesis is required for tumor development.

Orlistat inhibits pancreatic lipase in addition to systemic FASN. To determine whether FA synthesis specifically in the liver is required for tumor development, we used adenovirus associated virus (AAV) to knockdown FASN in hepatocytes. Importantly, to achieve hepatocyte specific knockdown, we used an AAV with high liver tropism (AAV-DJ) and expressing shFASN from the albumin promoter (AAV-DJ-Albumin-shFASN-RFP*,* referred to as AAV-shFASN) (Figure S2F). AAV-shFASN or the control virus AAV-shScrmbl (AAV-DJ-Albumin-shScrmbl-RFP*)* was injected into the tail-vein of 6 to 8 week-old L-dKO and control mice. Tumor burden was assessed in mice sacrificed at 20 weeks of age. FASN knockdown was confirmed by immunoblotting, qPCR and immunofluorescence (IF) (Figure S2G-I). L-dKO mice infected with AAV-shFASN exhibited significantly reduced hepatocyte proliferation, tumor burden and serum liver damage markers (ALT, AST and

LDH), compared to L-dKO mice treated with AAV-shScrmbl (Figures 2C-F and S2I, J). Although AAV-shFASN was very specific, it did not infect the whole liver, and it is likely that the few tumors that emerged arose from 'evaders'. Importantly, AAV-shFASN did not have a detrimental effect in wild-type mice (not shown), indicating that in contrast to transformed hepatocytes, normal hepatocytes are not dependent on high levels of FASN expression. Wild-type mice lacking tumors presumably obtain sufficient FAs from the diet. Collectively, these data indicate that hepatic FA synthesis supports tumor development in mTOR-driven HCC.

mTOR promotes *de novo* sphingolipid synthesis: Glucosylceramide. The transcriptomic and proteomic analyses described above suggested that mTOR promotes lipid synthesis in addition to FA synthesis. In particular, enzymes mediating sphingolipid (SL) and glycerophospholipid (GPL) synthesis were up-regulated in the liver of young L-dKO mice (4, 8, or 12 weeks) and in tumors of 20 week-old L-dKO mice (Figure 1C).

The rate-limiting reaction in *de novo* SL biosynthesis is the condensation of serine and palmitate catalyzed by Serine Palmitoyltransferase (SPT) to generate 3-keto-sphinganine (3kSN) (Aguilera-Romero et al., 2014) (Figure 1C). 3kSN is then reduced to yield the long chain base (LCB) sphinganine (Sa). LCB is N-acylated, with fatty acid chains of different lengths, by CerS to produce dihydroceramide (DHCer). The FAs used for sphingolipid synthesis are produced by the FA elongases (ELOV1-7). Dihydroceramide Desaturase (DES1-2) adds a double bond to complete the synthesis of ceramide. Ceramide undergoes head group modifications in the Golgi compartment to yield sphingomyelin (SM) or glucosylceramide (GlcCer). GlcCer synthesis is catalyzed by GCS (*Ugcg*) that transfers glucose from uridine diphosphate (UDP) glucose to ceramide. Ceramide can also be converted to sphingosine (So) by ceramidases. SL biosynthetic enzymes, including SPT and Ceramide Synthase (CerS1-6), are implicated in NAFLD (Pagadala et al., 2012) and cancer (Ogretmen and Hannun, 2004). L-dKO mice exhibited increased expression (mRNA and protein) of sphingolipid anabolic enzymes, in particular SPT, ELOV (1, 4, 6 and 7), CerS (2, 3, 5 and 6), DES1 and 2, and GCS (Figure 1C). Expression of enzymes that mediate the reverse, catabolic

steps was not altered, with the exception of nSMase and GBA that were increased and decreased, respectively. Importantly, these changes collectively favor GlcCer synthesis. Sphingosine Kinase 1 (SPHK1) and Sphingosine Lyase 1 (SGPL1) that mediate sphingosine 1 phosphate (S1P) metabolism downstream of ceramide were also up-regulated (see Discussion). Altered expression of SPT (*Sptlc1)*, Glucosylceramide Synthase (GCS, *Ugcg*), Sphingomyelin Phosphodiesterases (*Smpd3* and *Smpd1*), and *Sphk1* was confirmed by immunoblotting, qPCR or IF (Figures 1A, 3A and S3A, B). These findings suggest that *de novo* SL synthesis is increased in hepatocytes from L-dKO mice, likely leading to GlcCer accumulation.

To determine whether the above changes in expression lead to changes in lipid accumulation, we performed longitudinal unbiased quantitative lipidomic analyses on liver samples from L-dKO mice and age-matched littermates. Lipid enrichment analyses revealed that L-dKO mice exhibit enhanced accumulation of DHCer, ceramide, and GlcCer (Figures 3B and S3C-D; Table S3). As suggested by the above transcriptomic and proteomic analyses, GlcCer displayed the most pronounced accumulation. The observed GlcCer accumulation in L-dKO mice was validated by IF analysis (Figure S3E). Thus, livers of L-dKO mice accumulate sphingolipids, with particularly high levels of GlcCer.

To confirm that *de novo* SL synthesis, as opposed to SL salvage pathways that can also produce elevated levels of GlcCer, was high in L-dKO mice, we performed longitudinal metabolomic analyses on liver samples from L-dKO mice and control littermates. Indeed, sphinganine (Sa), an intermediate in *de novo* SL synthesis, was increased in L-dKO mice (Figure 3C). Moreover, metabolomic analyses indicated that the level of the amino acid serine, which is required for the first step in *de novo* SL synthesis, was consistently reduced in liver samples from L-dKO mice. Alanine levels were not similarly depleted (Figure S3F), suggesting that the reduction in serine is due to consumption by *de novo* SL synthesis. Thus, along with the observed increase in fatty acid synthesis, L-dKO mice display increased hepatic *de novo* SL synthesis.

Sphingolipid (GlcCer) is required for liver tumor development.

To determine whether the observed increase in *de novo* SL synthesis is required for tumor development, 8 week-old L-dKO and littermate control mice were treated with the SPT inhibitor myriocin. Myriocin or drug vehicle alone was administered every other day, for 12 weeks. Myriocin treatment reduced tumor burden and improved liver function in L-dKO mice (Figures 4A and S4A-B). Lipidomic analyses of tumors from L-dKO mice and of whole liver lysates from control mice confirmed the effect of myriocin (Figure 4B and Table S4). Principal component and lipidomic analyses indicated that myriocin-treated L-dKO mice became more control-like (Figures 4C). Myriocin had no effect on AKT-pSer473 and S6-pSer235/6 (Figure S4C), suggesting that myriocin acted independently of mTOR signaling. Myriocin treatment also reduced proliferation, as assessed by PCNA expression (Figure S4C). Liver and body weights of myriocin-treated mice were unchanged (Figure S4D). Notably, expression of SPT, the rate-limiting enzyme in SL synthesis, is elevated in many human tumors (Figure 4D). Thus, *de novo* SL synthesis supports tumor growth.

The above proteomic and lipidomic analyses indicated that L-dKO mice preferentially accumulate GlcCer (Figures 1C and 3B). Furthermore, expression of the GlcCer synthesis enzyme GCS correlates with tumorigenicity (Liu et al., 2013) and a GCS inhibitor blocked proliferation in various cancer cell lines (Huang et al., 2011; Wang et al., 2015). To determine whether GlcCer accumulation or synthesis is important for tumor development *in vivo*, we knocked down GCS in hepatocytes in L-dKO mice. AAV-shGCS (AAV-DJ-Albumin-shGCS-RFP) or the control virus AAV-shScrmbl was injected into tail-vein of 6 to 8 week-old L-dKO and control mice. Hepatic GCS knockdown was confirmed (Figure S2K). Strikingly, chronic GCS inhibition significantly reduced hepatocyte proliferation, tumor burden and serum liver damage markers, as assayed at 20 weeks of age (Figure 2C-F). Thus, GlcCer synthesis is required for tumor development.

mTOR promotes glycerophospholipid synthesis: Cardiolipin.

The transcriptomic and proteomic analyses described above also suggested that mTOR promotes glycerophospholipid (GPL) synthesis (Figure 1C). GPL synthesis begins with the acylation of glycerol 3-phosphate by GPAT to

generate lysophosphatidic acid (LPA). LPA is then converted to phosphatidic acid (PA) by AGPAT. PA is converted to diacylglyceride (DAG), by the PAP family of enzymes. In the 'Kennedy pathway', DAG and choline or ethanolamine are condensed to yield phosphatidylcholine (PC) or phosphatidylethanolamine (PE), respectively (Gibellini and Smith, 2010). Alternatively, DAG is conjugated to CDP by CDP-DAG Synthase (CDS) to produce the liponucleotide CDP-DAG. CDP-DAG is used for the synthesis of phosphatidylinositol, phosphatidylserine, and cardiolipin (CL). CLs are synthesized exclusively in mitochondria (Schlame et al., 2000). Phosphatidylglycerophosphate Synthase 1 (PGPS1) catalyzes the rate-limiting step in CL synthesis, condensing DAG and glycerol-3-phosphate to produce phosphatidylglycerolphosphate (PGP). PGP is dephosphorylated by the PTEN-like mitochondrial phosphatase PTPMT1, generating phosphatidylglycerol (PG) (Zhang et al., 2011). Cardiolipin Synthase 1 (CLS1) attaches PG to DAG, producing immature CL that is then remodeled in a series of reactions.

The transcriptomic and proteomic analyses described above and immunoblotting revealed increased hepatic expression of the GPL synthesis enzymes GPAT, AGPAT, LIPIN, CDS, PGPS1, CLS1 and PTPMT1 in L-dKO mice (Figures 1C, 5A), suggesting increased synthesis of GPLs, cardiolipin in particular. Indeed, lipidomic analysis revealed elevated levels of the GPLs phosphatidylinositol (PI), lysophosphatidylcholine (lysoPC) and CL, with accumulation of CL(18:≤1) being the most pronounced (Figure 3B). Importantly, the levels of PC and PE, both products of the Kennedy pathway, were reduced (Figure S3C). This suggests that CL is preferentially synthesized in L-dKO mice. Thus, again consistent with the observed increase in fatty acid synthesis, L-dKO mice display increased hepatic synthesis of GPLs, in particular via the biosynthetic pathway leading to CL.

Increased cardiolipin accumulation is associated with enhanced respiration.

CLs stabilize the complexes of the electron transport chain (ETC), thereby supporting oxidative phosphorylation (OxPhos) (Duncan et al., 2016). Expression of ETC complexes was unchanged in L-dKO mice (Figure S5A).

However, hepatocytes from L-dKO mice exhibited hyper-tubular mitochondria and pronounced cristae, as determined by EM analysis and IF (Figures 5B-C, S5B). Hyper-tubular mitochondria and pronounced cristae are associated with improved mitochondrial function (Cogliati et al., 2013). To investigate mitochondrial function, in particular OxPhos, we measured the oxygen consumption rate (OCR) and mitochondrial reserve capacity of primary hepatocytes. Primary hepatocytes from L-dKO mice displayed enhanced OxPhos and increased mitochondrial reserve capacity, compared to wild-type hepatocytes (Figure 5D). Thus, CL accumulation correlates with enhanced mitochondrial function. The increase in CL may improve mitochondrial function to sustain the enhanced metabolic needs of tumor cells.

mTORC2 promotes fatty acid, sphingolipid and cardiolipin accumulation.

The above suggests that hyperactive mTOR signaling promotes lipid synthesis and cancer. To confirm that tumor development is indeed mTOR dependent, 8 week-old L-dKO and control mice were chronically treated with the ATP competitive mTOR inhibitor INK128, or drug vehicle alone, for 12 weeks. Chronic INK128 administration reduced mTOR signaling (mTORC1 and mTORC2) (Figure S6A), and reduced both tumor burden and incidence and liver damage (Figures 6A-C, S6B), confirming that tumor development was mTOR dependent. Of note, chronic INK128 administration also reduced hepatic TG content and hepatocyte size (Figures 6D, S6C).

Hepatic mTORC1 and mTORC2 are activated in L-dKO mice (Figure 1A). To determine whether mTORC1 and/or mTORC2 signaling controls FA and lipid synthesis in L-dKO mice, we performed acute pharmacological inhibition studies. Eight week-old L-dKO and control mice were treated acutely (24 hr) with rapamycin, INK128 or drug vehicle alone. Acute rapamycin treatment inhibited mTORC1, whereas acute INK128 administration inhibited both mTORC1 and mTORC2 (Figure S6D), as expected (Hsieh et al., 2012). Acute INK128 treatment reduced both expression of ACC, FASN, and SCD (Figures 6E; 6F quantified in S6E) and, compared to INK128-treated controls, hepatic TG content in L-dKO mice (Figure S6F). Acute INK128 treatment increased overall hepatic TG in L-dKO and control mice compared to mice

treated with drug vehicle alone, likely due to enhanced lipolysis in adipose tissue (Kumar et al., 2010) as suggested by increased serum free FA levels (Figure S6G). Inhibition of mTORC1 alone (rapamycin) had little-to-no effect on expression of hepatic FA synthesis enzymes and hepatic TG content (Figures 6E-F and S6E). However, rapamycin insensitive mTORC1 substrates may still be involved in FA synthesis (Peterson et al., 2011). To investigate further a possible role of mTORC1 in the regulation of FA synthesis, we examined hepatic expression of FA synthesis enzymes in liver-specific *Tsc1* knockout (L-*Tsc1*^{KO}) mice. L-*Tsc1*^{KO} mice exhibited activated mTORC1, but not mTORC2, and unaltered expression of FA biosynthetic enzymes (Figure 6G). These data suggest that hepatic FA synthesis is likely activated by mTORC2.

To confirm that mTORC2 promotes *de novo* FA and lipid synthesis, TG accumulation and liver cancer development, we deleted *Rictor*, an essential core component of mTORC2, in L-dKO mice. *Tsc1*$^{loxP/loxP}$*; Pten*$^{loxP/loxP}$ mice were crossed with *Rictor*$^{loxP/loxP}$ mice expressing *Alb-Cre*, to generate triple knockout (L-TriKO) mice lacking *Tsc1, Pten* and *Rictor* (Figure S7A). *Rictor* loss was confirmed at mRNA and protein levels (Figure S7B-C). Immunoblots revealed markedly reduced hepatic mTORC2 signaling in L-TriKO mice *(Tsc1*$^{loxP/loxP}$*; Pten*$^{loxP/loxP}$*; Rictor*$^{loxP/loxP}$*; Alb-Cre)* compared to control mice *(Tsc1*$^{loxP/loxP}$*; Pten*$^{loxP/loxP}$*; Rictor*$^{loxP/loxP}$*)*, while mTORC1 signaling was unchanged (Figure S7C), confirming the L-TriKO mouse model. Importantly, L-TriKO mice displayed reduced expression of ACC, FASN, SCD and their transcriptional activator SREBP1c (mature form), and reduced hepatic TG content (Figure 7A-B), compared to L-dKO mice. Expression of the SL synthesis genes *Sptlc1* and *Smpd3* was also reduced in L-TriKO mice, compared to L-dKO mice (Figure 7A, C). Similarly, immunoblotting of primary hepatocytes from knockout and control mice showed reduced CLS1 expression in L-TriKO mice (Figure S7D). Lipidomic analyses of livers from L-TriKO, L-dKO and control mice revealed reduced SL and GPL levels, including GlcCer and CL(18:≤1), respectively, specifically in L-TriKO mice (Figure 7D and Table S5). Similarly, L-TriKO mice displayed reduced hepatic sphinganine and normalized serine levels (Figures 7E, S7E). Collectively, the above indicates that mTORC2 activates *de novo* FA and lipid synthesis.

Importantly, in addition to reduced FA and lipid synthesis, L-TriKO mice also displayed reduced tumorigenicity (Figures 7F-I, S7F-G). We note that the few tumors that arose in L-TriKO mice exhibited increased mTORC2 activity, as assessed by AKT-pSer473 (Figure S7H), suggesting that such tumors were due to 'escapers' in which *Rictor* was not deleted. Thus, it is mTORC2 that is lipogenic and oncogenic in L-dKO mice.

To examine the oncogenic role of mTORC2 in another mouse model of NAFLD progressing to HCC, liver specific *Rictor* knockout mice (LiRiKO) and littermate controls were chronically fed a choline deficient, high-fat diet (CD-HFD) and treated with the hepatic pro-carcinogen diethylnitrosamine (DEN) (Wolf et al., 2014). Four out of five LiRiKO mice failed to develop liver tumors and exhibited reduced expression of lipogenic enzymes, as compared to control mice (n=3) (Figure S8A-B). Of note, tumors that arose in control mice displayed elevated AKT-pSer473, FASN and SPT expression, suggesting that tumors require lipogenic enzymes for growth (Figure S8B). The above underscore the critical role of mTORC2 in lipogenesis and HCC.

Lipogenesis is up-regulated in human hepatosteatosis progressing to HCC.

L-dKO mice display hepatosteatosis progressing to HCC. To examine the relevance of this model to human HCC, we investigated mTORC2 activity and expression of FA and lipid biosynthetic enzymes in human liver cancer cell lines. Human liver cancer cell lines with high AKT-pSer473 also exhibit increased expression of FA and lipid synthesis proteins and vice versa (Figure S8C). To investigate the functional significance of this correlation, HCC cells exhibiting high (Hep 40) and low (PLC) mTORC2 activity were treated with a FASN (C75) or SPT (myriocin) inhibitor. At a high dose (200 nM), C75 killed practically all cells in both cell lines, demonstrating a general dependence of tumor cells on *de novo* FA synthesis (Figure S8D). At lower doses (10 nM or 50 nM), Hep 40 cells were more sensitive to FASN inhibition (50% reduction) compared to PLC cells. Remarkably, myriocin administration killed Hep 40 cells but had no effect on PLC cells, at all doses examined (Figure S8D, E). Thus, similar to our findings in L-dKO mice, FA and lipid

biosynthesis correlates with mTORC2 activity and is required for proliferation in human cancer cells.

To address the clinical relevance of our findings to HCC development in patients, we performed proteomic analysis on HCC and matched non-tumor biopsies from 18 patients. In 12 patients (one of which presented two separate tumors) HCC had progressed from hepatosteatosis, whereas in the remaining six patients HCC had progressed from viral (HCV or HBV) infection (Table S6). Strikingly, the tumor tissue from the 12 steatotic patients displayed high levels of FA and lipid biosynthetic enzymes, and these levels exceeded the level in the matched non-tumor samples. In comparison, enzyme levels were low in the virus infected (non-steatotic) patients and unchanged or reduced in the HCC sample relative to the matched non-tumor tissue (Figures 8A, S8F and Table S7). Thus, FA synthesis and lipogenesis are upregulated in human HCC that progressed from steatosis, as in L-dKO mice.

To confirm that lipogenesis is upregulated in steatotic tissue, we examined liver biopsies from ten hepatosteatotic (NAFLD or NASH), cancer-free, patients and from two healthy individuals. Immunoblot analyses revealed that the rate-limiting enzymes of FA (FASN and ACC) and GlcCer (GCS) synthesis were increased in 40% and 80% of the patients, respectively, as compared to healthy controls (Figure 8B). Patients exhibiting hepatosteatosis that do not show increased *de novo* lipogenesis may take up FA from the diet (Donnelly et al., 2005). Importantly, as observed in L-dKO mice, increased FASN, ACC and GCS expression correlated with high mTORC2 activity, as assessed by AKT-pSer473 (Figure 8B). Furthermore, AKT-pSer473 positively correlated with GlcCer lipid levels in human biopsies, as determined by immunoblotting and lipidomic analyses (Figure 8C). Thus, mTORC2 may promote sphingolipid synthesis (GlcCer) also in human hepatosteatosis that could in turn contribute to HCC development. The above results suggest that hepatosteatosis patients would benefit from early intervention with lipogenesis inhibitors, or with an mTOR inhibitor(s), to prevent progression to HCC.

Discussion

Diverse mutations activate mTOR signaling to promote tumorigenesis, but tumorigenic mechanisms downstream of mTOR remain poorly defined, in particular for mTORC2. We examined tumorigenesis in an mTOR-dependent HCC mouse model (L-dKO) and in human patients. We report that mTORC2 promotes hepatosteatosis and ultimately cancer via fatty acid (FA) and lipid synthesis.

Similar to patients who develop NAFLD that progresses to HCC (Postic and Girard, 2008), L-dKO mice exhibit enhanced FA synthesis and thereby develop hepatosteatosis, liver damage and HCC. FASN knockdown (or orlistat treatment) blocked carcinogenesis. Notably, FASN inhibition did not have a detrimental effect in wild-type hepatocytes, exposing a vulnerability specific to cancer cells.

How do FAs promote carcinogenesis? First, FAs are required for lipid synthesis, which may in turn be necessary for carcinogenesis. We observed that lipid synthesis pathways, in particular *de novo* SL and GPL synthesis, are up-regulated in L-dKO mice, and that *Sptlc1* expression is high across various human tumors including HCC. Importantly, inhibition of the first reactions in *de novo* SL synthesis or GlcCer synthesis prevented HCC in L-dKO mice, indicating that up-regulation of lipid synthesis is essential for tumorigenesis. Second, enhanced FA synthesis causes hepatosteatosis, a pathological accumulation of FAs, which may in turn promote inflammation (i.e. NASH) that progresses to HCC (Park et al., 2010). For example, FAs could be used to produce the bioactive sphingolipid S1P that signals to recruit macrophages (Liang et al., 2013). Consistent with the notion that FAs promote tumor development via inflammation, L-dKO mice exhibited up-regulated *Sphk1* and increased S1P levels, which correlated with increased STAT3 phosphorylation. The immune system plays an important role in hepatosteatosis to HCC progression (Kondylis et al., 2015). We note that L-dKO mice exhibited both innate and adaptive immune cell infiltration, and that in L-dKO mice hepatosteatosis seems to precede inflammation. The role of inflammation in mTOR driven tumors, and in particular its promotion by FAs, requires further study.

How does up-regulation of SL and GPL synthesis (in particularly of GlcCer and CL, respectively) promote cancer? First, SL and GPL are structural components of membranes and may thereby support tumor growth simply as building blocks. Ceramide glycosylation by GCS yields the membrane component GlcCer, but also eliminates excess ceramide and its anti-proliferative effect (Ogretmen and Hannun, 2004). Importantly, L-dKO mice preferentially accumulate GlcCer. Thus, the essential role of GlcCer synthesis in tumorgenicity may be two-fold, one is to produce a building block, the other is to eliminate ceramide. It is possible that a more complex glycosphingolipid (ganglioside), produced from GlcCer is critical for tumorigenesis. Second, CL in the inner mitochondrial membrane stabilizes complexes of the ETC thereby enhancing energy production and satisfying the increased metabolic needs of tumor cells (Peck et al., 2016). Importantly, we observed increased CL synthesis, enhanced mitochondrial cristae and mitochondrial energy production in liver of L-dKO mice. Third, lipids provide second messengers in oncogenic signaling pathways. For example, phosphorylated forms of phosphatidylinositol mediate growth factor signaling. It remains to be determined whether the increased lipid synthesis observed in L-dKO mice affects second messenger levels in oncogenic signaling pathways.

How does mTORC2 regulate FA synthesis? L-dKO mice display increased levels of mature SREBP1c and increased expression of SREBP1c target genes *Acaca*, *Fasn*, *Scd* and *Elovl6*. Pharmacological or genetic ablation of mTORC2 signaling in L-dKO mice (L-TriKO) prevented SREBP1c processing, decreased *Acaca*, *Fasn*, and *Scd* expression, and thereby reduced *de novo* FA synthesis. Thus, mTORC2 promotes *de novo* FA synthesis via SREBP1c, as shown previously (Hagiwara et al., 2012). mTORC2 or its effectors may also phosphorylate FA synthesis enzymes directly, as recently proposed for ACLY (Chen et al., 2016). Finally, mTORC2 increases expression of the FA transporter CD36, allowing up-take of FA from the bloodstream.

mTORC1 has also been proposed to stimulate FA synthesis (Ricoult and Manning, 2013), for example, in breast cancer cell lines (Ricoult et al., 2015). However, mTORC1 alone is insufficient to promote FA synthesis *in vivo*. First,

L-*Tsc1*^{KO} mice (constitutively active mTORC1) exhibit reduced FA levels and are even protected from age and diet induced hepatosteatosis (Cornu et al., 2014; Kenerson et al., 2015; Kenerson et al., 2011; Yecies et al., 2011). Second, activation of mTORC1 is insufficient to activate expression of lipogenic genes (Titchenell et al., 2016). Third, liver specific *Raptor* knockout mice (abrogated mTORC1 activity) exhibit unaltered hepatic FA levels, even when fed a high fat diet (Umemura et al., 2014). Thus, mTORC2 may play a more important role than mTORC1 in hepatic FA synthesis.

How does mTORC2 activate lipid synthesis? We observed that mTORC2 controls expression of critical lipid synthesis genes, suggesting a mechanism at the level of transcription. However, mTORC2 is functionally at ER-mitochondria contact sites (Betz et al., 2013), where GPL synthesis and exchange occurs. Thus, mTORC2 or its kinase effectors could directly phosphosphorylate and activate GPL synthesis enzymes. In skeletal muscle, AKT phosphorylates ACLY thereby improving mitochondrial function (Das et al., 2015). The mechanism by which mTORC2 activates lipid synthesis remains to be determined. Yeast TORC2 stimulates sphingolipid synthesis via post-translational regulation of SPT and ceramide synthase (Berchtold et al., 2012; Muir et al., 2014; Roelants et al., 2011).

Does mTORC1 play a role in lipid synthesis in L-dKO mice? mTORC1 stimulates pyrimidine biosynthesis via phosphorylation of CAD and transcriptional activation of the pentose phosphate pathway (Ben-Sahra et al., 2013; Robitaille et al., 2013). The pyrimidines uridine diphosphate (UDP) and cytidine diphosphate (CDP) are required for the rate-limiting reactions in GlcCer and GPL synthesis, respectively. Indeed, L-dKO mice display increased CAD phosphorylation. Thus, our data favor a model in which mTORC1 and mTORC2 converge on lipid synthesis to drive tumorigenesis.

Collectively, we demonstrate that mTORC2 promotes tumorigenesis via FA and lipid synthesis. Several FA and lipid blocking therapies are being evaluated in ongoing clinical trials (Beloribi-Djefaflia et al., 2016). Our data suggests that cancer patients exhibiting high mTORC2 signaling, particularly steatotic patients potentially progressing to HCC, should be considered for such therapies.

Author Contributions

Conceptualization, Y.G., H.R. and M.N.H.; Methodology, Y.G., S.M., P.J., J.R., I.R, H.R., E.D., M.H.H and M.N.H.; Investigation, Y.G., M. C., S.K.H., I.R.; Writing – Original Draft, Y.G. and M.N.H.; Funding Acquisition, Y.G., M.N.H. and H.R.; Resources, H.R. and M.N.H.; Supervision, M.N.H. and H.R.

Conflict of Interest

The authors declare no conflict of interest.

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Figure legends

Figure 1. L-dKO mice develop HCC and exhibit enhanced *de novo* FA and lipid synthesis.

(A) Immunoblots of liver extracts from *ad-libitum* fed 4, 8, 12, and 20 week-old L-dKO and control mice (n=6 per group). SREBP1c (p) precursor (m) mature.

(B) Representative H&E (Hematoxylin and Eosin), SR (Sirius Red), PAS (Periodic Acid Schiff) staining of HCCs (20 week) L-dKO and control mice. Asterisks and arrows indicate HCC and HCC border (fibrosis), respectively; white scale bar, 100 μm.

(C) Lipid biosynthetic enzymes detected by immunoblotting or proteome analyses of livers from young mice (4, 8 and 12 week; n=6 per group), or detected in immunoblotting, proteome or transcriptomic analyses from HCCs (n=12). Color coded bars indicate relative expression from young mice on the

left and HCC on the right (STAR Methods). n.c., not changed; n.d., not detected; down, down-regulated; up, up-regulated. ACLY-pSer455 and enzymes experimentally inhibited, FASN (shFASN/orlistat), SPT (myriocin) and GCS (shGCS) are indicated. pE/H, phoshoethanolamine/hexadecanal.
(D) Representative images and quantification of HCCs stained with Oil-Red-O (ORO) (n=3); au, arbitrary unit; white scale bar, 100 μm.
(E) Hepatic triglyceride (TG) from *ad-libitum* fed L-dKO and control mice (≥n=4). Data are represented as mean ± SEM; p value by two-tailed unpaired t-test. See also Figure S1 and Tables S1-S2.

Figure 2. Inhibition of FASN or GCS prevents hepatocellular carcinoma.
(A) Tumor number and size and **(B)** serum ALT, AST and LDH levels of L-dKO mice chronically treated with orlistat or the drug vehicle.
(C-F) L-dKO mice infected with AAV-shFASN (n=4), AAV-shGCS (n=6) or control AAV-shSCRMBL (n=5). Tumor number and size **(C),** representative whole-liver images and histological analyses **(D)**; scale bar, 1 cm; arrows indicate tumors; scale bar, 100 μm**.** Number of Ki67 positive hepatocytes per 10X magnification (n=3) and **(E)** serum ALT, AST and LDH levels from L-dKO mice infected with AAV-shScrmbl (n=5), AAV-shFASN (n=4) or AAV-shGCS (n=6) **(F)**. Data are represented as mean ± SEM; p value by two-tailed unpaired t-test. See also Figure S2.

Figure 3. L-dKO mice accumulate SLs and GPLs in the liver.
(A) Relative hepatic mRNA (quantitative PCR) from 4, 8 and 20 week L-dKO and control mice (n=6 per group); au, arbitrary unit.
(B) Hepatic lipid z-score enrichment expressed as L-dKO/control mice of indicated ages (n=6 per group; STAR Methods); up-regulated (up-reg.) and down-regulated (down-reg.). GlcCer, glucosylceramide; SM, sphingomyelin; CL, cardiolipin; Cer, ceramide; PS, phosphatidylserine; PI, phosphatidylinositol; CerP, ceramide phosphate; LysoPC, lyso-phosphatidylcholine; OH, hydroxyl; DH, dihydro.
(C) Hepatic sphinganine in 4, 8, 12 and 20 week-old mice (n=6). Data are represented as mean ± SEM; p value by two-tailed unpaired t-test. See also Figure S3 and Table S3.

Figure 4. mTOR promotes *de novo* SL synthesis, thereby tumor development.

(A) Tumor number and size in 20 week-old L-dKO mice after chronic myriocin or drug vehicle administration.

(B) Volcano plot of the major lipid species regulated in livers from myriocin-treated mice compared to drug vehicle-treated mice (L-dKO/control). GlcCer in blue. Black line indicates significant from non-significant alterations for a threshold of 0.75 Log_2-Fold.

(C) Principal component analysis (PCA) of lipidomic analysis from myriocin-treated mice. Discrimination between L-dKO and control mice driven by principal component 1 (dashed circles). Principle component 2 drove the separation between myriocin-and vehicle-treated groups (closed circle).

(D) *Sptlc1* expression (TPM, STAR Methods) across various non-tumorous and tumorous tissues. Each dot represents information from one patient. Data are represented as mean ± SEM; p values by two-tailed unpaired t-test. See also Figure S4 and Table S4.

Figure 5. mTOR promotes CL synthesis and OxPhos in hepatocytes.

(A) Immunoblots of liver extracts from 4, 8, 12, and 20 week-old L-dKO and control mice. Arrow specifies PTPMT1.

(B) Representative confocal images of primary hepatocytes, perfused from 8 week-old L-dKO and control mice, stained by MitoTracker-Red. Arrows indicate tubular mitochondrial networks. Nuclei, blue (DAPI); scale bar, 10 μm.

(C) Representative electron micrographs of hepatocytes from L-dKO and control mice (n=3). Arrows indicate mitochondria cristae. Scale bar, 500 nm.

(D) Oxygen Consumption Rate (OCR) and Extracellular Acidification Rate (ECAR) analyses of primary hepatocytes perfused from 8 week-old L-dKO and control mice (n=3). The time at which a given compound was added is indicated; background contained no cells. Data are represented as mean ± SEM. See also Figure S5.

Figure 6. mTOR inhibition prevents FA accumulation and HCC.

(A-C) L-dKO mice chronically treated with INK128 (INK) or a vehicle (n=7 per group). Representative whole-liver images (scale bar, 1 cm, arrows indicate tumors), liver tumor number and size **(A)** and incidence, **(B)** serum ALT, AST and LDH levels (U/L) and **(C)** hepatic triglyceride (TG) **(D)**.
(E-F) Liver extracts from 8 week-old L-dKO and control mice (n=4) acutely treated with rapamycin (rapa) or INK128 (INK) or a vehicle (veh^r and veh^i, respectively) mRNA **(E)** immunoblots **(F)**; au, arbitrary unit.
(G) Immunoblots of liver lysates from L-Tsc1, L-dKO and control mice (n=4 per group); n.s., not significant. Data are represented as mean ± SEM; p values by two-tailed unpaired t-test. See also Figure S6.

Figure 7. mTORC2 promotes FA, lipid synthesis and tumorigenesis.
(A) Immunoblots of liver extracts from 20 week-old L-dKO, L-TriKO and littermate control mice (n=4 per group). Arrow specifies mature SREBP1c (m) from precursor (p).
(B-C) 20 week-old L-dKO (n=6), L-TriKO (n=4) and pooled controls (n=8). Hepatic triglyceride (TG) **(B)** Relative mRNA **(C)**; au, arbitrary unit.
(D) Hepatic lipid enrichment in L-dKO/control and L-TriKO/L-dKO mice (n=4 per group).
(E) Hepatic sphinganine in 20 week-old L-dKO and L-TriKO mice, normalized to control mice (n=4 per group) (STAR Methods).
(F-H) 20 week-old L-dKO and L-TriKO mice. Liver tumor number, size **(F)** and incidence **(G)**.
(H) Representative histological analyses from livers of L-TriKO and control mice; scale bar, 100 μm.
(I) Survival proportions of L-dKO and L-TriKO mice (Kaplan-Meier, **** $p<0.0001$ for both Mantel-Cox and Gehan-Breslow-Wilcoxon tests). Data are represented as mean ± SEM; p values by two-tailed unpaired t-test. See also Figure S7 and Table S5.

Figure 8. Lipogenesis is up-regulated in human hepatosteatosis progressing to HCC, and correlates with mTORC2 activity.
(A) Protein expression in HCC compared to a non-HCC region from the same patient. Each box represents data from one HCC. n.c., not changed;

n.a., not applicable; down, down-regulated; up, up-regulated in proteome (STAR methods).

(B) Immunoblots from liver biopsies from healthy individuals (ctrl, n=2), NAFLD (n=5) and NASH (n=5) patients.

(C) Nonlinear regression (R square) of hepatic glucosylceramide (GlcCer) level and AKT-pSer473 in human liver biopsies. Data are represented as mean ± SEM; p value by one-tailed unpaired t-test. See also Figure S8 and Tables S6-S7.

STAR METHODS

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and request for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Michael N. Hall (m.hall@unibas.ch).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Patient Needle-Core Biopsies. Patients were recruited in the Clinic for Gastroenterology and Hepatology of the University Hospital Basel, Switzerland. They gave written informed consent to donate liver biopsy specimens for research. Study protocol was approved by the Ethics Committee of the Canton Basel, Switzerland. The biopsies were performed under ultrasound guidance using a coaxial needle technique allowing repetitive sampling from the same part of a focal lesion with a full-core biopsy instrument (BioPince; Angitech). Diagnosis of HCC was made by histopathological analysis. The gender and the age of hepatosteatosis and HCC patients can be found in Table S6.

Animals. Liver-specific tuberous sclerosis complex 1 (*Tsc1*) and phosphatase and tensin homolog (*Pten*) double knockout mice were obtained

by crossing *Tsc1*$^{loxP/loxP}$ mice with *Pten*$^{loxP/loxP}$ mice (Horie et al., 2004) to transgenic mice expressing Cre recombinase under the control of the hepatocyte-specific albumin promoter (*Alb-Cre*), to generate liver specific double knockout (L-dKO) mice. Liver-specific *TSC1* knockout mice (L-*Tsc1*) were generated as described (Cornu et al., 2014). *Tsc1*$^{loxP/loxP}$*; Pten*$^{loxP/loxP}$ mice were crossed to *Rictor*$^{loxP/loxP}$*; Alb-Cre* (Hagiwara et al., 2012) to obtain *(Tsc1*$^{loxP/loxP}$*; Pten*$^{loxP/loxP}$*; Rictor*$^{loxP/loxP}$*; Alb-Cre)* liver specific triple knockout (L-TriKO mice) mice. L-TriKO mice were born according to Mendelian ratios and gained weight normally (not shown). As controls, male littermate *Tsc1*$^{loxP/loxP}$ mice or *Tsc1*$^{loxP/loxP}$*; Pten*$^{loxP/loxP}$ mice or *Tsc1*$^{loxP/loxP}$*; Pten*$^{loxP/loxP}$*; Rictor*$^{loxP/loxP}$ mice (which do not express Cre-recombinase) were used. Mice were maintained on mixed genetic background (C57BL/6J, 129/SvJae, BALB/cJ). Both L-dKO mice *(Tsc1*$^{loxP/loxP}$*; Pten*$^{loxP/loxP}$*; Alb-Cre)* and L-TriKO *(Tsc1*$^{loxP/loxP}$*; Pten*$^{loxP/loxP}$*; Rictor*$^{loxP/loxP}$*; Alb-Cre)* mice were born viable at the expected Mendelian ratio and displayed normal fertility. PCR genotyping was performed as described (Cornu et al., 2014). Mice were maintained under temperature and humidity-controlled conditions, lights on at 6:00am and off at 6:00pm. In all experiments, mice were sacrificed between 6:00 to 7:00am and were *ad-libitum* fed, unless mentioned otherwise. All experiments were conducted with male mice, and performed in accordance with federal guidelines and were approved by the Kantonales Veterinaeramt of Kanton Basel-Stadt.

Choline deficient (CD) high-fat diet (HFD) and diethylnitrosamine (DEN) treatment. 4 week-old liver specific *Rictor* knockout (LiRiKO, *Rictor*$^{loxP/loxP}$*;*

Alb-Cre) and control (*Rictor*$^{loxP/loxP}$*)* mice were intraperitoneally injected with DEN (25 mg/kg bw) once a day for one week, and were *ad-libitum* fed with CD-HFD for about 10 months. Afterwards, mice were sacrificed and liver tumors were counted, as described below.

Human liver cancer cell lines. All human liver cells were grown in DMEM high glucose media supplemented with 10 % FBS, glutamine (GlutaMAX, Gibco), non-essential amino acids (Gibco) and penicillin-streptomycin (Gibco). For colony growth assays, Hep 40 and PLC cells were seeded in 12 well plate (Corning) and grown to reach 80 % confluency. C75 or myriocin were solubilized in DMSO and added to fresh pre-warmed media in 50ml tubes (Falcon). Aliquots from the above media containing inhibitors (C75 or myriocin or the drugs vehicle alone) were then added to the proper wells. 24 hr post treatment cells were fixed for 5 min with 10 % formalin and stained for 30 min with 0.05 % Crystal violet dye, washed and analyzed. Quantification was performed using Fiji. The sex and the age of the patients who were the source of the cells are indicated in Table S6.

METHODS DETAILS

Virus administration.

AAV-albumin-shRNA-FASN-RFP was used for the knockdown of the gene *FASN.* AAV-albumin-shRNA-GCS-RFP was used for the knockdown of the gene *Ugcg.* AAV-albumin-shRNA-Scrmbl-RFP was used as a control. All viruses were purchased from Vector BioLabs. Mice were infected with AAVs

by tail-vein injection (5*10^11 viral genome, unless otherwise mentioned). Knockdown sequences were confirmed both *in vitro* (not shown) and *in vivo*. To examine the effect on tumor growth mice were sacrificed about three months post infection.

Whole-body metabolic analysis. RER (Respiratory Exchange Rate), locomotor activity and food consumption were measured in 30 min intervals, for the indicated time using a comprehensive laboratory animal monitoring system (CLAMS, Linton Instrumentation and Columbus Instruments). Measurement was performed over 72 hr, after providing 24 hr of acclimatization. Mice had free access to food and water. Body fat composition was assessed in *ad-libitum* fed mice, by nuclear magnetic resonance imaging (Echo MRI mouse, Echo Medical Systems, Houston, USA).

Pharmacological treatments. For chronic mTOR inhibition, INK128 or the drug vehicle alone was orally given to 8-week-old L-dKO mice (n=7) and littermate control (n=7) mice, at dose of 1 mg/kg bw, every other day for 12 weeks. INK128 was dissolved in 5 % 1-methyl-2-pyrrolidinone, 15 % polyvinylpyrrolidone K30, and 80 % water, as described (Hsieh et al., 2012). Rapamycin was dissolved as published (Cornu et al., 2014) in 5 % (vol/vol) PEG-400, 4 % (vol/vol) ethanol, and 5 % (vol/vol) Tween 80, and was injected intraperitoneally. For acute mTOR inhibition studies, rapamycin or INK128 or the drugs vehicles were administered to 8-10 weeks-old L-dKO and littermate controls (n=4 per group), twice (6 am and 6 pm) over 24 hr, at

doses indicated above. Orlistat (Xenical) was orally given to 6 to 8 week-old L-dKO (n=4) and control mice (n=5) at a dose of 120 mg/kg bw, for 12 weeks, daily, as described (Sounni et al., 2014). Myriocin was intraperitoneally administrated to 8 week-old L-dKO (n=5 myriocin, n=6 vehicle) and control (n=5 myriocin, n=4 vehicle), at a dose of 0.3 mg/kg bw, every other day, for 12 weeks. For assessment of liver tumor burden, in all cases unless otherwise indicated, mice were sacrificed at the 20th week of age. Mice were euthanized and their livers quickly removed. Subsequently, externally visible tumors (>0.2 mm) were counted and measured. Were indicated tumors were resected and subsequently used for biochemistry, histology, and/or OMICS analyses: (phospho)proteomics, lipidomics, metabolomics.

BrdU (5-Bromo-2'-deoxy-Uridine) examination. To evaluate cell proliferation *in vivo* in tumors, L-dKO and control mice at 16 weeks of age (at which time tumor and non-tumor regions can be readily discriminated) were intraperitoneally injected with 100 mg/kg bw BrdU once a day, at 6:00pm for three days. At 6:00am of the fourth day, mice were sacrificed and livers were removed. Liver lobes, containing both tumor and non-tumor regions, were fixed in 4 % paraffin and were then used for immunofluorescence imaging using the BrdU in situ detection kit. HCCs were confirmed by histological analyses (H&E) of serial sections.

Serum parameters. Prior to liver dissection, blood was collected from the inferior vena cava using a 25-gauge needle. Blood was placed immediately into a heparin-coated tube (BD microtainer) to obtain serum. Subsequently,

50 µl of serum was diluted three times and loaded to a Cobas c 111 analyzer (Roche). Serum parameters: circulating transaminases (ALT, AST), LDH and albumin were measured. Plasma free-fatty acid levels were determined using a commercial kit (HR Series NEFA-HR (2) and Cayman).

Isolation of primary cells and cell culture. For isolation of primary mouse hepatocytes, 8-10 week-old L-dKO and controls were used. Primary hepatocytes were obtained by liver perfusion. Shortly, peritoneal cavity was opened and the inferior vena cava was cannulated using a 23-gauge needle catheter. Cannula was clamped using a surgical clip. Liver was then perfused with pre-warmed (37 °C) Ca^{2+} and Mg^{2+} free-$HBSS^{++}$ containing 1 mM EGTA, at a rate ranging from 5 to 10 mL/min, prior to chest dissection. The aorta was ligated and the portal vein was transected. Liver was then perfused with pre-warmed (37 °C) $HBSS^{++}$ with Ca^{2+} and Mg^{2+} containing collagenase digestion solution (collagenase type II and DNase I (10 µg/mL)), at a rate of 7.5 mL/min. The perfused and digested liver was then carefully dissected and placed in a petri dish containing ice-cold $HBSS^{++}$. Glisson capsule was then gently teased with forceps to get the cell suspensions. Cell suspensions were filtered through gauze to remove undigested or connective tissue and centrifuged 650 × g for 5 min. Thereafter, re-suspended with $HBSS^{++}$ and followed by 3 min at 35 × g centrifugation to obtain parenchymal cells (pellet) and non-parenchymal cells (supernatant). Parenchymal cell suspensions, containing vital hepatocytes, were used for culture. Briefly, hepatocytes were resuspended in pre-warmed culture media (DMEM high glucose with 10 % fetal bovine serum (FBS)). Primary hepatocyte

maintenance supplements, including dexamethasone ITS (insulin, transferrin, selenium complex) and glutamine were added to the media.

OCR and ECAR measurements. Measurements were performed with an XF96 Extracellular Flux Analyzer (Seahorse Bioscience of Agilent) following manufacturer instructions. 2×10^4 hepatocytes were seeded into 96 culture plate (Seahorse Bioscience of Agilent) overnight or measured 1 hr post-seeding. Media was exchanged prior to the measurement. Oligomycin inhibits ATP synthase (complex V), FCCP uncouples oxygen consumption from ATP production, and rotenone and antimycin A inhibit complexes I and III, respectively. All drugs were obtained from Seahorse Bioscience of Agilent.

FACS analysis. To obtain immune cells (non-parenchymal), livers from 4, 8 and 20 week-old littermate L-dKO and control mice were perfused as described above. The non-parenchymal cell suspension was resuspended in $HBSS^{++}$ supplemented with 0.5 % BSA. Then, non-parenchymal cell suspension was filtered through a 100 μm cell strainer (Corning) and centrifuged for 500 g for 10 min and incubated in an Fc-block (BD Biosciences), and stained with CD45R (B220) PerCP-Cyanine5.5 (Thermo Fisher) and Live/Dead Fixable Dead Cell Kit (Thermo Fisher). Stained immune cell were analyzed using FACS Canto II (BD Biosciences).

RNA isolation and quantitative reverse transcriptase PCR. Total RNA was isolated from 50 mg of mouse livers. Liver samples were homogenized

for 30 s bead beating in lysing matrix D tubes (Q-Biogene) containing 1 mL of TRIzol reagent (Sigma). After chloroform extraction and centrifugation, samples were mixed with 600 µL of 70 % ethanol, and the extraction was continued with the RNeasy kit (Qiagen). DNase digestion was performed using RNase-Free DNase Set (Qiagen). cDNA synthesis was performed using SuperScript III reverse transcriptase (Invitrogen). Quantitative real-time PCR was done using the fast SYBR green mix (Applied Biosystems) and quantitated using Applied Biosystems StepOnePlus Real-Time PCR Systems (Applied Biosystems). Relative expression levels were determined by normalizing each CT values (duplicate or triplicate runs of each sample) to cyclophilin expression using the ΔΔCT method. The sequence for the primers used in this study are indicated in Table S8.

Protein isolation and Immunoblotting. For mouse samples, at 6:00am mice were euthanized, livers were rapidly dissected, flash-frozen into liquid nitrogen, and stored at -80 °C. For protein extraction, liver tissues were homogenized using a polytron, in ice-cold lysis buffer (100 mM Tris (pH 7.5), 2 mM EDTA, 2 mM EGTA, 0.5 M mannitol) supplemented with 1 % Triton X-100, protease and phosphatase inhibitor mixture tablets (Roche). Lysates were centrifuged at 10,000 × g for 10 min at 4 °C, afterwards pelleted cell debris was removed. Total protein concentration was assessed (BCA Protein Assay; Thermo Scientific). Subsequently, 20 µg of proteins were loaded on SDS/PAGE and transferred to a nitrocellulose membrane. For human samples, liver biopsies were snap frozen into liquid nitrogen and stored at -80 °C. Subsequently, liver biopsies were pulverized using a

precooled in-house-constructed metal mortar (‘cryogenic grinding’, as described below). Protein extraction for proteome and phosphoproteome analyses was performed using a urea based lysis buffer. Afterwards, lysates were vortexed, sonicated, centrifuged and protein concentration was assessed using Bradford Protein Assay (see section ‘proteome and phosphoproteome’). The antibodies used for immunoblotting, immunofluorescence or immunohistochemistry can be found in the Key Resources Table. For detection, Supersignal west pico chemiluminescent substrate or Supersignal west femto maximum sensitivity substrate (Pierce) was used.

Histology. Mice were euthanized, livers quickly dissected and fixed in 4% paraformaldehyde. After overnight dehydration through several steps of ethanol, tissues were then embedded into paraffin wax blocks. Afterwards, embedded-tissues were cut into 4 µm-thick sections placed on SuperFrost slides (Thermo Scientific). Slides were then stained with: Hematoxylin and Eosin (H&E) or Periodic Acid Schiff (PAS) Stain Kit (Mucin Stain) or Picro Sirius Red Stain Kit (Connective Tissue Stain), according to manufacturers’ protocols.

Immunofluorescence (IF) and immunohistochemistry (IHC).

For IF or IHC, mouse liver samples were fixed with 4 % neutral buffered paraformaldehyde and paraffin embedded according to standard protocol. After de-paraffinization and antigen retrieval steps 4 µm-thick serial sections used for immunostaining. Primary antibodies used were against: RFP, FASN,

Ki67 or AKT-pSer473. Alexa Fluor secondary antibodies from Invitrogen Life Technologies were used. DAPI was used for nuclear counterstaining.

Immunocytochemistry. For immunocytochemistry, freshly isolated primary hepatocytes, obtained by liver perfusion of L-dKO or control mice as described above, were placed on glass coverslips (thickness number 1,5 from VWR) in media containing 12-well culture plate. After cells were attached and equally confluent, coverslips were used for immunocytostaining. Briefly, coverslips were placed in a homemade box-holder to keep humidity. After a quick washing step using pre-warmed PBS, cells were incubated for 45 min with MitoTracker red CMXRos diluted in pre-warmed media and placed in 37 °C. Subsequently, cells were washed, fixed, permeabilized and were immediately used for staining with anti-GCS or -SPT specific antibodies. Alexa Fluor secondary antibodies from Invitrogen Life Technologies were used. DAPI nuclear counterstaining was used. Coverslips were mounted on a glass slide and sealed using nail polish. Slides were then visualized using a scanning confocal “LSM800 Inverted“ with AirScan.

Frozen sections. Mouse livers were dissected into the different lobes and immediately immersed in Optimum Cutting Temperature (O.C.T.) compound and frozen in 2-methylbutane precooled in liquid nitrogen. Afterwards, tissues were cut into 10-µm-thick sections using a cryostat microtome, placed on glass slides on SuperFrost slides (Thermo Scientific), air dried, and stored at -80 °C. Subsequently, slides were warmed at RT and stained with ORO (Sigma) according to standard protocol. Frozen sections stained by ORO

dye were imaged using a Leica upright widefield microscope (DM6000B) and neutral lipid droplet size and number were quantified using Fiji. Detection of GlcCer was done in a lipid preservative method. Frozen liver sections from 8 and 20 week-old L-dKO, L-TriKO and control mice were warmed at RT and washed twice with PBS, subsequently incubated for 1 hr with 5 % goat serum for blocking. Anti-glucosylceramide antibody (GlycoBiotech^{TM}) was diluted with PBS/1% BSA and left for overnight at 4 °C. The same buffer was used to dilute an Alexa Fluor® 488 conjugate secondary antibody, followed by 2 hr incubation at RT. Stained sections were mounted with Vectashield H-1000 (Vector Laboratories, Burlingame, CA). Images were taken using a wide-filed DeltaVision microscope (Olympus IX71) and were deconvoluted.

Image processing. IHC, H&E, SR, PAS and ORO images were all taken using a Leica upright widefield microscope (DM6000B) and scanned using a Zeiss Axio Scan.Z1 Slide Scanner. Hepatocyte size was calculated from liver samples that were stained with H&E using ImageJ (Fiji). Number and size of hepatic lipid droplets were quantified from liver frozen-sections stained for ORO using ImageJ. Number of Ki67 positive hepatocytes, per 10X magnification field, was performed using a wide-filed DeltaVision microscope (Olympus IX71).

Hepatic triglycerides (TG) measurement. Hepatic TG extraction was performed as described (Hagiwara et al., 2012). Briefly, ~ 50 mg of mouse liver tissue was homogenized in a cold room, for 30 s (x4, with a 3 min break on ice in between, to prevent heating) bead beating in lysing matrix D tubes

(Q-Biogene) containing 1 mL chloroform:methanol (2:1). Tissue lysate was transferred into a glass tube and 1 mL of distilled water was added. Subsequently, tubes were vigorously vortexed for 2 min and centrifuged at 800 × g for 10 min at 4 °C. Then, the upper aqueous phase was discarded and the lower solvent phase was dried under nitrogen gas at 50 °C, followed by resuspension with 1 mL of chloroform. TG were then separated by Solid Phase Extraction (SPE) column (Interchim) and dissolved in chloroform/Triton X-100 (1 %). Triglyceride levels determined using a commercial kit according to manufacturer instructions (TG PAP 150; Biomérieux).

Proteome and phosphoproteome. For time-course proteome analysis 4, 8 and 12 week-old L-dKO and control mice were overnight fasted. At 6:00am mice were euthanized and livers dissected (n=6 per group). For analysis of liver tumors, livers from 20 week-old L-dKO mice (n=4) were dissected and separated into individual lobes and tumors (3 tumors per mouse) were micro-dissected, whole-liver samples from 20 week-old control mice (n=6) were used as control. Tissues were immediately snap-frozen in liquid nitrogen. For protein extraction, we performed a 'cryogenic grinding', in which a frozen liver biopsy was crushed into a fine powder, using an in-house-constructed metal mortar, pre-cooled on dry ice. Subsequently, crushed liver tissues were homogenized using a polytron. The lysates were transferred into a cooled 1.5-mL tube containing 150-400 µL lysis buffer (50 mM Tris-HCl (pH 8.0), 8 M urea, 150 mM NaCl, 1 mM PMSF, protease and phosphatase inhibitor mixture tablets (Roche), 100 mM sodium pyrophosphate/β-

glycerophosphate/NaF/NaN_3/ para-nitrophenylphosphate). The lysates were vortexed vigorously for 5 min, then sonicated in a VWR Ultrasonic cleaner bath (USC300T) for 1 min. Sonication was repeated three times with 2 min cooling phases in between. Samples were then centrifuged at 2500 × g for 10 min at 15 °C to remove cell debris. Protein concentration in the supernatants was determined by the Bradford Protein Assay. Proteins were reduced with 2.5 mM DTT for 1 hr at 37 °C, and alkylated with 50 mM iodoacetamide for 30 min at room temperature in the dark.

The urea concentration was reduced to 4 M with 25 mM Tris-HCl, pH 8.0. Proteins were digested with two rounds of endoproteinase LysC (1:100w/w, Wako) at 37°C for 2 hr. The urea concentration of the LysC digest was reduced to approximately 1.2 M with 100 mM Tris-HCl, pH 8.0. Subsequently, trypsin digestion (1:100 w/w) was for 2 hr at 37 °C, followed by a second round of trypsin digestion (1:100 w/w) overnight at 37 °C. Digestion was stopped by adding TFA to 0.4 % (vol/vol) final concentration. The digest was centrifuged at 12,000 rpm for 10 min at RT and the pellet was discarded. For strong cation exchange chromatography (SCX), the peptides were desalted on a C18 reverse-phase SepPak cartridge (Waters). The peptide load was kept to about 5 % (w/w) of the weight of the column packing. The cartridge was primed with 5 mL 100 % acetonitrile, washed with 5 mL 50 % AcCN containing 0.5 % AcOH and equilibrated with 10 mL 0.1 % TFA. The peptides were applied to the SepPak cartridge and washed with 20 mL of 0.1 % TFA. Finally, the cartridge was washed with 1 mL of 0.5 % AcOH and the peptides were eluted with 4 mL of 80 % AcCN / 0.5 % AcOH. The peptide

concentration was estimated, afterwards peptide elute was dried in a SpeedVac.

SCX (Strong Cation Exchange) chromatography. SCX peptide fractionation was done on a HiTrap SP cartridge (GE Healthcare). A HiTrap SP cartridge was washed twice with 1 mL of SCX buffer A (5 mM KH_2PO_4 (pH 2.65), 30 % AcCN). Then, the cartridge was equilibrated twice with 1 mL of SCX buffer B (5 mM KH_2PO_4 (pH 2.65), 30 % AcCN containing 500 mM KCl), followed by re-eqilibration with 2 mL of SCX buffer A. The dried peptides were dissolved in 2 mL of SCX starting buffer (5 mM KH_2PO_4 (pH 2.65), 30 % AcCN) and applied onto the HiTrap SP cartridge. The flow-through was collected. Bound peptides were desorbed in a stepwise manner with 1 mL each of SCX buffer A containing 50 mM, 100 mM, 150 mM, 250 mM, 350 mM, and 500 mM KCl. Fractions were collected individually and the peptide concentration was estimated at 280 nm. The fractions were dried in a SpeedVac and peptides were then desalted on SepPak cartridges (the size of the cartridge was adjusted to the peptide load). The peptide concentration was measured at 280 nm. 20 % of each fraction was used for subsequent proteome analysis by LC-MS/MS (see below).

Phosphopeptide enrichment. Desalted peptides from the individual SCX fractions were dissolved in TiO_2-binding buffer to obtain a peptide concentration of 1 µg/ µL (Kettenbach and Gerber, 2011). TiO_2 beads (GL Sciences Inc.) equilibrated in binding buffer were then added to the peptide solution. Adsorption of the peptides to the TiO_2 beads was done at RT for 1

hr with vigorous shaking. The beads were pelleted at 3000 rpm for 2 min and washed three times with 50 %AcCN/0.1 % TFA. Bound peptides were eluted with 50 mM K_2HPO_4, pH 10. The eluted peptides were immediately acidified with 5 % formic acid. The enriched phosphopeptide pool was desalted on Stage Tips (Thermo Scientific) according to manufacturer's recommendations, and dried in a SpeedVac.

LC-MS/MS analysis for (phospho)proteome. The dried phosphopeptides were dissolved in 20 µL of 0.1 % AcOH, 0.005 % TFA. Peptides from the proteomes and phosphoproteomes were analyzed by capillary LC-MS/MS using a homemade separating column (0.075 mm × 18 cm) packed with Reprosil C18 reverse-phase material (2.4 µm particle size, Dr. Maisch, Ammerbuch-Entringen, Germany). The column was connected on line to an Orbitrap FT hybrid instrument (Thermo Scientific, Reinach, Switzerland). The solvents used for peptide separation were 0.1 % acetic acid in water/0.005 % TFA (solvent A) and 0.1 % acetic acid/0.005 % TFA and 80 % acetonitrile in water (solvent B). 2 µl of peptide digest was injected with a Proxeon Easy-LC capillary pump (Thermo Scientific) set to 0.3 µl/min. A linear gradient from 0 to 40 % solvent B in solvent A in 190 min was delivered with the nano pump at a flow rate of 300 nL/min for the proteome, while a shorter linear gradient of 95 min under identical conditions for the phosphoproteome was used. At the end of the linear gradient, the percentage of solvent B was increased to 75 % in ten min. The eluting peptides were ionized at 2.5 kV. The mass spectrometer was operated in data-dependent mode. The precursor scan was done in the Orbitrap set to a 60,000 resolution, while the

fragment ions were mass analyzed in the LTQ instrument. A 'top ten method' for the phosphoproteome, and a 'top twenty method' for the proteome were run so that the ten or twenty most intense precursors were selected for fragmentation.

Protein identification and data processing. The LC-MS/MS data were searched with Proteome Discoverer 1.4 (Thermo Scientific) set to Mascot and Sequest HT search engines with 10 ppm precursor ion tolerance, while the fragment ions were set to 0.6 Da tolerance. Modifications were set to: carbamidomethyl-cysteine, protein N-terminal acetylation, oxidized methionine, and phosphorylation for serine, threonine, and tyrosine. The peptide search matches were set to 'medium confidence', which corresponds to 5 % false discovery rate. For the searches, the Swiss-Prot KB database set to *M. musculus* was used for Mascot, while for Sequest HT, a local databank was constructed by extracting all *M. musculus* entries from UniProtKB. Two missed cleavages were allowed during the searches. The peptide false-discovery rate was set to 2 %.

Label-free proteome quantification. The LC-MS/MS RAW files were analyzed with the Progenesis software (Non-linear Dynamics, Waters). Alignment was accepted when at least 80% of all features could be matched. The search results from Proteome Discoverer were then imported into the aligned Progenesis files. Only phosphopeptides were further evaluated whose site localization probability was ≥75%. The intensities of all identified features were exported via Excel into a FileMaker database.

Proteome of human HCC biopsies. Human HCC biopsies were measured by sequential window acquisition of all theoretical mass spectra (SWATH), in which data-independent acquisition is coupled with peptide spectral library match (Gillet et al., 2012). Per biopsy, 30 µg of peptides were used for SWATH measurement and 100 µg of peptides were used for library preparation. For library generation, 100 µg of peptides from each of the biopsies were pooled and subjected to high-pH reverse phase fractionation by 5 injections of 200 µg on an Agilent 1260 Infinity HPLC. The 36 collected fractions were subsequently pooled into 12 fractions by concatenation.

SWATH analysis and library preparation. Biopsies were analyzed on a Thermo Scientific QExactive Plus instrument coupled to an Easy nLC 1000 capillary pump. 10 % of iRT peptide mix (HRM kit, Biognosys) was added to both SWATH and library samples prior to injection. Proteome was analyzed by capillary LC-MS/MS using a PicoFrit separating column (0.075 mm × 38 cm) (New Objective) packed with Reprosil C18 reverse-phase material (2.4 µm particle size; Dr. Maisch). For SWATH measurements, one Full MS-SIM scan at resolution 70.000 was followed by 40 mass windows of dynamic size ranging from 400 to 1600 m/z with 4 kDa overlap at resolution 17.500. For library measurements, the mass spectrometer was operated in data-dependent mode and the precursor scan was done at a 70,000 resolution. A top-20 method was run.

Data analysis (SWATH). The library was generated with MaxQuant (version 1.5.1.2) using the default settings except that the mass tolerance of the instrument was set to 10 ppm and the minimal ratio count for quantification was set to 1. The Uniprot SwissProt database (17th August 2015) including the iRT fusion peptide was used for the searches. The SWATH runs were analyzed with Spectronaut (version 9.0.11240.2) (Biognosys) with the default settings used.

Human data sets (transcriptome). Gene expression data from RNA sequencing was obtained from the Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx) projects (STAR Methods). Tissue types are sorted by median expression. To compare expression levels, we used the transcript per million unit (TPM), a measure of overall transcript abundance of genes.

Lipid extraction protocols. Lipid extraction was performed using a modified MTBE protocol. Briefly, for time-course lipidome analysis fresh liver samples from 4, 8, 12 and 20 (cancer tumors were obtained) week-old L-dKO and control mice (n=6 per time point) were used. For assessment of mTORC2-regulated lipids, 20 week-old L-TriKO, L-dKO and control mice were used (n=4 per group). To examine the *in vivo* effect of myriocin, lipidomics of liver samples from myriocin or the drug vehicle treated L-dKO (n=5 myriocin, n=6 vehicle) and control (n=5 myriocin, n=4 vehicle) mice were performed. As described above, in all cases, tissues were immediately snap-frozen in liquid nitrogen. Subsequently, tissues were pulverized into a

fine powder in an in-house-constructed metal mortar, pre-cooled on dry ice ('cryogenic grinding', as described above). Of that, 30-35 mg ground liver tissue was weighted in a pre-chilled 2 mL Eppendorf Safe-Lock tube and resuspended in 100 µl H_2O and transferred into a 2 mL Eppendorf tube. Then, 360 µl methanol was added and vortexed. A mixture of lipid standards (see Key Resources Table) plus 50 µl 1.4 mm Zirconium glass beads (Bertin Technologies, France) were added and the pulverized tissue was homogenized using a Cryolysis System (Bertin Technologies, France) (program: 6200-3x45-045) cooled to 4 °C. MTBE (1.2 mL) was then added and the sample was incubated for one hr at room temperature with a shaking (750 rpm). Phase separation was induced by adding 200 µl H_2O. After 10 min of incubation at RT, the sample was centrifuged at 1000 x g for 10 min (RT). The upper (organic) phase was transferred in a 13 mm screw cap glass tube and the lower phase was extracted with 400 µl artificial upper phase (MTBE/methanol/water (10:3:1.5, v/v)). The two upper phases were combined and the total lipid extract was divided in 3 equal aliquots (one for phospholipids (TL), one for sterols (S) in 2 mL amber vials and one for sphingolipid (SL) detection in a 13 mm glass tube) and dried in a Centrivap at 50°C or under a nitrogen flow. The TL and S aliquots were ready to be analyzed by mass spectrometry and were kept at -80°C. The SL aliquot was deacylated to eliminate phospholipids by methylamine treatment (Clarke method). 0.5 mL monomethylamine reagent (MeOH/H2O/n-butanol/Methylamine solution (4:3:1:5 v/v) was added to the dried lipid, followed by sonication (5 min). Samples were then mixed and incubated for one hr at 53°C and dried (as above). The monomethylamine treated lipids

were desalted by n-butanol extraction. 300 µl H_2O saturated n-butanol was added to the dried lipids. The sample was vortexed, sonicated for 5 min and 150 µl MS grade water was added. The mixture was vortexed thoroughly and centrifuged at 3200 x g for 10 min. The upper phase was transferred in a 2 mL amber vial. The lower phase was extracted twice more with 300 µl H_2O saturated n-butanol and the upper phases were combined and dried (as above).

For analysis of human biopsy samples, lipids were extracted from the same lysates used for immunoblot analysis (described above). Human biopsy lysates (60-80 µl) were extracted with MTBE/methanol. The aliquots were dried, then treated with 2 mg of urease (Sigma, U4002, 50,000-100,000 units/g) in 750 µl 20 mM ammonium acetate, pH 7-7.5 for 1.5 hr at 30 °C to remove the remaining urea that co-isolated with the lipid fraction. After drying, the aliquot for sphingolipid analysis was treated with methylamine, dried, and both aliquots were desalted by butanol extraction.

Phospholipid and sphingolipid detection on a triple quadrupole TSQ Vantage (ThermoFischer Scientific). LC-MS or HPLC grade solvents were used and the samples were pipetted in a 96 well plate (final volume = 100 µl). Positive mode solvent: Chloroform/Methanol/Water (2:7:1 v/v) + 5mM Ammonium Acetate. Negative mode solvent: Chloroform/Methanol (1:2 v/v) + 5mM Ammonium Acetate. The TL and SL aliquots were resuspended in 250 µl Chloroform/methanol (1:1 v/v) and sonicated for 5 min. The TL were diluted 1:50 in negative and positive mode solvents and the SL were diluted 1:10 in positive mode solvent and infused onto the mass spectrometer.

Tandem mass spectrometry for the identification and quantification of phospho- and sphingolipid molecular species was performed using multiple reaction monitoring (MRM) with a TSQ Vantage Triple Stage Quadrupole Mass Spectrometer (Thermo Fisher Scientific) equipped with a robotic nanoflow ion source, Nanomate HD (Advion Biosciences, Ithaca, NY). The collision energy was optimized for each lipid class. The detection conditions for each lipid class are listed below. Ceramide species were also quantified with a loss of water in the first quadrupole. Each biological replicate was read in 2 technical replicates (TR). Each TR comprised 3 measurements for each transition. Lipid concentrations were calculated relative to the relevant internal standards and then normalized to the total phosphate content of each total lipid extract, as described in (Ding et al., 2013). Data was then normalized to age-matched control.

Determination of total phosphorus. A standard curve was constructed using 0, 2, 5, 10 and 20 µl of 3 mM KH_2PO_4 brought to 20 µl with H_2O. The dried total lipid extract was resuspended in 250 µl chloroform/methanol (1:1 v/v) and 50 µl were placed into a 13 mm disposable pyrex tube. The solvent was completely evaporated and 20 µl of water was added to each sample 20 µl of water and 140 µl of 70 % perchloric acid was then added to all tubes. Tubes were heated at 180 °C for 1 hr in a fume hood and cooled at RT for 5 min. Then 800 µl of freshly prepared H_2O/1.25 % NH_4-Molybdate/1.66 % ascorbic acid (5:2:1 v/v) were added. Tubes were heated at 100 °C for 5 min with a marble on each tube to prevent evaporation and were cooled to RT. 2

x 100 µl of each sample was then transferred into a 96-well microplate and the absorbance at 820 nm was measured.

Metabolite extraction protocol. Pulverized liver tissues were obtained as described above (5-8 mg) and resuspended in 200 µl extraction solvent (ethanol/water/diethylether/pyridine/ammonium hydroxide 4.2N (15:15:5:1:0.018)). To that, a mixture of lipid standards (see Key Resources Table) and 50 µl 1.4 mm Zirconium glass beads (Bertin Technologies, France) were added. The pulverized tissue was homogenized using a Cryolysis System (Bertin Technologies, France) (program: 6200-3x45-045) cooled at 4 °C. The sample was incubated for 20 min on ice, centrifuged at 21000 x g for 2 min and the supernatant was transferred to an Eppendorf tube. The pellet was extracted a second time with 200 µl of extraction solvent. The combined supernatants were centrifuged at 21000 x g for 5 min to remove remaining cell debris and split into two equal aliquots in 300 µl glass inserts. The metabolite extract was dried in a Centrivap at 50 °C for 30 min and then 60 °C for 2 hr.

Derivatization of amino groups. Dried lipid extract was resuspended in 10 µl 0.1 % formic acid, 70 µl borate buffer (200 mM boric acid pH 8.8 (with NaOH), 10mM tris (2-carboxyethyl)phosphine (TCEP), 1 mM ascorbic acid, 35.7 µM $^{13}C^{15}N$-Valine (internal standard)). The mixture was sonicated for 5 min and 20 µl of 10mM AQC (2.85 mg/mL in acetonitrile) was added. The sample was incubated for 15 min at 55 °C with shaking (750 rpm) followed by overnight incubation at 24 °C. The quantification of the metabolites was

performed using multiple reaction monitoring (MRM) (See below) with a TSQ Vantage Triple Stage Quadrupole Mass Spectrometer (Thermo Fisher Scientific). Area of each metabolite was normalized to the respective internal standard.

LC-MS/MS-metabolomics. After incubation, the sample was centrifuged as above and the supernatant transferred in a new insert. 10 µl were loaded onto a NUCLEOSHELL RP 18 (Particle size: 2.7 µm, length: 100 mm, Diameter: 2 mm) (MACHEREY-NAGEL) and a gradient (see below) was applied: solvent A: milliQ water + 0.1 % formic acid, solvent B: isopropanol + 0.1 % formic acid, at a flow of 300 µl/min.

Detection of Lipids by MS/MS (related to lipidome analyses)

Lipid Class	Standard	Polarity	Mode	m/z ion	CE
Phosphatidylcholine $[M+H]^+$	PC31:1	+	Product ion	184.07	30
Phosphatidylethanolamine $[M+H]^+$	PE31:1	+	Neutral ion loss	141.02	20
Phosphatidylinositol $[M-H]^-$	PI31:1	-	Product ion	241.01	44
Phosphatidylserine $[M-H]^-$	PS31:1	-	Neutral ion loss	87.03	23
Cardiolipin $[M-2H]^{2-}$	CL56:0	-	Product ion	acyl chain	32
Ceramide $[M+H]^+$	C17Cer	+	Product ion	264.30	25
Dihydroceramide [M+H]+	C17Cer	+	Product ion	266.40	25
Hexacylceramide $[M+H]^+$	C8GC	+	Product ion	264.30	30
Hexacyldihydroceramide [M+H]+	C8GC	+	Product ion	266.40	30
Sphingomyelin [M+H]+	C12SM	+	Product ion	184.07	26

MRM list for detection of sphingoid bases (metabolomics)

Q1	Q3	CE
456.322	171.055	20
456.358	171.055	20
470.338	171.055	20
472.353	171.055	20
536.501	171.055	30
538.304	171.055	30
550.304	171.055	30

552.32	171.055	30

Gradient Nucleoshell RP 18 column

N°	Time	A%	B%	C%	D%	µl/min
0	0	99	1	0	0	300
1	12	90	10	0	0	300
2	14.5	60	40	0	0	300
3	18.5	20	80	0	0	300
4	20	0	100	0	0	300
5	22.5	0	100	0	0	300
6	24	99	99	0	0	300
7	30	99	99	0	0	300

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis – mouse phosphoproteome and proteome: The data was analyzed with the Perseus software, version 1.4.0.2 (Tyanova et al., 2016). For mouse data, volcano plots based on ANOVA two sample t-test were performed. S0 and FDR were set to 0.5 and 5 % respectively and number of randomizations were kept at 250. For the hierarchical clustering, only proteins that were significantly deregulated in at least one time point (4, 8 or 12 weeks) were included. Normalization was performed using Z-scoring within a given time point. Finally, unsupervised hierarchical clustering was performed, the distance was set to Pearson, the linkage to average, and the maximal numbers of clusters to 300 (Tyanova et al., 2016). In data obtained from tumors (20 weeks), up- or down regulated proteins (Figure 1C) were included if detected in at least 11 tumors (out of 12 tumors measured), and deregulated in at least 75% of total detections. Binarization of the data was done based on the volcano plot analysis. Additionally, we included data exceeding a threshold for Log2 fold change of +/-1. 'Black and white' cases (i.e., proteins that were detected either in mutant or in control, but not

in both) were included only if the detection was true in 3/3 control runs and 0/3 tumor runs and vice versa.

Human biopsies proteome – the data was analyzed using Perseus as described above. Volcano plots based on ANOVA two sample t-test were performed, S0 and FDR were set to 0.1 and 2% respectively, number of randomizations we kept at 250. Binarization of the data was done using thresholds for Log_2 fold change of +/- 0.7 if the volcano plot analysis returned a significant change otherwise we used a threshold for Log2 fold change of +/-1. 'Black and white' situations were also included if the detection was true in 3/3 control runs and 0/3 tumor runs and vice versa. Unsupervised hierarchical clustering was performed after Log2 ratios of tumor versus non-tumor data were normalized using z-scoring. The distance was set to Pearsons or Spearmans, the linkage to average and k-means algorithm was disabled (Tyanova et al., 2016).

Statistical analysis – **lipidome:** To generate categories of minor, medium or major species, we used 68 biological replicas from liver samples of wild type mice (all were sacrificed at 6:00am). Within individual lipid species we defined the categories based on median raw abundances. The data was analyzed with the Perseus software, version 1.4.0.2. For the volcano plots, an ANOVA two-sample t test was performed, adjusting S0 to 0.1, number of randomizations to 250, and FDR to 5 %. For the heat maps, only lipid species that were significantly deregulated in at least one time point were included. Z-scoring was performed with grouping within same time points.

Finally, for unsupervised hierarchical clustering, the distance was set to Euclidian, the linkage to average, and the maximal numbers of clusters to 300.

Immunoblots for AKT2-pS473 and AKT were quantified using Fiji. Sample size was chosen according to our previous studies and published reports in which similar experimental procedures were described. No blinding of investigators was done. All data are shown as mean ± SEM. Sample n numbers are indicated in figure legends. For mouse experiments, n number represents number of animals used in a group. For cell culture experiments, n number indicates number of independent experiments. Fold changes in Figure S6E were calculated from 3 independent experiments.

To statistically determine the significance between two groups, unpaired Student's *t* test was performed, unless otherwise indicated.

Data analysis. Statistical analyses and data plotting were performed using Prism Graph Pad 6.0. Unless mentioned otherwise, statistical significance was defined as <0.05. An unpaired t-test assuming Gaussian distribution was used to compare between two groups. Kaplan-Meier method was used for survival analysis. Data represent mean ± SEM.

List of Supplemental Tables

Table S1. Related to Figure 1. Enrichment analyses by Pathway Maps.

Table S2. Related to Figure 1. Proteome, phosphoproteome and RNAseq from young (4, 8 and 12 week-old) and 20 week-old mice (HCCs).

Table S3. Related to Figure 3. Longitudinal lipidomic analyses.

Table S4. Related to Figure 4. Myriocin lipidomics analyses.

Table S5. Related to Figure 7. mTORC2-regulated lipid species - lipid enrichment analysis.

Table S6. Related to Figure 8. Human patients information.

Table S7. Related to Figure 8. Lipogenesis biosynthetic enzymes in patient HCCs.

Table S8. Related to STAR methods. Primer sequences.