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The Role of the Tumor Suppressor Kinase LKB1 in Xenobiotic Metabolism

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Abstract of the Dissertation

The Role of the Tumor Suppressor Kinase LKB1 in Xenobiotic Metabolism

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The liver kinase-B1 (LKB1) is a serine/threonine kinase that has numerous roles in human disease. LKB1 was originally identified as a tumor suppressor kinase that, when inactivated, is responsible for the human inherited disorder Peutz-Jeghers syndrome which is characterized by benign gastric polyposis and increased cancer predisposition. More recently, inactivating LKB1 somatic mutations in humans have been found in 15-35% of non-small cell lung carcinomas and in 20% of cervical carcinomas. LKB1 functions as a master kinase upstream of 14 different substrates kinases and these substrates have critical roles in cell growth, polarity, and metabolism. Previous studies show that in specialized metabolic tissues such as the liver LKB1 and its downstream substrate AMPK control cholesterol, lipid, and glucose metabolism. However,

LKB1's role in other biological processes in the mammalian liver is poorly understood.

To identify pathways deregulated upon loss of LKB1 signaling a microarray screen was employed using mice with an inducible liver-specific deletion of *Lkb1*. Unexpectedly, one of the most prominently deregulated pathways upon hepatic LKB1 loss is the xenobiotic metabolism pathway. Xenobiotic metabolism occurs primarily in the liver and serves to remove endogenous and exogenous compounds from the organism. Mice lacking hepatic LKB1 have reduced expression of many phase I xenobiotic metabolism genes, collectively known as the cytochrome P450 family (CYP). In particular, expression of CYP2E1, an important mediator of drug interactions, was markedly reduced upon LKB1 loss. LKB1-deficient livers exhibit a decreased response to hepatocarcinogens compared to wild-type controls. In addition, mice lacking hepatic LKB1 are refractory to all stages of liver carcinogenesis promoted by repeated high-dose administration of the hepatocarcinogen carbon tetrachloride. Thus, it appears that LKB1 is an important regulator of xenobiotic metabolism and attenuation of this pathway could be useful in mediating toxicity of drugs or other exogenous compounds.

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CHAPTER 1: Introduction

LKB1 is a tumor suppressor gene

The rare autosomal-dominant inherited cancer disorder Peutz-Jeghers syndrome (PJS) occurs in approximately 1 in every 120,000 births . PJS is characterized by mucocutaneous hyperpigmentation, benign hamartomatous polyps in the gastrointestinal tract, and affected individuals have an increased risk of cancer malignancies [1]. Linkage analysis using short-sequence-repeat polymorphism markers identified a region on chromosome 19p13.3 containing a putative PJS gene [2]. Further mapping revealed a single gene mutated in PJS kindreds, encoding a previously uncharacterized serine/threonine kinase STK11, also known as Liver Kinase B1 (LKB1) [3-5].

Patients with PJS are ten times more likely than the general population to develop cancer, display similar cancer risk between males and females, and have a 48% chance of dying from cancer by age 57 [6, 7]. There may be a sequential neoplastic transformation from benign hamartomas to adenomas and finally to carcinomas that may rely on the loss of the remaining wild-type *LKB1* allele [2]. To further investigate the role of LKB1 in tumor suppression, several groups created knockout mice for *Lkb1*. Mice homozygous for loss of LKB1 activity die in mid-embryogenesis (E8.5-E11) from vascularization and placental development defects [8-10]. However, as in patients with PJS, mice heterozygous for *Lkb1* develop gastrointestinal polyps with 100% penetrance and clinically mimic the hamartomas seen in human patients. PJS patients are susceptible not just to gastrointestinal cancer but also at

other sites including breast, lung, and pancreas (Figure 1) [11]. Some of these tumor sites have been modeled in mice with tissue-specific deletion of *Lkb1*.

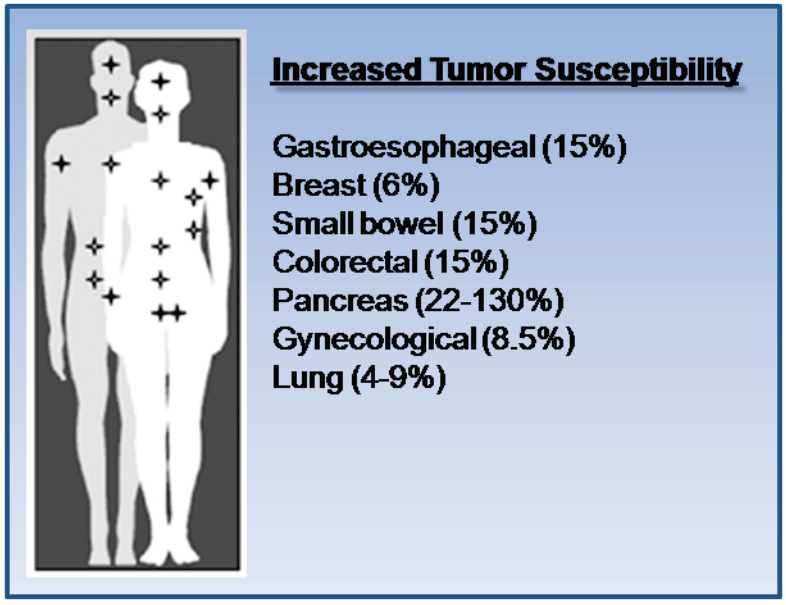


Figure 1. Tumor spectrum in Peutz-Jeghers syndrome patients. Increased risk of cancer of each tumor type is cited in parentheses. several different tumor types. *Adapted from Sanchez-Cespedes, 2007.*

Most mutations in *LKB1* are small insertion/deletion events or single-base substitutions that result in protein truncation or loss of kinase activity [12]. These are all heterozygous loss-of-function mutations and as such mutation of the second allele of *LKB1* has been demonstrated in all malignancies found in PJS patients, and whether loss of the second allele is required for hamartoma formation remains controversial [9, 13, 14]. Though missense mutations are not common in PJS patients, those

found tend to cluster to the kinase domain and extensive analysis of these mutations reveal that all result in loss of LKB1 kinase activity. Thus genetically, the function of LKB1 as a tumor suppressor is directly connected to its kinase activity. Indeed point mutations that have been found in PJS kindreds in the catalytic lysine and catalytic aspartic acid which govern ATP binding, these principal mutations are employed by investigators trying to make kinase-dead alleles of many other kinases [15-17]. In vivo, LKB1 is found in a 1:1:1 heterotrimeric complex with the pseudokinase Ste20-related adaptor (STRAD) and scaffolding protein mouse protein 25 (MO25). LKB1 protein is stabilized through that interaction and, unlike most other kinases, is activated through phosphorylation-independent mechanisms [18-21]. This mechanism of allosteric regulation of LKB1 highlights that cancer mutations occurring not only in the kinase domain of LKB1 but that other mutations affecting complex assembly and interaction with substrates and regulators are also important [22].

LKB1 activates AMPK to control cell growth via mTOR signaling

Given that the kinase activity of LKB1 was central to its tumor suppressor function, several laboratories set about to identify substrates of LKB1 which may mediate its tumor suppressor function. Multiple independent approaches identified the AMP-activated protein kinase (AMPK) as a direct substrate [23, 24]. LKB1 directly phosphorylates AMPK on the critical threonine in its activation loop, resulting in a 1000X stimulation in its kinase activity. Unlike the little information known about

the cellular function of LKB1 at the time, AMPK had been extensively studied and was thought to be a key modulator of cell metabolism under conditions of low energy [25]. A connection between cellular metabolism and tumorigenesis was first proposed in the early 1900s by Otto Warburg, who postulated that tumor cells rely on glycolysis rather than generating energy by oxidation of pyruvate. It is only in the last decade that the molecular mechanisms controlling cell growth and metabolism have begun to be decoded. An essential requirement for all cells is the ability to couple proliferative signals from growth factors with nutrient availability so that cell division only occurs when nutrients are freely available. In that regard, AMP-activated protein kinase (AMPK) serves as the primary energy-sensing regulator of cellular response to low ATP levels. AMPK phosphorylation of its downstream targets results in the down-regulation of energy-consumptive processes and up-regulation of ATP-producing catabolic pathways until energy balance is restored [26, 27].

LKB1 is the upstream kinase capable of AMPK in vivo and as such is a critical mediator of cellular response to low energy [21, 23, 28]. It appears that LKB1 dictates most of the AMPK activation in all tissues examined where LKB1 has been genetically knocked out. In some endothelial cells, T cells, and hypothalamic neurons AMPK activity may be modulated by calcium and calmodulin-dependent protein kinase kinase (CAMKK) although only in response to changes in calcium levels and not energy levels [29-32]. Notably, LKB1 also phosphorylates a total of 14 downstream substrate kinases, although only AMPK α1 and α2 are activated by low ATP levels (Figure 2). The remaining 12 AMPK-related kinases are less well-defined; substrates such as MARKs are

required for early embryonic partitioning and polarity while the SIKs may be involved in metastasis [33-36].

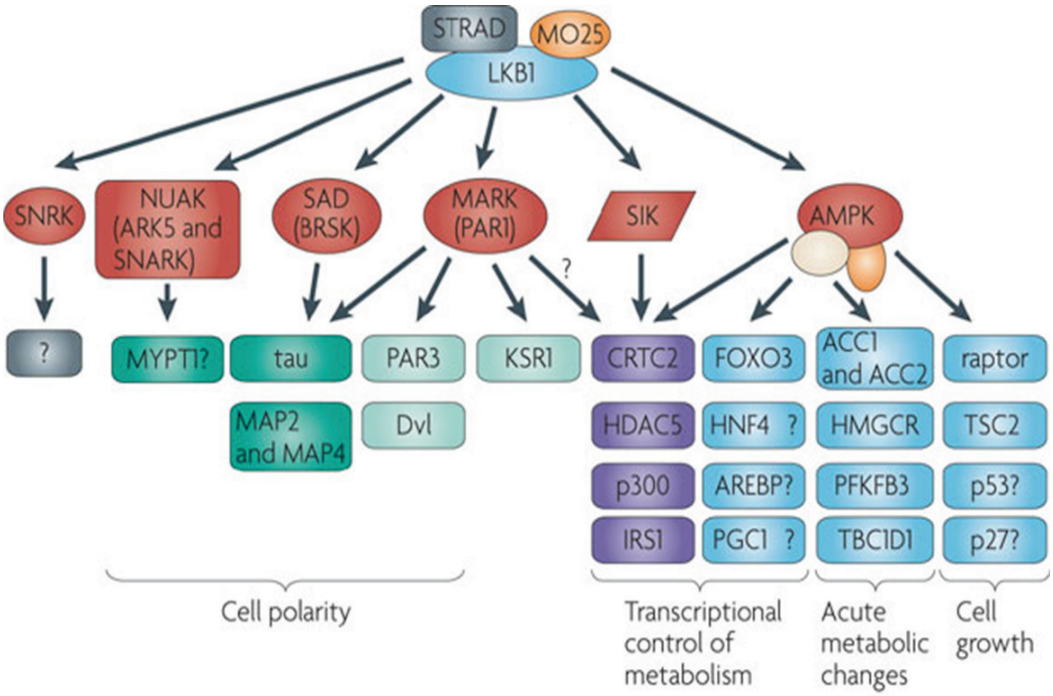


Figure 2. LKB1-dependent kinase signaling. LKB1 is complexed with its two regulatory subunits and directly phosphorylates and activates 14 AMPK-related kinases. Downstream substrates of the AMPKRs are shown, with question marks next to substrates for which further *in vivo* data are needed.

LKB1 and AMPK exert their role as mediators of cell growth and division at least in part through regulation of mammalian target of rapamycin complex 1 (mTORC1). Highly conserved in all eukaryotes, mTOR is serine/threonine kinase that is a central regulator controlling cell growth and protein synthesis and is deregulated in most human cancers [37]. mTOR is found in two functionally discrete complexes, mTORC1 and mTORC2. The mTORC1 complex includes regulatory-associated protein of mTOR (raptor) and this scaffolding recruits downstream substrates to control protein synthesis. mTORC1 induces the synthesis

of many growth regulators such as MYC and cyclin D1. mTORC1 is nutrient sensitive and can be inhibited by the bacterial macrolide rapamycin. mTORC2 is complexed with the rapamycin-insensitive companion of mTOR (rictor) and is not inhibited by rapamycin treatment or nutrient deprivation.

In cells undergoing glucose or amino acid deprivation mTORC1 signaling is inhibited via two distinct phosphorylation events[38]. AMPK can inhibit mTORC1 by directly phosphorylation and activating the negative mTORC1 regulator tuberin, which is the product of the Tuberous Sclerosis Complex 2 tumor suppressor (TSC2) [39]. More recently, AMPK has been shown to phosphorylate the mTORC1 subunit raptor, which leads to subsequent 14-3-3 binding and inactivation [40]. Thus the LKB1-AMPK pathway turns off the mTORC1 pathway when intracellular ATP levels are low [41]. Interestingly, mutations in LKB1, PTEN, NF1, and TSC2 are responsible for a family of inherited cancer syndromes collectively known as phakomatoses. Clinically, loss of function in any one of these results in syndromes characterized by hamartomas and aberrant mucocutaneous pigmentation defects (Figure 3). As each of these tumor suppressors function upstream of mTORC1 the underlying mechanism of cell-autonomous hyperactivation of mTORC1 results in tumors uniquely reliant upon mTORC1 signaling [42].

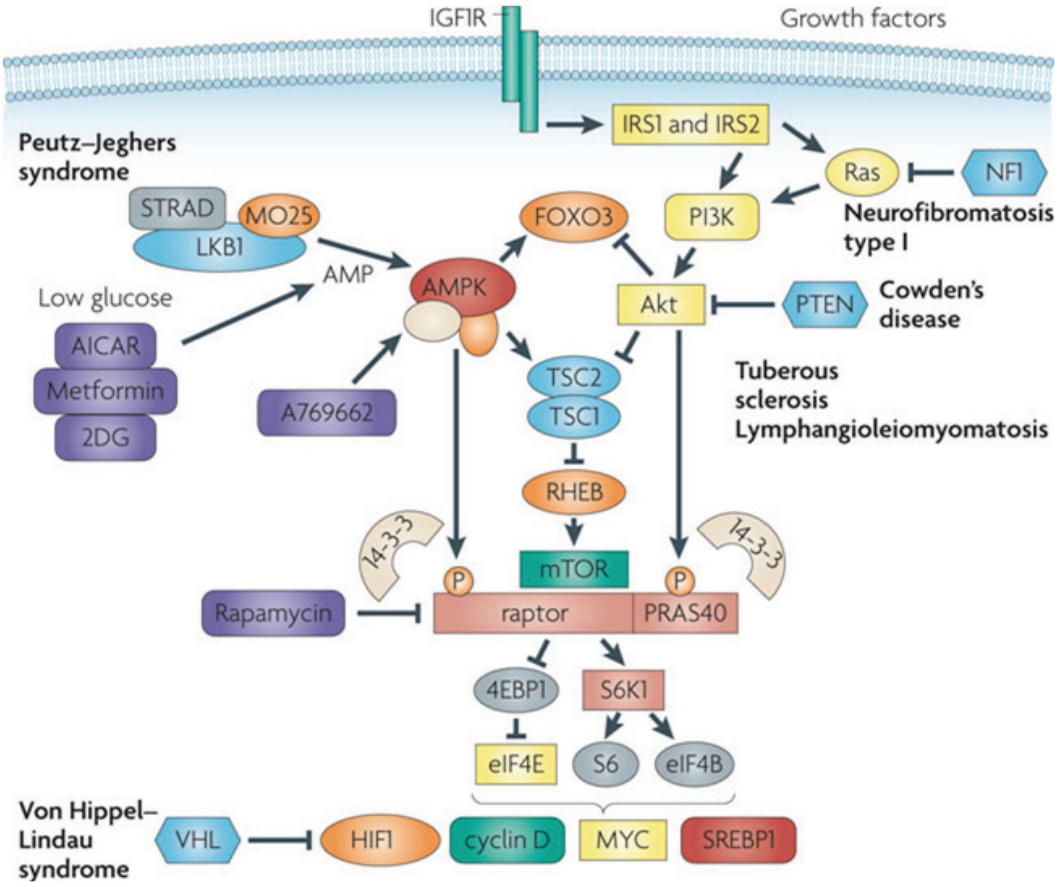


Figure 3. AMPK and PI3K signaling converge to antagonize mTORC1 signaling. AMPK mediates mTORC1 activity through phosphorylation of TSC2 and Raptor. Oncogenes are shown in yellow, tumor suppressor genes in blue, while chemical compounds modulating kinase activity are shown in purple. Note that many hamartoma and cancer predisposition syndromes show hyperactivation of mTORC1.

LKB1-AMPK pathway plays key roles in organismal metabolism

Physiologically, the LKB1-AMPK pathway is activated under conditions of energy stress such as fasting, calorie restriction, or exercise. AMPK is activated in adipocytes, hepatocytes, and the hypothalamus in response to low glucose and upon refeeding this activity is decreased [43-45]. Conversely, overnutrition and hyperglycemia can suppress LKB1-AMPK signaling, perhaps contributing to increased cancer risk in obese or poorly-controlled diabetic populations. AMPK also plays a role in skeletal and cardiac muscle hypertrophy, glucose metabolism, and exercise. Acute resistance exercise in untrained human subjects decreases mTORC1 signaling via AMPK activation while trained human subjects show blunted AMPK activation during exercise [46, 47]. Muscle-specific LKB1 knockout mice display a markedly decreased ability for voluntary treadmill running and glucose uptake after muscle contraction is severely limited [48, 49]. Incredibly, chronic AMPK activation by orally bioavailable AMP mimetic 5-aminoimidazole-4-carboxamide riboside (AICAR) treatment leads to a “super endurance” phenotype with a 45% increase in running capacity in untrained mice [50].

Pharmacological intervention in the LKB1-AMPK pathway has garnered intense scrutiny as a target of type 2 diabetes and other abnormal metabolic syndromes. Metformin is the most widely used type 2 diabetes therapeutic and is thought to act by decreasing hepatic gluconeogenesis, thus lowering blood sugar levels [51]. Metformin and its more potent analog, phenformin, are mitochondrial poisons that inhibit complex I of the respiratory chain resulting in a decline in ATP production.

Intact LKB1-AMPK signaling is absolutely required for the blood glucose-lowering effects of metformin [52]. Similarly AICAR and A769662, the only known small molecule capable of binding directly to AMPK, are able to activate AMPK signaling and lower blood glucose [53, 54].

In the liver the LKB1-AMPK pathway plays important roles in glucose and lipid metabolism. Circulating hormones such as leptin and adiponectin are able to activate AMPK and suppress mTORC1 signaling and gluconeogenesis [55]. Repression of gluconeogenesis by AMPK is mediated through direct phosphorylation of transcription factors and coactivators that control transcription of gluconeogenic enzymes such as PEPCK and G6Pase [56]. A key positive regulator of lipogenesis, SREBP1, accumulates during mTORC1 activation and drives lipid formation. Mice lacking hepatic LKB1 have elevated SREBP1 levels resulting in increased lipid buildup and hepatic steatosis (fatty degeneration of the liver) [52]. While the maintenance of glucose and lipid metabolism homeostasis by the LKB1 pathway is critical for the suppression of diabetes and other metabolic syndromes it is also likely to be important for the tumor-suppressive effects of LKB1.

The role of LKB1 in sporadic human cancers and mouse models of cancer

Recent studies have discovered that LKB1 mutations are common in 15-35% of non-small cell lung carcinomas (NSCLC) which represents the majority of lung cancer diagnosis [57]. Human NSCLC cell lines and primary tumors revealed loss of AMPK activity and mTORC1

hyperactivation [58]. These findings suggest that a functional LKB1/AMPK/mTOR pathway is important for suppressing tumor formation in the lung. Of particular interest is the discovery of K-ras oncogene co-mutation with LKB1 in NSCLC [59]. In order to create a lung environment in mice similar to those found in human primary tumors, mice carrying a conditional Cre-lox activated allele of K-ras were crossed to mice carrying conditional Cre-lox inactivated alleles of LKB1. Adult mice were treated with adenovirus-Cre recombinase to simultaneously induce oncogenic K-ras and delete LKB1 expression. These double mutant mice have rapidly accelerated tumor growth and are highly metastatic [57]. Importantly, some of the carcinomas formed are squamous tumors, which had never before been observed in genetically engineered mouse models of lung cancer, marking the first instance of a genetically defined lung cancer model recapitulating all subtypes of the human disease.

Using a similar Cre-Lox strategy to conditionally delete LKB1 in prostate epithelial cells, loss of LKB1 resulted in male mice with predisposed to hyperplasia which progressed into prostate intraepithelial neoplasia (PIN) within 2-4 months of LKB1 recombination. Also observed in this model were other genitourinary lesions such as bulbourethral gland cysts, hyperplasias of the urethra, and seminal vesicle squamous metaplasias. Immunohistochemical analysis of the resultant PIN revealed overexpression of β-catenin and downstream Wnt target genes and an increase in p-Akt. Interestingly this model revealed an unexpected surge of p-AMPK and suppression of mTORC1in PIN lesions, which the authors hypothesize may be due to increased PDK1 activity [60].

PJS is strongly associated with pathologically distinct neoplasias in the female reproductive tract. In human sporadic cases of cervical cancer, at least 20% of tumors harbor somatic mutations in *LKB1*, marking the first recurrent genetic alteration found in cervical cancer [61]. In aged *Lkb1* heterozygous female mice, 53% display uterine neoplasias. Intrauterine injections of adenovirus-Cre recombinase resulted in loss of LKB1 in the uterine lumen and provoked well-differentiated, polarized yet highly invasive metastatic endometrial adenocarcinomas [62]. Rapamycin treatment in this model not only slowed tumor growth but could lead to the regression of pre-existing tumors [63]. Intriguingly, cervical cancer cell lines treated with AICAR undergo growth arrest or cell death. Human papillomavirus virus oncogene transcription was also suppressed with AICAR treatment, raising the possibility that cervical cancers arising from chronic HPV infection may be susceptible to treatment with AMPK activators [64].

In order to study the role of LKB1 loss in mammary tumors, a life-cycle specific conditional β-lactoglobulin Cre recombinase (BLG-Cre) system was used. Virgin female mice expressed very low levels of BLG-Cre and therefore LKB1 recombination was low while lactating mice expressed very high levels of BLG-Cre. Female mice were allowed to progress through two pregnancies and then were monitored for tumor development. After a latency of 11-21 months, 19% of these mice scored positively for at least one mammary gland tumor. These tumors share some histological features with female PJS patients diagnosed with breast cancer [65].

As mutations of *Lkb1* in sporadic tumors such as NSCLC is linked to tobacco carcinogen exposure, it is not surprising that Lkb1-deficient

mice treated with 7,12-dimethylbenz(a)anthracene (DMBA) are especially prone to tumor formation. Mice heterozygous for *Lkb1* are prone to loss of the wild-type allele and to squamous tumor development in both the skin and the lung following DMBA treatment. Mice with an epidermal-specific deletion of *Lkb1* are also susceptible to DMBA-promoted squamous cell carcinomas (SCC). Untreated mice with epidermal deletion of *Lkb1* also develop SCC after a long latency period. Surprisingly, while DMBA treatment initiates A-T transversions in codon 61 of H-ras in other mouse models of SCC there is no evidence of activating H-ras mutations on an LKB1-deficient background.

Hepatocellular carcinoma

After lung, stomach, and colorectal cancers, hepatocellular carcinoma ranks the fourth most common cause of cancer death worldwide in 2009 according to the World Health Organization. It is well known that infection with hepatitis B or C virus and ingestion of the rice fungal contaminant aflatoxin B1 are important risk factors for the development of HCC [66]. In Western society where HBV and HCV infection rates are low the most common risk factor is chronic alcohol abuse [67]. In most patients diagnosed with HCC treatment such as surgical resection, radiation, and chemotherapy are often ineffective and only palliative care may be given. The disease progression often takes decades and HCC is associated with chronic liver disease such as cirrhosis and chronically active hepatitis [68]. However, 5%-30% of HCC case studies lacked such a readily identifiable risk factor [69].

In recent years it has been recognized that this “cryptogenic” form of HCC is attributable to nonalcoholic steatohepatitis which is one of the hepatic symptoms of metabolic syndrome, a collection of physical problems such as obesity, diabetes, dyslipidemia, and insulin resistance [70]. Epidemiological data suggests that diabetes is associated with risk of HCC development by a 2-fold to 4-fold increase and that HCC risk in diabetics synergizes with HCV infection and chronic alcohol exposure [71, 72]. Overnutrition by excess consumption of fatty acids and glucose can lead to increased expression of signaling molecules such as EGF, FGF, and NFκB in addition to suppressing the LKB1-AMPK signaling pathway leading to mTOR activation [73]. Intriguingly, diabetics taking biguanides like metformin or thiazolidinediones have a 70% reduced HCC risk compared to diabetics relying on dietary control, insulin, or sulfonylureas [74].

In addition to energy homeostasis the liver is the primary location of detoxification of endogenous and exogenous compounds, a process collectively known as xenobiotic metabolism. While other tissues such as lung, intestine, and skin express many xenobiotic metabolism genes it is the liver that bears the responsibility for detoxifying ingested compounds. This depends on the anatomical location of the liver as the portal vein brings drugs and chemicals directly to the liver from the gut in a highly concentrated form [75]. The metabolism of xenobiotics in the liver can be divided into four phases (Figure 4). The phase I oxidative metabolism system is mediated by cytochrome P450 (CYP) family and the flavin mixed-function oxidase family.

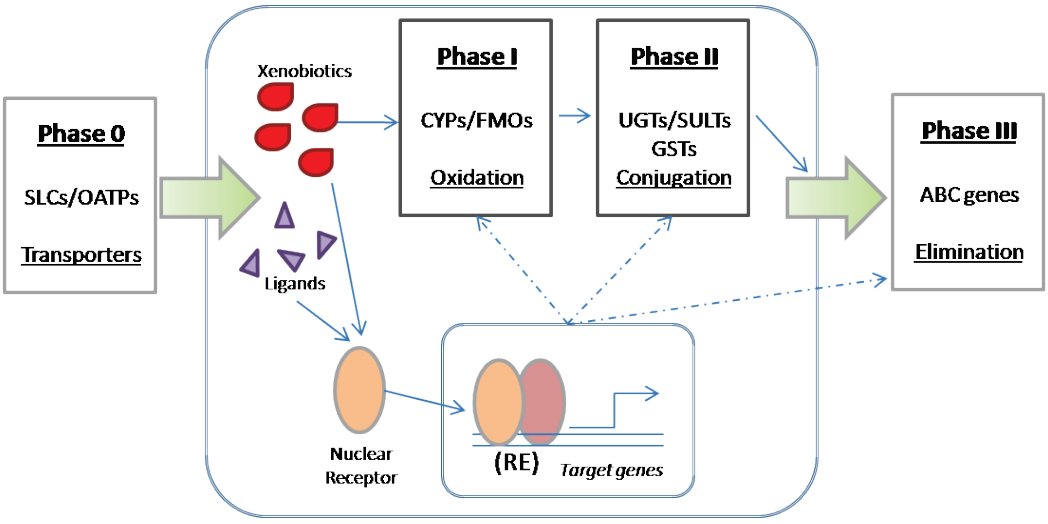


Figure 4. Schematic of the four phases of xenobiotic metabolism. In phase 0, xenobiotics or ligands are transported across the hepatocyte membrane. Recognition of these substrates by nuclear hormone receptors is critical for the regulation of genes involved in xenobiotic metabolism. Upon activation, nuclear receptors alter gene expression via a conserved response element (RE) in the promoter region of target genes. In Phase I, CYPs or FMOs mediate oxidation of xenobiotics to hydrophobic metabolites. Conjugation with sulfate, glutathione, or glucuronide by Phase II enzymes converts metabolites into hydrophilic forms for elimination by Phase III transporters.

Phase I metabolism is often the critical step in the unintentional formation of genotoxic or cytotoxic metabolites from much less toxic precursor compounds. In phase II, activated hydrophobic metabolites are conjugated to glutathione, sulfate, or glucuronide to form hydrophilic metabolites for elimination via the phase III ATP-dependent export pumps [76]. In humans, less than 10 CYPs account for greater than 90% of all drug oxidation [75]. One of these enzymes, CYP2E1, metabolizes and activates many small molecules such as ethanol, carbon tetrachloride, and acetaminophen to metabolites much more toxic than the original

compound [77]. Constant exposure to toxic intermediate metabolites promotes HCC formation in rodent models [78].

Peutz-Jeghers syndrome patients exhibit a wide variety of tumor types at sites other than the gastrointestinal tract which have been recapitulated in genetically defined mouse models. Notably, hepatocellular carcinoma (HCC) has not been reported among PJS patients. This is surprising given that LKB1 disruption in the liver has profound effects on energy metabolism and LKB1 loss in the lung, mammary gland, endometrial tissue, and prostate have all been shown to increase tumorigenesis. Nonetheless, little work has been done to examine the role of LKB1 in HCC. Comparative genomic hybridization analysis of 50 primary human HCC samples characterized the loss of 19p, where *Lkb1* resides, in 42% of samples [79]. Single-strand conformation polymorphism PCR analysis of 80 primary human HCC tumors found one missense mutation in the kinase domain of LKB1 (1% of cases studied) and 6 cases (22%) of LKB1 loss of heterozygosity. In contrast, 9 of the 80 HCCs profiled had mutations in *p53* (11%) and only one case with both LKB1 LOH and p53 mutation (1%) [80]. Epigenetic silencing of LKB1 in HCC seems unlikely as methylation profiling of the *Lkb1* gene in 28 primary human HCC tumors revealed that *Lkb1* remains unmethylated in tumor tissue compared to normal adjacent liver [81].

The body of work herein probes a novel pathway deregulation after LKB1 loss in the liver using conditional deletion of *Lkb1* alleles in adult mice. Unexpectedly, I discovered one of the most prominently deregulated pathways upon hepatic LKB1 loss is the xenobiotic metabolism pathway. Key findings include loss of phase I xenobiotic metabolism gene expression and the discovery that LKB1-deficient livers

display a distinct lack of response following acute treatment with the hepatocarcinogens diethylnitrosamine and carbon tetrachloride. Furthermore, I have compelling evidence that loss of LKB1 is hepatoprotective against chemical-induced cholangiocarcinoma development using chronic, high-dose administration of carbon tetrachloride.

CHAPTER 2: Deregulation of xenobiotic metabolism after loss of LKB1 signaling in the liver.

Microarray analysis of livers lacking LKB1 expression

Previous work examining the role of hepatic LKB1 signaling has shown that it is crucial to homeostatic regulation of energy metabolism through control of gluconeogenesis and lipogenesis. One of the most commonly prescribed type 2 diabetes therapeutic, metformin, suppresses blood glucose levels by shutting off hepatic gluconeogenesis through signals mediated by an intact LKB1-AMPK signaling pathway; this pathway is required for metformin to be effective [52]. In order to understand what other pathway effector role LKB1 might have in the liver I took a microarray-based approach to find genes whose expression was altered upon LKB1 loss. Age-matched adult male mice with either homozygous wild-type or *Lkb1*flox/flox (henceforth referred to as +/+ and L/L, respectively) alleles were administered via tail vein injection adenovirus expressing Cre recombinase under the cytomegalovirus promoter to delete exons 3-6 of *Lkb1*(Figure 5) [8]. Mice were allowed to recover from viral infection for two weeks to allow any gene expression changes due to immune response to diminish. Liver samples were processed for downstream applications and data analysis was performed as described in *Materials and Methods*.

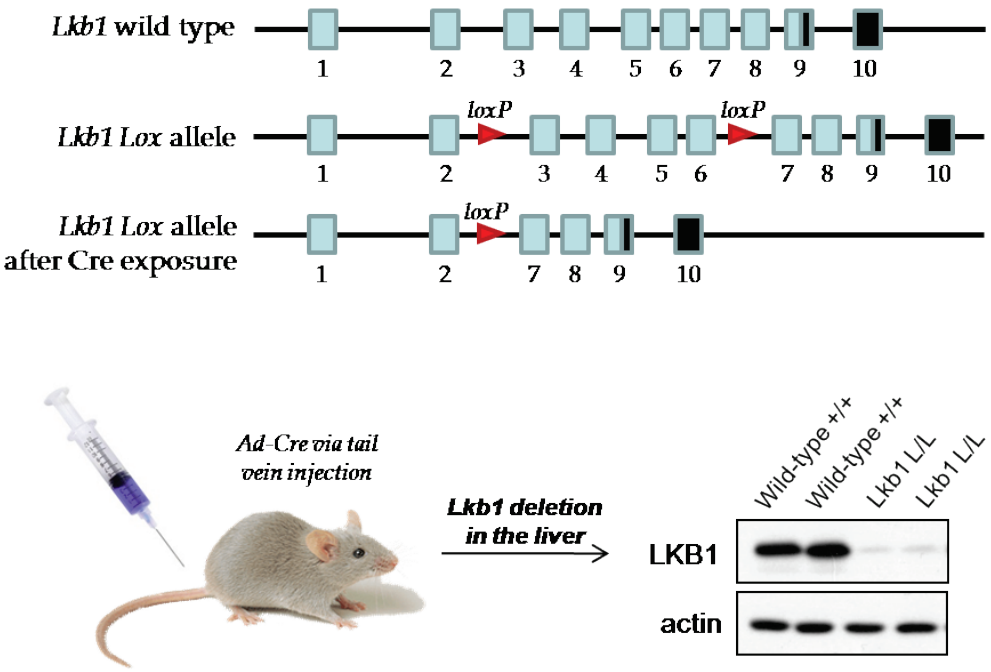


Figure 5. Schematic illustrating Lkb1 targeting strategy. Administration of Cre-recombinase mediates deletion of exons 3 through 6 in the LKB1 gene with 3' untranslated regions are shown in black. The transcript from this allele removes exons 2-6 resulting in a translational frameshift, eliminating the kinase domain of LKB1.

Consistent with previous findings this method of deleting LKB1 is highly effective at abolishing LKB1 protein in the liver, which results in the inactivation of AMPK as seen a decrease in AMPK phospho-Thr^{172} and concomitant lack of phosphorylation of the AMPK downstream substrate Raptor on Ser^{792} (Figure 6a). As loss of LKB1-AMPK signaling is known to de-repress expression of genes involved in gluconeogenesis I further validated loss of LKB1 signaling by examining the expression of two genes involved in gluconeogenesis, glucose-6-phosphatase (G6Pase) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha

(PGC1α). From the microarray results G6Pase and PGC1α were up-regulated approximately 3.2-fold and 2.5-fold, respectively, in the LKB1-deficient livers. PGC1α expression was reexamined using qPCR (Figure 6b).

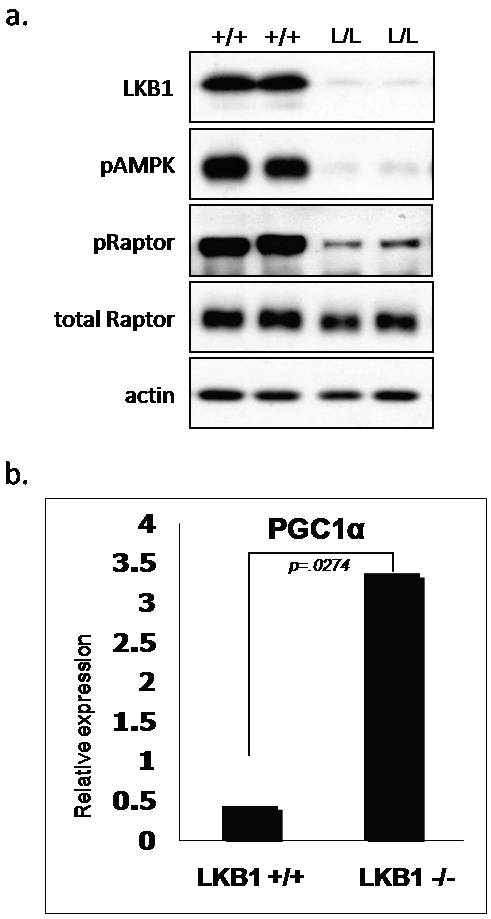


Figure 6. Loss of LKB1 in the liver deregulates AMPK signaling. (a) Total liver lysates of wild type or LKB1 L/L mice. Deletion of Lkb1 leads to a decrease in phosphorylation of AMPK and subsequently of Raptor. (b) Quantitative RT-PCR validating loss LKB1 signaling by derepression of the gluconeogenic gene PGC1α upon disruption of the LKB1-AMPK signaling pathway.

To ascertain what other genes might be regulated by LKB1I ranked the results of the microarray analysis to look at genes highly altered in livers that lack hepatic LKB1 (Table 1). Intriguingly, several phase I xenobiotic metabolism genes, cytochrome P450s (CYPs), were among the most deregulated in LKB1 knockout livers while phase II genes, glutathione-S-transferases (GSTs) were among the most up-regulated. LKB1 disruption greatly increased expression of two different CYPs, Cyp17a1 and Cyp2b9 14-fold and 3.5-fold respectively. These two CYPs have no known function in metabolism of specific xenobiotic compounds; rather Cyp17a1 is involved in the biosynthesis of adrenal and gonadal hormones such as cortisol and testosterone and Cyp2b9 is a glucocorticoid-responsive CYP [82, 83].

20 Most down-regulated genes upon LKB1 loss		Fold change
Mgst1	microsomal glutathione S-transferase 1	0.025
Lcn13	lipocalin 13	0.087
Serpina4-ps1	pseudogene 1	0.091
Hsd3b5	delta-isomerase 5	0.112
Serpina12	serine (or cysteine) peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 12	0.154
Aqp8	aquaporin 8	0.175
Arrdc3	arrestin domain containing 3	0.176
Cyp4a12b	cytochrome P450, family 4, subfamily a, polypeptide 12B	0.205
Mug1	murinoglobulin 1	0.227
Mug2	murinoglobulin 2	0.228
Cyp2c50	cytochrome P450, family 2, subfamily c, polypeptide 50	0.229
Cyp8b1	cytochrome P450, family 8, subfamily b, polypeptide 1	0.235
Cml4	camello-like 4	0.236
Cyp2c37	cytochrome P450, family 2. subfamily c, polypeptide 37	0.237
Ear2	eosinophil-associated, ribonuclease A family, member 2	0.248
Parp12	poly (ADP-ribose) polymerase family, member 12	0.248
Enpp2	ectonucleotide pyrophosphatase/phosphodiesterase 2	0.263
Accn5	amiloride-sensitive cation channel 5, intestinal	0.269
Mup4	major urinary protein 4	0.274
Mup2	major urinary protein 2	0.288

20 Most up-regulated genes upon LKB1 loss		Fold change
Mmd2	monocyte to macrophage differentiation-associated 2	17.981
Nnmt	nicotinamide N-methyltransferase	15.229
Tff3	trefoil factor 3, intestinal	14.169
Cyp17a1	cytochrome P450, family 17, subfamily a, polypeptide 1	14.065
Slpi	secretory leukocyte peptidase inhibitor	13.146
Akr1b7	aldo-keto reductase family 1, member B7	12.758
Egln3	EGL nine homolog 3 (C. elegans)	9.041
Tspan3	tetraspanin 3	7.315
Cth	cystathionase (cystathionine gamma-lyase)	6.732
Ren2	renin 2 tandem duplication of Ren1	6.065
Cenpm	centromere protein M	5.989
Pdrg1	p53 and DNA damage regulated 1	5.471
Robo1	roundabout homolog 1 (Drosophila)	5.235
Ly6d	lymphocyte antigen 6 complex, locus D	5.231
Got1	glutamate oxaloacetate transaminase 1, soluble	5.064
H19	H19 fetal liver mRNA	4.939
Ly6d	lymphocyte antigen 6 complex, locus D	4.881
Gtdc1	glycosyltransferase-like domain containing 1	4.852
Tuba8	tubulin, alpha 8	4.636
Igf2	insulin-like growth factor 2	3.919

Table 1. The top 20 genes whose expression is down- or up-regulated upon hepatic LKB1 loss.

Significantly down-regulated in livers without LKB1 signaling were many CYPs involved in xenobiotic metabolism (Figure 7). For example, Cyp2c37 and Cyp2b10 are involved in metabolism of the non-genotoxic hepatocarcinogen phenobarbitol. Cyp2e1 metabolizes many small organic molecules such as ethanol and carbon tetrachloride while Cyp1a2 is involved in metabolism of polycyclic aromatic hydrocarbons, caffeine, aflatoxin B1, and acetaminophen. It is of particular note that CYPs involved in metabolism of compounds that are known to cause hepatocellular carcinoma in both humans and rodents (such as ethanol and aflatoxin B1) or acute liver failure (such as acetaminophen) are significantly down-regulated in the absence of LKB1.

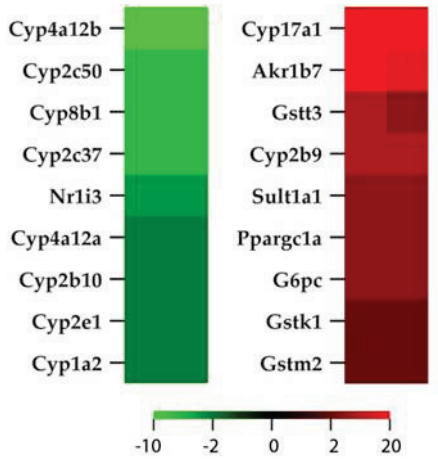


Figure 7. Heat map of deregulated xenobiotic metabolism genes. Transcriptional profiles from parallel harvested livers of ad libitum fed LKB1 L/L or LKB1 +/+ littermate mice were compared. Data illustrates the fold change in mRNA expression of a subset of genes altered in the LKB1-deficient livers as compared to the control LKB1+/+ livers. Genes involved in xenobiotic metabolism are highly deregulated after loss of hepatic LKB1. Many Phase I CYP enzymes are down-regulated while Phase II conjugation enzymes are up-regulated. (Nrli3=CAR)

Based on these findings, I then expanded the array analysis data set to include mice given an acute treatment with carbon tetrachloride (CCl_4) or mineral oil vehicle and AICAR (Figure 8). CCl_4 treatment for 24 hours or AICAR treatment for 6 hours did not robustly alter the expression of the down-regulated CYPs in either +/+ or L/L livers. These treatments did increase expression of several Phase II xenobiotic metabolism genes such as Gstm2 and Gstt3 but only in +/+ livers while L/L livers remained unresponsive. It is surprising to note the divergent expression patterns of Phase I and Phase II enzymes given many of these genes share common mechanisms of transcriptional regulation via specific response elements in their promoter regions [76].

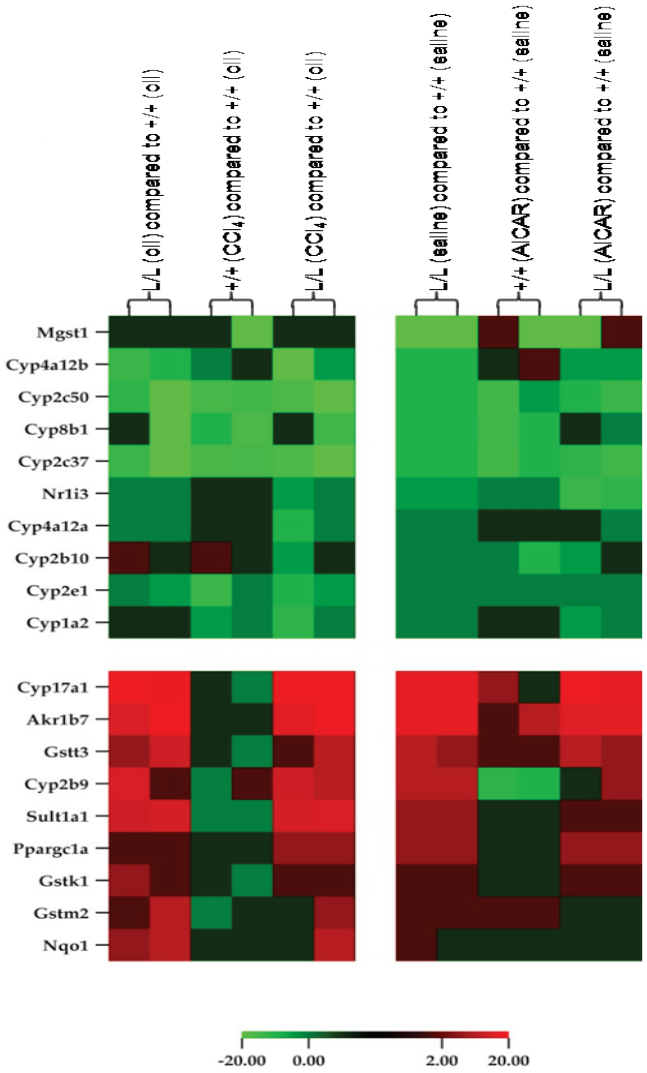


Figure 8. Heat map containing genes up-regulated (red) and down-regulated (green) upon LKB1 loss combined with treatment of the hepatointoxicant CCl_4 or the AMPK-activating chemical AICAR.
Data illustrates the fold change in mRNA expression of a subset of genes altered in the LKB1-deficient livers as compared to the control LKB1+/+ livers treated with CCl_4 or AICAR. Unlike wild-type livers, LKB1-deficient livers are incapable of down-regulating expression of the gluconeogenesis gene PGC1α (Ppargc1a) upon AICAR treatment. CCl_4 treatment results in the down-regulation of many Phase II genes in wild-type, but not L/L, livers.

Alteration of xenobiotic metabolism genes

In order to validate the array results, RNA prepared from these same mice were used in a qPCR analysis of several differentially regulated genes (Figure 9). Indeed, down-regulation of CYPs 2b10, 2c37, and 2c50, and the nuclear hormone receptor Car and up-regulation of Cyp2b9, Gsta1, Gstm2 and PGC-1α (the positive control for loss of LKB1-AMPK signaling) is confirmed using this approach.

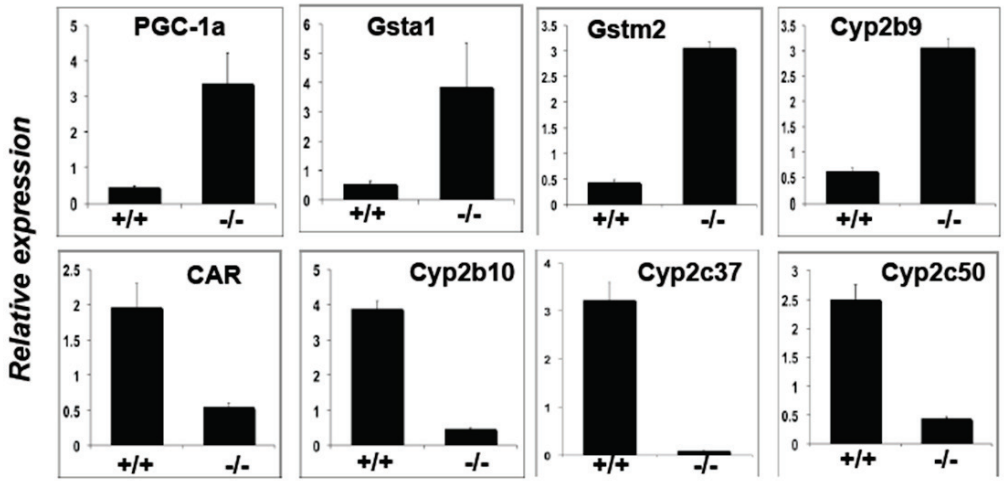


Figure 9. qPCR analysis of xenobiotic metabolism genes.
Quantitative RT-PCR validating mRNA expression of indicated genes in LKB1+/+ or LKB1 L/L livers as in microarray samples in A. PGC1-a is a positive control for previously observed effects of LKB1 deletion in liver. All analyses have a p value of < .05.

In addition to mRNA transcript levels, western blot analysis indicates that protein levels of many of these xenobiotic metabolism genes are deregulated upon LKB1 loss (Figure 10). Consistent with our microarray and qPCR results, mice lacking hepatic LKB1 protein show

reduced levels of CYP2E1, CYP1A1/1A2, and increased expression of POR compared to their wild-type counterparts. POR is the electron donor for all CYP genes and its up-regulation may be an attempt at compensation for CYP protein reductions and thus loss of catalysis of endogenous and exogenous compounds in the L/L livers [84]. Here I also observe the suicide substrate effect of CCl4 on CYP2E1 (lanes 1 and 2 versus lanes 5 and 6).

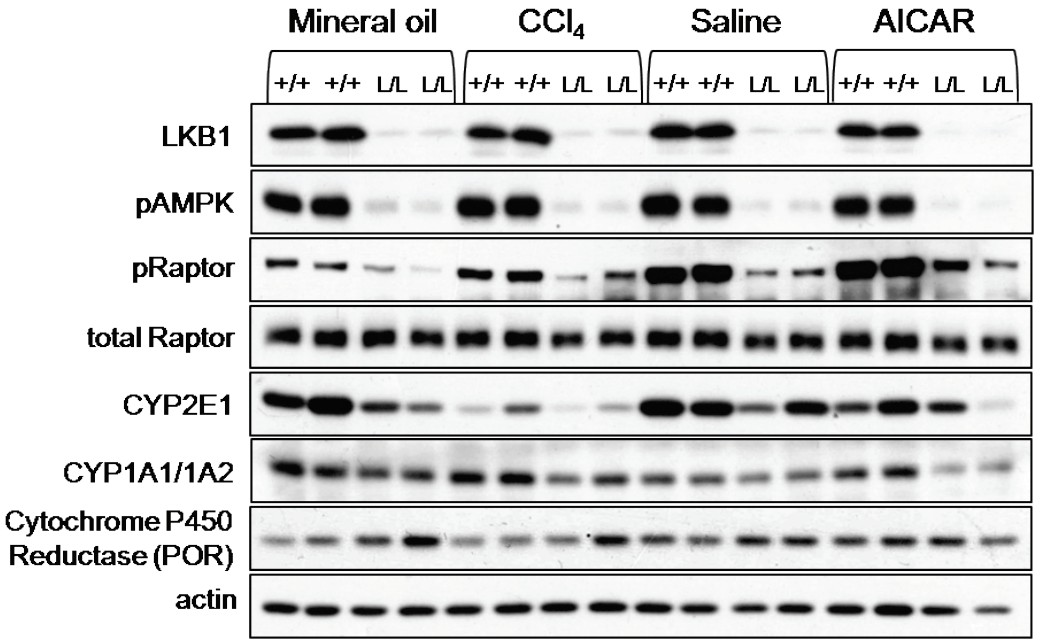


Figure 10. Western blot analysis of selected genes from the microarray analysis. Total cell lysates from livers from ad libitum fed mice of the indicated genotypes were immunoblotted with the indicated antibodies. LKB1 deletion results in reduced phosphorylation of AMPK and its downstream substrate raptor, in addition to near complete loss of CYP2E1 protein. In addition, LKB1 loss leads to decreased CYP1A1/1A2 and increase POR protein.

Gene ontology and pathway analysis

In order to more fully understand the impact of the microarray results, significantly altered genes were subject to further analysis by the online databases the Gene Ontology and the Kyoto Encyclopedia of Genes and Genomes. The Gene Ontology (GO) is a curated program that developed three “vocabularies” or ontologies that describe gene products in terms of associated biological processes, cellular components, and molecular function [85]. The Kyoto Encyclopedia of Genes and Genomes (KEGG) is a database integrating information from 16 other databases into broadly categorized systems information, genomic information, and chemical information. These programs group the thousands of genes whose expression were altered as seen by microarray analysis and group them into pathways or process clusters based on known information about each gene. This approach is useful to visualize broad-spectrum changes within a sample set.

Using GO begets a greater understanding how groups of genes work in concert to alter biological outcomes based not only by expression but by function and location (Table 2). For instance the most common location of CYP enzymes, the endoplasmic reticulum and the microsome, are significantly altered cellular components upon LKB1 loss. In addition, three separate categories of the molecular function oxidoreductase activity are also highly altered in LKB1-deficient livers.

ID	Group name	Count in group	p-value	Ontology
GO:0003824	catalytic activity	112	0.0E+00	molecular function
GO:0030170	pyridoxal phosphate binding	12	0.0E+00	molecular function
GO:0016491	oxidoreductase activity	32	1.0E-10	molecular function
GO:0019842	vitamin binding	14	1.0E-10	molecular function
GO:0016829	lyase activity	14	1.7E-09	molecular function
GO:0004497	monooxygenase activity	13	2.4E-09	molecular function
GO:0048037	cofactor binding	15	8.5E-09	molecular function
GO:0005783	endoplasmic reticulum	29	7.2E-08	cellular component
GO:0019752	carboxylic acid metabolic process	23	1.4E-07	biological process
GO:0006082	organic acid metabolic process	23	1.4E-07	biological process
GO:0003674	molecular_function	211	5.7E-07	molecular function
GO:0005792	microsome	12	7.7E-07	cellular component
GO:0042598	vesicular fraction	12	9.5E-07	cellular component
GO:0006520	amino acid metabolic process	15	1.1E-06	biological process
GO:0005506	iron ion binding	16	1.4E-06	molecular function
GO:0005576	extracellular region	42	1.6E-06	cellular component
GO:0009063	amino acid catabolic process	7	1.6E-06	biological process
GO:0006807	nitrogen compound metabolic process	19	1.8E-06	biological process
GO:0004867	serine-type endopeptidase inhibitor activity	9	1.8E-06	molecular function
GO:0005737	cytoplasm	104	1.8E-06	cellular component
GO:0009055	electron carrier activity	12	2.3E-06	molecular function
GO:0000096	sulfur amino acid metabolic process	5	2.4E-06	biological process
GO:0016616	oxidoreductase activity, CH-OH group of donors, NAD or NADP as acceptor	9	3.0E-06	molecular function
GO:0044444	cytoplasmic part	69	3.2E-06	cellular component
GO:0004857	enzyme inhibitor activity	12	4.5E-06	molecular function

Table 2. Gene Ontology terms highly altered upon LKB1 loss in the liver. Note that many groups contain xenobiotic metabolism genes; such as the molecular function “catalytic activity”, the cellular components “endoplasmic reticulum” and “microsome”, and the biological process “organic acid metabolic process”.

KEGG pathway analysis perhaps more clearly illustrates the differences between wild-type and LKB1-deficient livers (Table 3). Independent of any treatment, L/L livers consistently have “Metabolism of xenobiotics by cytochrome P450” ranked within the top three deregulated pathways. Frequently, pathways such as arachidonic and lineolic acid metabolism are also deregulated. These three pathways cross-talk using several CYP family members from the xenobiotic metabolism pathway and therefore are also expected to be highly deregulated.

Comparison	KEGG ID	Term	Count in class	Count in KEGG	p-value
L/L to +/+	mmu00970	Aminoacyl-tRNA biosynthesis	11	44	3.19E-07
	mmu00980	Metabolism of xenobiotics by CYP P450	8	76	1.80E-06
	mmu00590	Arachidonic acid metabolism	8	88	2.59E-06
	mmu00910	Nitrogen metabolism	6	23	4.70E-06
	mmu00272	Cysteine metabolism	5	64	7.41E-06
+/+ oil to +/+ CCl4	mmu03010	Ribosome	18	132	0.00E+00
	mmu00980	Metabolism of xenobiotics by CYP P450	11	76	7.28E-07
	mmu00240	Pyrimidine metabolism	12	104	6.70E-06
	mmu00590	Arachidonic acid metabolism	10	88	8.88E-06
	mmu00591	Linoleic acid metabolism	8	45	1.56E-04
L/L CCl4 to +/+ oil	mmu00980	Metabolism of xenobiotics by CYP P450	13	76	4.00E-10
	mmu00970	Aminoacyl-tRNA biosynthesis	13	44	5.21E-07
	mmu00720	Reductive carboxylate cycle (CO2 fixation)	6	33	2.34E-06
	mmu00591	Linoleic acid metabolism	9	45	2.84E-06
	mmu04612	Antigen processing and presentation	10	104	3.74E-06
+/+ AICAR to +/+ saline	mmu00980	Metabolism of xenobiotics by CYP P450	10	76	1.77E-08
	mmu00591	Linoleic acid metabolism	7	45	1.88E-05
	mmu00590	Arachidonic acid metabolism	7	88	4.00E-05
	mmu00040	Pentose & glucuronate interconversions	5	26	5.29E-05
	map00500	Starch and sucrose metabolism	6	77	1.21E-04
L/L AICAR to +/+ saline	map00100	Biosynthesis of steroids	13	28	1.79E-08
	mmu00980	Metabolism of xenobiotics by CYP P450	17	76	1.13E-06
	mmu00970	Aminoacyl-tRNA biosynthesis	22	44	1.18E-05
	map00600	Sphingolipid metabolism	12	40	3.51E-05
	map04910	Insulin signaling pathway	22	77	7.05E-05

Table 3. KEGG pathways highly altered upon LKB1 loss. KEGG analysis of microarray data showing pathways deregulated upon loss of hepatic LKB1 signaling. Loss of LKB1 in the liver with or without additional CCl4 treatment highly deregulates the pathway termed "Metabolism of xenobiotics by cytochrome P450s". Treatment of +/+ animals with CCl4 also alters this pathway.

Interestingly, +/+ livers treated with AICAR also display a deregulated xenobiotic metabolism pathway. Selected features within

this group include CYP genes that are known targets of the constitutive androstane receptor, a nuclear receptor involved in transcription of some Phase I, II, and III genes. Wild-type livers are able to appropriately respond to AICAR treatment by altering expression of genes involved in metabolism of sugars; particularly in the sucrose and pentose metabolism pathways. L/L livers are unable to respond in this manner due to lack of intact AMPK signaling.

LKB1-deficient livers have attenuated response to carcinogen treatment

In order to determine what biological outcome deregulation of the cytochrome P450 genes may have I used two known hepatotoxic chemicals, diethylnitrosamine (DEN) and carbon tetrachloride (CCl_4) in wild-type and $LKB1^{lox/lox}$ mice as the first step to understanding what impact loss of these CYPs had on the mouse liver. DEN requires activation through primarily through CYP2E- and some CYP2A6-catalyzed α-hydroxylation, forming reactive metabolites that alkylate DNA [86]. CCl_4 is a suicide substrate of CYP2E1, being first activated to the trichloromethyl radical which destroys the heme group of CYP2E1 and then reacts with molecular oxygen to form the trichloromethylperoxyl radical; both radicals lead to lipid peroxidation or attack on other cellular macromolecules [87, 88].

Consistent with previous studies LKB1 deletion in the liver leads to loss of AMPK activity, concomitant decreased phospho-Raptor and increased mTORC1 activity as seen by an increase phosphorylated

4EBP1 (Figure 11) [52]. In vehicle-treated animals it is immediately apparent that there is a suppression of basal DNA-damage response signaling as seen by the reduction in activated p53 phospho-Ser^{18} (Figure 11, lanes 1 versus 2). DEN treatment dramatically increased activated p53, while CCl_4 treatment subtly increased activated p53. Expression of p21, a p53 target gene, is suppressed under basal conditions in L/L animals but at this timepoint following chemical intoxication there is no increase in p21 protein levels in either DEN or CCl_4-treated livers. There is precedent for p53-independent induction of p21 in situations including normal tissue development and cellular differentiation [89]. Interestingly, p21 can still be induced in p53-null mice following liver intoxication with CCl_4 and the authors of that study conclude that the transcription factor C/EBPα may be controlling it's expression [90].

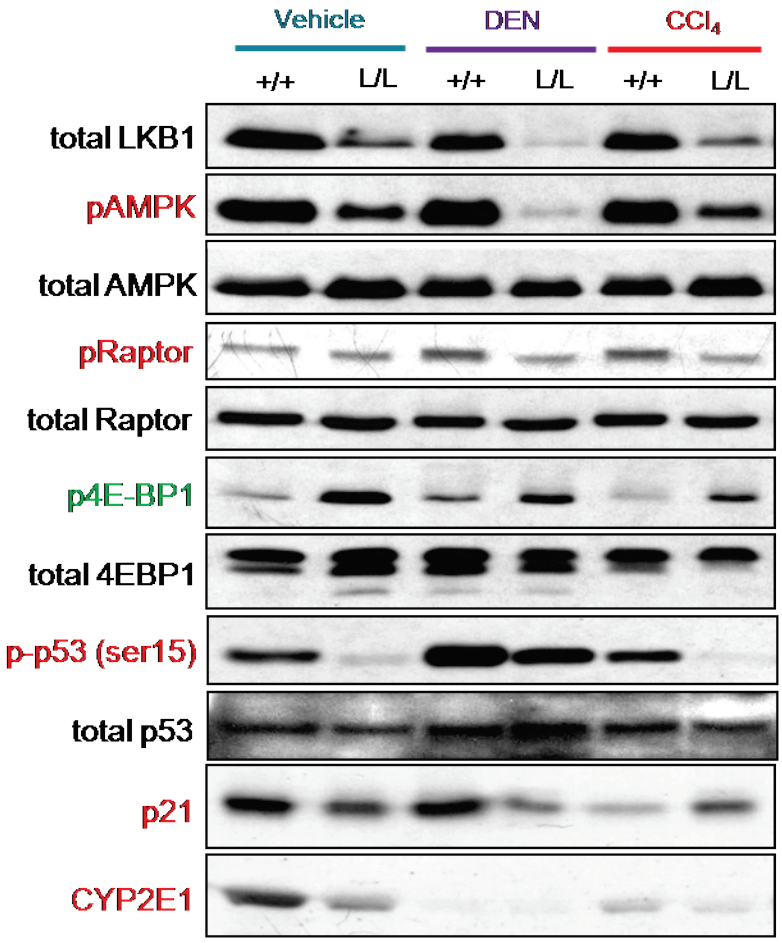


Figure 11. Signaling in mice treated acutely with hepatocarcinogens. Total liver cell lysates harvested from mice 48 hours after treatment with the indicated compounds. Mice lacking hepatic LKB1 have decreased phosphorylation of AMPK and Raptor (red) and concomitant increased mTORC1 signaling as seen by increased phosphorylated 4E-BP1 (green). Signaling through active p53 is also basally reduced in L/L livers and attenuated after carcinogen treatment. Protein levels of CYP2E1, the enzyme largely responsible for metabolism of these two carcinogens, is decreased in L/L livers.

In this acute treatment, protein levels of CYP2E1 are basally reduced in vehicle-treated L/L livers and protein expression is vastly decreased upon treatment with DEN or CCl_4. CCl_4 is a known suicide substrate of CYP2E1 causing heme group destruction or electrophilic protein damage, resulting in increased ubiquitin-conjugation and elimination of the damaged protein [91]. Interestingly, from this data it also appears that DEN or one its metabolites may be a suicide substrate of CYP2E1 as well.

Decreased DNA-damage response signaling is not due to loss of AMPK-mediated phosphorylation of p53

It was recently reported that phosphorylation of the tumor suppressor p53 was increased by activated AMPK [92]. Jones *et al* found that AMPK activation induces phosphorylation of p53 on Ser^{15} and mediated an AMPK-dependent cell cycle checkpoint upon glucose deprivation. In order to gauge the contribution of genotoxic and cytotoxic chemical-induced damage compared to loss of AMPK signaling I probed these livers for activation of the DNA damage response pathway upstream of p53 (Figure 12a). In addition to reduced p53 phospho-Ser15, LKB1-deficient livers also display decreased activated Chk1, Chk2, and Rad17. These results indicate that the reduced active p53 phospho-Ser15 is not due to defective AMPK signaling but rather that the damage response pathway as a whole is suppressed in L/L livers.

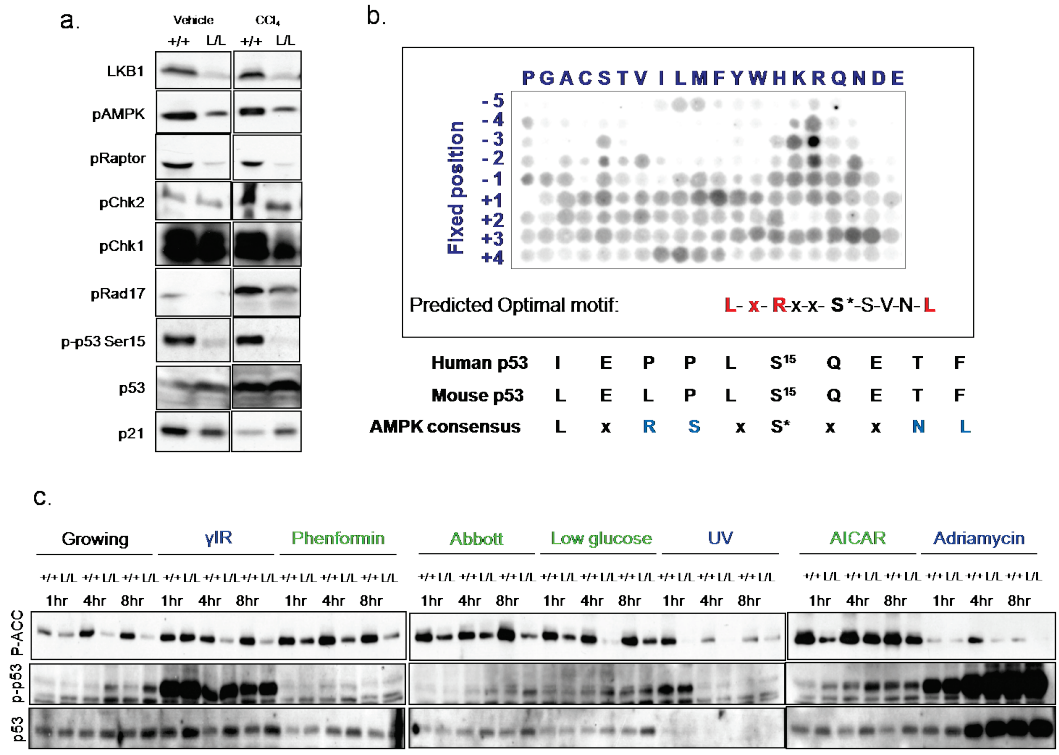


Figure 12. DNA damage response and AMPK. (a) Many members of the DNA damage response pathway are basally suppressed in livers with LKB1 loss and CCl_4 intoxication does not activate the DNA damage response pathway in L/L livers. (b) The AMPK predicted optimal consensus motif does not match the sequence surrounding Ser15 of p53. (c) Loss of LKB1 in MEFs does not alter p53 activation in response to DNA-damaging treatments (blue) but increases phosphorylated p53 after AMPK-activating treatments (green).

In addition, by using the same bioinformatic approach that identified both LKB1 as the upstream kinase of AMPK and AMPK as the upstream kinase of Raptor, I was able align the preferred amino acid sequence for AMPK phosphorylation with the residues surrounding serine 15 of mouse p53 (Figure 12b) [23, 40]. Based on that alignment (and similar

alignments for other known phosphorylated serines in the p53 protein, data not shown) it is unlikely that p53 is a direct target of AMPK phosphorylation.

In order to more clearly define the role of LKB1-AMPK signaling in p53 activation, I used primary non-immortalized MEFs either wild-type or deleted for LKB1 and subjected them to a variety of DNA-damaging and AMPK-activating stimuli (Figure 12c). There is no genotype-specific response to any of the DNA-damaging treatments: gamma irradiation, ultraviolet irradiation, or adriamycin. There is a small but noticeable increase in phospho-p53 in L/L cells over +/+ cells at 4 hours and 8 hours of glucose deprivation and at all timepoints in during AICAR treatment. This result is perhaps expected based on previous studies indicating that LKB1 L/L MEFs undergo apoptosis when deprived of glucose and that AMPK-deficient MEFs show increased levels of basal phosphorylation of p53 at serine 15 [52, 93].

Signaling through p53 may be active in untreated +/+ livers due to the lack of exogenous substrates (due in part to the extremely well-defined rodent chow) for members of the CYP family, particularly CYP2E1. When exogenous substrates for CYP2E1 are not present within the liver, CYP2E1 switches its “substrate load” to endogenous fatty acids causing lipid peroxidation and establishing a low level of oxidative stress within the hepatocytes [94]. I assessed total protein oxidation levels in +/+ and L/L livers and discovered that livers lacking hepatic LKB1 contained less oxidized proteins than their wild-type counterparts, perhaps explaining the basal suppression of p53 signaling in L/L livers (Figure 13).

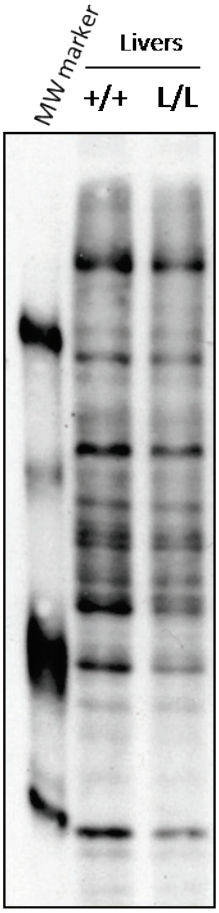


Figure 13. Total protein oxidation in wild-type and LKB1-deficient livers. . Loss of LKB1 leads to a reduction CYP2E1 enzyme and subsequently a lower amount of total protein oxidation in untreated livers.

Hepatocyte response to acute carcinogen treatment

To confirm the biological output of reduced metabolism of DEN and CCl_4, I performed immunohistochemistry (IHC) on liver sections from each genotype and treatment. Untreated animals of both genotypes have little apoptotic or necrotic hepatocytes as seen by terminal deoxyribonucleotidyl transferse (TdT)-mediated biotin-16-dUTP nick-end labelling (TUNEL). DEN and CCl_4-treated +/+ animals have increased TUNEL positive nuclei while L/L livers have far fewer positively stained cells (Figure 14i-vi). As seen by Ki67 positive nuclei, the proliferative index of L/L livers is markedly reduced compared to +/+ livers treated with DEN or CCl_4 (Figure 14vii-xii). To determine if L/L livers were shunted into senescence rather than apoptosis after treatment with hepatointoxicants, I performed staining for senescence-associated β-galactosidase positive cells. Livers lacking hepatic LKB1 are overall less senescent than their wild-type counterparts regardless of treatment. DEN and CCl_4 treatment increases senescence-associated β-galactosidase positive areas in +/+ livers (Figure 15a). Interestingly, the biliary epithelia in +/+ livers is more proliferative than that of L/L livers after CCl_4 treatment (Figure 15b). Taken together, this data indicates that L/L livers show fewer signs of toxicity and undergo little proliferation when challenged with an acute dose of carcinogens.

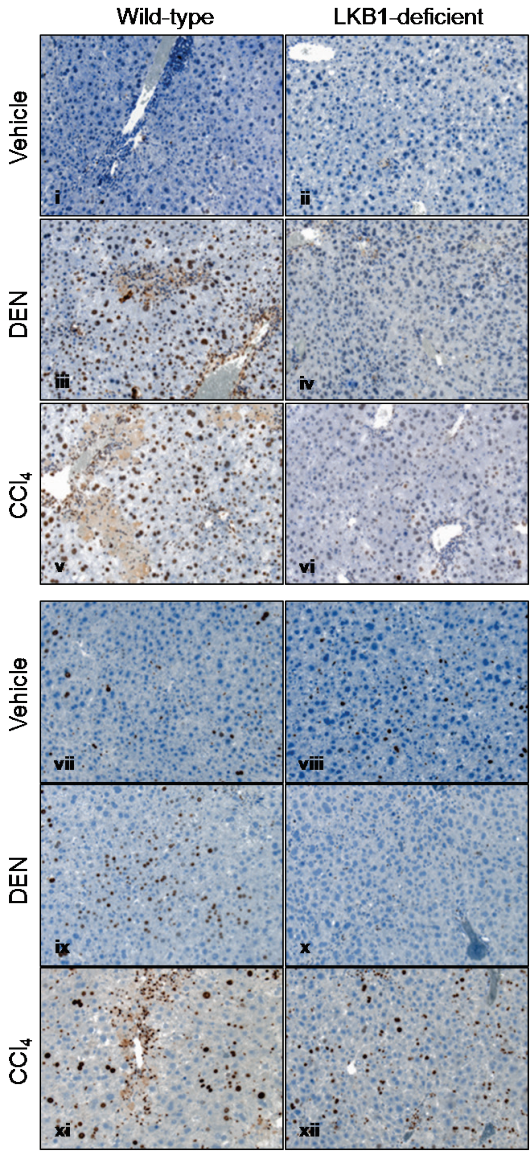


Figure 14. LKB1-deficient livers have reduced apoptosis and subsequent proliferation after carcinogen treatment. (i-vi) There are very few TUNEL positive cells in either genotype without carcinogen treatments. After DEN or CCl_4 exposure, wild-type livers readily undergo apoptosis while LKB1-deficient livers do not. Similarly (vii-xii) carcinogen treatment increases Ki67-positive cells in +/+ but not L/L animals. Note that the most positively-scoring areas for both assays localize around bile ducts and blood vessels.

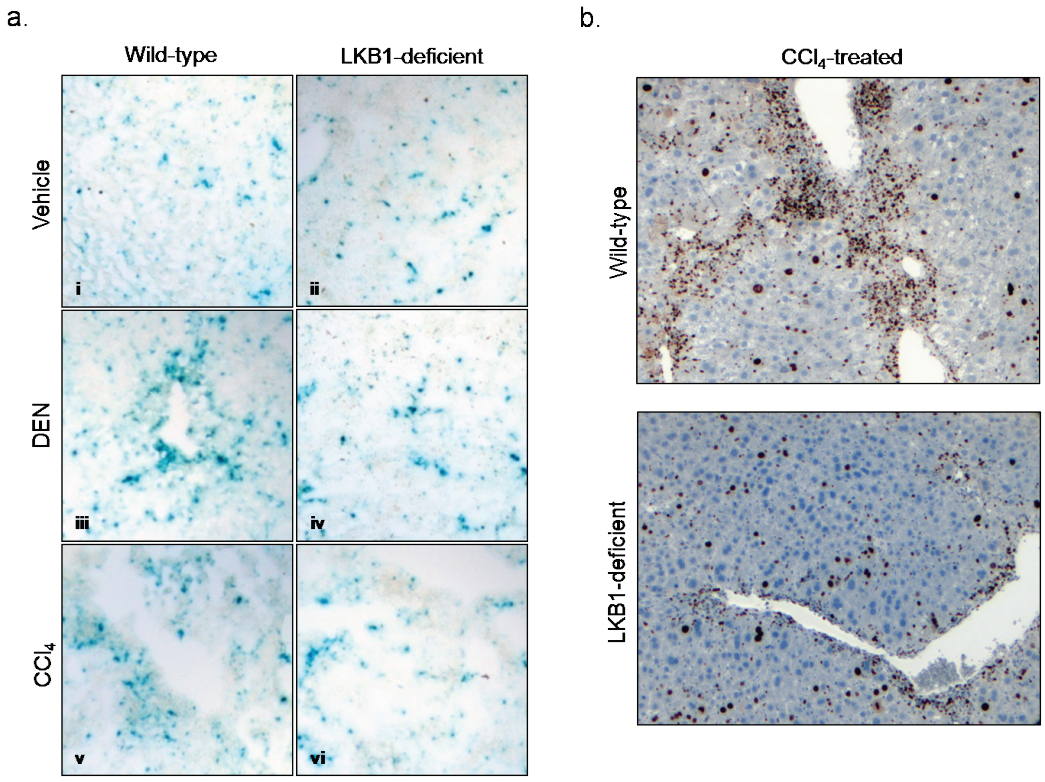


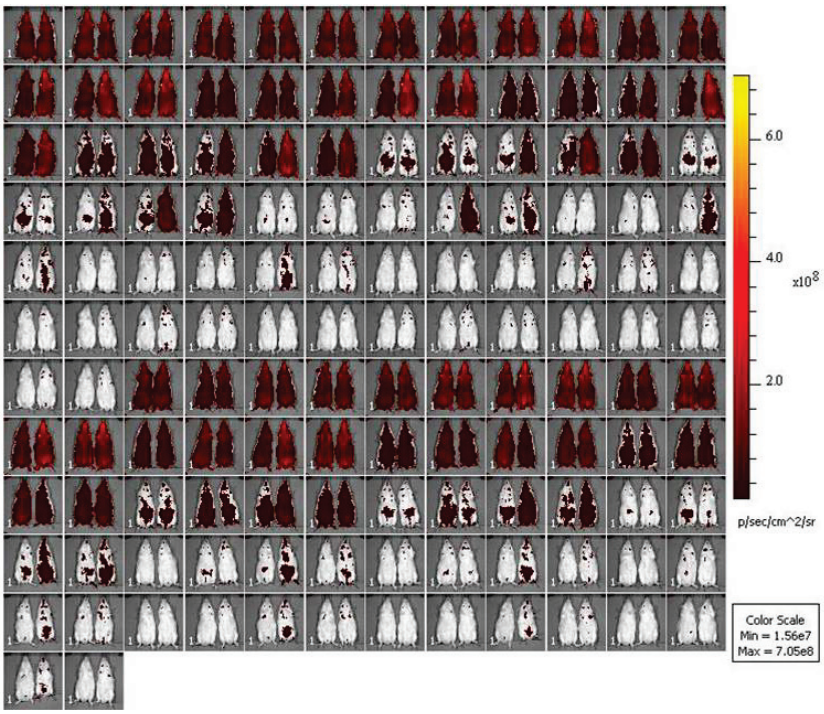
Figure 15. Loss of LKB1 does not promote a senescent phenotype. CCl_4 causes biliary epithelial cell proliferation. (a) Reduced senescence-associated β-galactosidase positive areas in LKB1-deficient livers treated with DEN or CCl_4 than their wild-type counterparts. Note that greatest concentration of senescent-positive area in located near blood vessels and bile ducts. (b) Dramatically increased biliary epithelial cell proliferation after CCl_4 intoxicant in +/+ but not L/L livers.

LKB1-deficient livers display delayed uptake and excretion of indocyanine green.

In order to determine if LKB1-deficient livers have decreased capacity for transport of small molecules across the hepatocyte membrane, ingress or egress, I treated +/+ and L/L mice with a single

bolus of indocyanine green (ICG) via tail vein injection. ICG is a widely used diagnostic tool for measuring hepatic blood flow and function. ICG is taken up from the blood stream exclusively by the hepatocytes and is excreted through the bile unmetabolized [95]. As LKB1 deletion occurred in adult mice whose liver architecture is already fully formed there should be no genotypic differences in hepatic blood flow, therefore any differences in dye output should be solely due to uptake and excretion by the hepatocytes. ICG fluoresces in the near-infrared at 805nm therefore a live-animal imaging system capable of detecting that wavelength is ideally suited to track dye clearance.

Five mice per genotype were given an ICG injection and then imaged repeatedly over a 6 day period. Mice lacking hepatic LKB1 display a delayed maximal dye uptake and clearance from these animals are also delayed, suggesting that both dye uptake and dye elimination are affected by LKB1 loss (Figure 16a, b). Interestingly, ICG ingress is dependent on the Oatp1 solute transport carrier and expression of Oatp1 is down-regulated in L/L livers (data not shown). Both genotypes exhibit ICG clearance within the 6 day window of observation. This suggests that uptake and elimination of small molecules may be delayed but is not abolished upon loss of LKB1 in the liver and likely has minimal impact on chemically-induced toxicity.



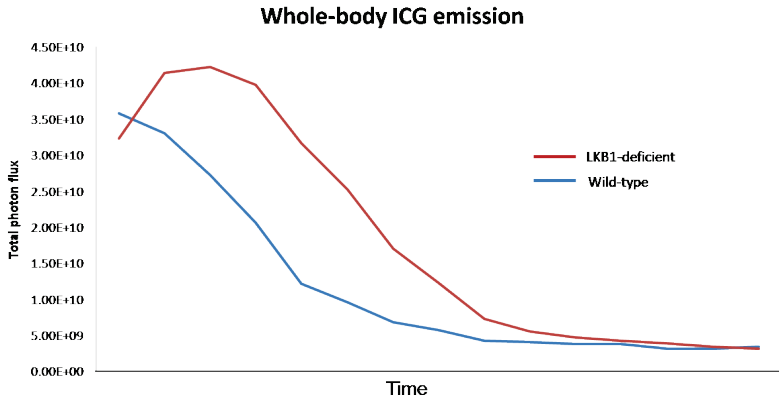


Figure 16. Indocyanine green clearance assay. Mice were imaged repeatedly during a period of 6 days after injection with dye. Clearance was monitored by decreasing fluorescent emission of ICG. Mice lacking hepatic LKB1 reach maximal dye uptake more slowly than wild-type mice and the dye concentration remains higher than wild-type mice for longer although the two genotypes reach full dye clearance by 6 days.

Discussion

This study has provided conclusive evidence that LKB1 is a regulator of xenobiotic metabolism in the liver. I show that many Phase I xenobiotic metabolism genes, the cytochrome P450 family, are significantly down-regulated upon LKB1 loss while many Phase II xenobiotic metabolism genes, such as glutathione-S-transferases, are up-regulated. It is intriguing that loss of LKB1 is able to uncouple transcriptional regulation of these two phases, as many of these genes share common mechanisms of transcriptional regulation via specific response elements in their promoter regions. It is possible that both of these changes increase the resistance of L/L livers to DEN and CCl_4; less toxic metabolite intermediates would be produced as there is less CYP2E1 enzyme to catalyze the reaction, while the increase in GSTs would hasten conjugation and elimination of any intermediates that get produced from the residual CYP2E1 in LKB1-deficient livers. Implications of lowered toxic or reactive intermediate formation caused by loss of CYP enzymes could have a striking influence on cancer risk, while and increased antioxidant response through increased GST expression could attenuate age-associated diseases such as Parkinson's or Alzheimer's disease.

Here I have also shown that AMPK likely does not directly phosphorylate p53 as there is no genotype-specific response to a variety of DNA-damaging stresses in +/+ versus L/L MEFs and thus does not contribute to the lack of p53 activation seen in L/L livers. Indeed, the entire DNA-damage response pathway is suppressed in animals lacking hepatic LKB1. Functionally, loss of CYP2E1 expression appears to

ameliorate the genotoxic and cytotoxic effects of DEN and CCl_4. L/L livers do not senesce or undergo apoptosis after chemical intoxication and there is no hepatocyte proliferation that would normally be seen after such an insult.

CHAPTER 3: AMPK activation changes xenobiotic expression profile

AMPK mediates expression of CAR target and non-target genes.

A small body of literature suggests that AMPK may mediate the activation of the constitutive androstane receptor (CAR), a nuclear hormone receptor responsible for transcriptional activation of several CYPs. Phenobarbitol treatment, an indirect CAR agonist, in hepatoma cell lines triggers a physical association of LKB1 and AMPK and subsequent increase in AMPK activity and decrease in expression of gluconeogenic enzymes such as G6Pase and PEPCK1 [96].

Human hepatoma cell lines can be forced into re-expressing many hepatocyte specific genes by metabolic selection on fructose-containing media. In such cells metformin, AICAR, and phenobarbitol all increase AMPK activity and induce transcription of the CAR target genes Cyp2b10 and Cyp3a11 [97]. Phenobarbitol and TCPOBOP (a direct CAR agonist) treatment in the AMPK DKO mice blunts induction of the two CYPs compared to wild-type animals [98]. AMPK activation by AICAR treatment causes an accumulation of nuclear CAR but translocation of CAR is not absolutely dependent on AMPK activity as the AMPK DKO mice have relatively normal CAR translocation upon phenobarbitol treatment. Our lab, using a bioinformatic approach to identify AMPK substrates, does not find that any sequence in CAR matches the AMPK recognition motif (data not shown), suggesting that AMPK probably does not directly

phosphorylate CAR. When these salient features from previous work are taken together it appears that there is cross-talk between AMPK metabolic signaling and CAR transcriptional activation but that the connection is indirect at best.

Consistent with these reports, our transcriptional profiling identified the aforementioned classic CAR target gene Cyp2b10 as being an LKB1-dependent transcript in liver. However, our findings suggest that LKB1-AMPK signaling controls xenobiotic response beyond activation of CAR alone. Two of the other CYPs also diminished in LKB1-deficient livers are Cyp2e1 and Cyp2c50, which have been previously shown to not be CAR target genes [99-101]. Cyp2e1 is ubiquitously expressed at high levels in hepatocytes and only recently have mechanisms of transcriptional control been identified. Cyp2e1 has probable response elements in the promoter region for HNF1α and β-catenin but does not appear to contain any known CAR response elements [102-104]. Perhaps even more convincingly, CAR knockout mice have normal expression of Cyp2e1 mRNA and protein as seen by northern blots, qPCR analysis, and western blots [99, 105].

I hypothesized that if loss of LKB1-AMPK signaling led to a repression of CYP gene expression that pharmacological activation of AMPK may increase their expression in animals and primary hepatocytes. Indeed, wild-type primary hepatocytes treated with either AICAR or metformin increased CYP2E1 protein abundance over untreated control hepatocytes (Figure 17). Indirect activation of CAR through treatment with phenobarbitol or direct activation using TCPOBOP caused a very slight increase in AMPK phosphorylation but there was no increase in phosphorylation of the AMPK substrate Raptor, indicating that AMPK-

mediated repression of the mTORC1 pathway is not increased with these treatments. Phenobarbitol or TCPOBOP did not increase CYP2E1 protein abundance, further confirming that CYP2E1 is not a CAR target gene.

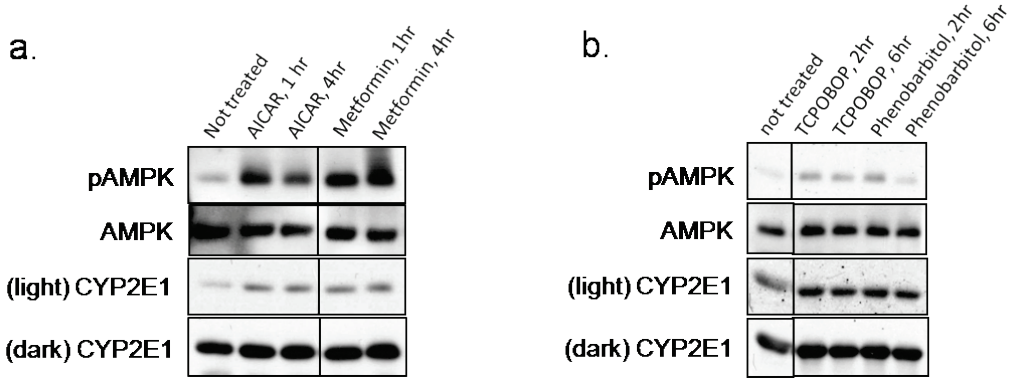


Figure 17. CYP2E1 expression and pharmacological activators. (a) The AMPK-activating drugs AICAR and metformin increase CYP2E1 protein levels in wild-type primary hepatocyte culture. (b) The direct CAR agonist TCPOBOP and the indirect CAR agonist phenobarbitol do not induce CYP2E1 in wild-type primary hepatocyte culture.

Next, qPCR analysis was performed in order to further characterize Cyp2e1 expression changes upon AMPK activation with AICAR and metformin or CAR activation with phenobarbitol and TCPOBOP (Figure 18).

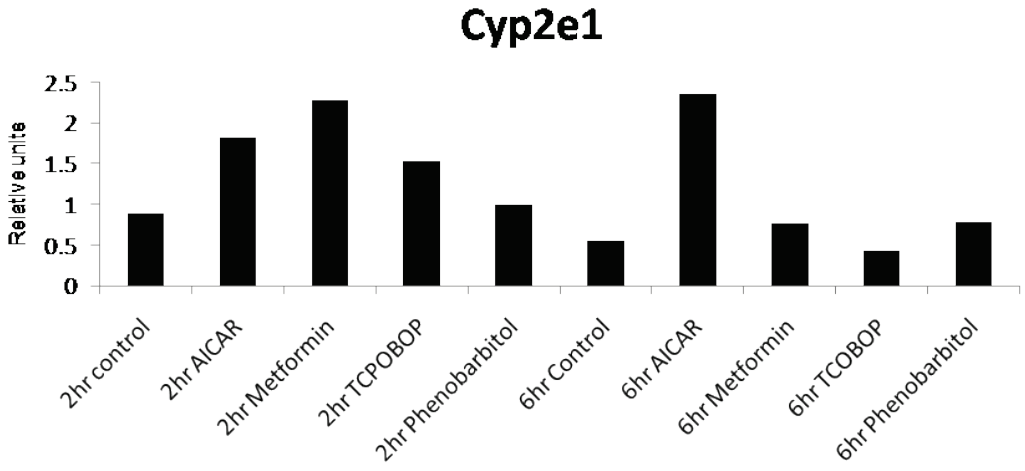


Figure 18. Cyp2e1 expression profiling after pharmacological activation of AMPK or CAR. Quantitative RT-PCR analysis of wild type primary hepatocytes treated with AICAR and metformin significantly increase Cyp2e1 mRNA levels while CAR activation through TCPOBOP and phenobarbitol do not. Note that increased metformin-dependent transcription is transient.

Fasting does not increase CYP2E1 in the mouse liver

Dietary restriction to 50% of normal food calories and fasting conditions in rats increase liver Cyp2e1 expression and potentiates hepatotoxicity with CCl_4 [106, 107]. However, during overnight fasting, AMP concentrations rise in adipose tissue but not muscle or liver and consequently there is AMPK activation only in adipose tissue [43]. Long-term calorie restriction in rats has been shown to decrease both active,

phosphorylated AMPK and total AMPK protein; perhaps as compensation for constitutively active low-nutrient signaling [44].

Because nearly all of the previous studies were performed in rats, I used liver lysates from fasted mice to determine what, if any, effect fasting had on AMPK activation and CYP2E1 protein level in the murine liver. Similar to the results found in rats, an 18-hour fast did not increase the phosphorylation of AMPK or the downstream target Raptor, indicating that AMPK activity is not induced by fasting (Figure 19). Loss of LKB1 in the liver further suppressed AMPK activation and fasting did not increase AMPK phosphorylation in these animals. Unlike the previous studies, fasting in mice does not increase CYP2E1 expression. Fasting (for 18 hours or less) and starvation (for 1 to 3 days) may potentiate different metabolic signaling; whether starvation in mice increases CYP2E1 is unknown.

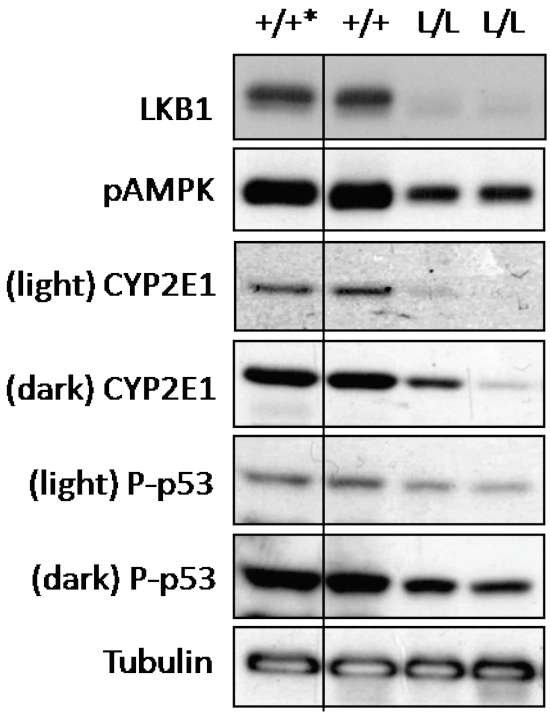


Figure 19. Fasting in mice does not induce CYP2E1. Total liver lysates from wild-type fed ad libitum (asterisk) and wild-type fasted animals show no activation of AMPK or induction of CYP2E1 after an 18hr fast. Fasting does not increase CYP2E1 in L/L livers.

To more directly examine whether acute AMPK activation was sufficient to promote CYP2E1 increase at the protein level, I used intraperitoneal injection of AICAR to induce AMPK activation in the liver. In mice with intact LKB1 signaling but not mice lacking hepatic LKB1, a single dose AICAR is capable of increasing AMPK-mediated phosphorylation of Raptor confirming activation of AMPK. In this experiment, CYP2E1 protein abundance increases 3 hours after treatment (Figure 20).

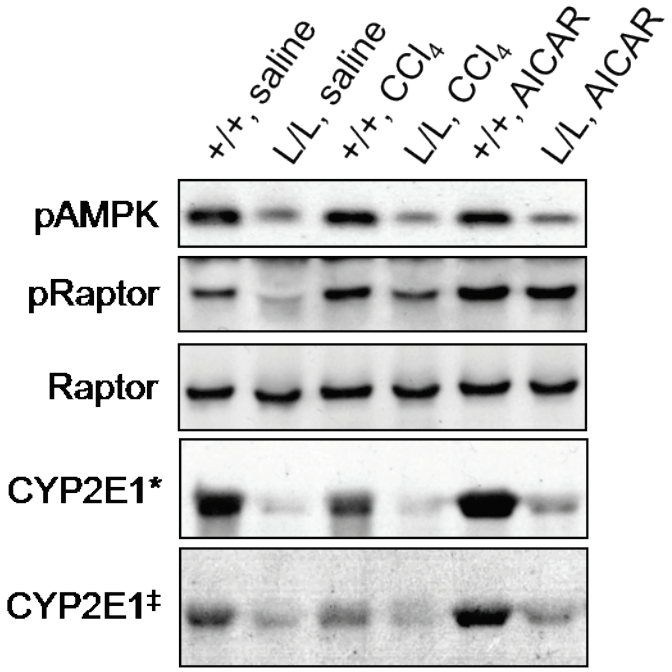


Figure 20. Brief AICAR treatment induces a burst of CYP2E1 expression. Total liver lysates from mice of the indicated genotype were sacrificed 3 hours after a single injection of AICAR display an increase in CYP2E1 protein level. CYP2E1 was detected by two antibodies: Abcam (*) and Millipore (‡).

Pre-treatment with metformin and AICAR before hepatointoxication

Because a brief AICAR treatment in wild-type mice caused an increase in CYP2E1 protein, I next tested whether a daily treatment regimen with AMPK activating drugs would cause an increase in CYP enzymes and potentiate response to a single, acute dose of CCl_4. By restricting the administration of metformin or AICAR to once daily, this

model reflects a dosage schedule that would be suitable for use in humans. Mice were given saline, metformin, or AICAR daily at 9am for one week, at 5pm on the 7th day mice were either given vehicle (oil) or CCl_4. On the morning of the 8th day mice were given a final dose of saline, metformin or AICAR. Mice were sacrificed 24 hours after administration of oil or CCl_4, which is 8 hours after the last dose of AMPK-activating drugs (Figure 21).

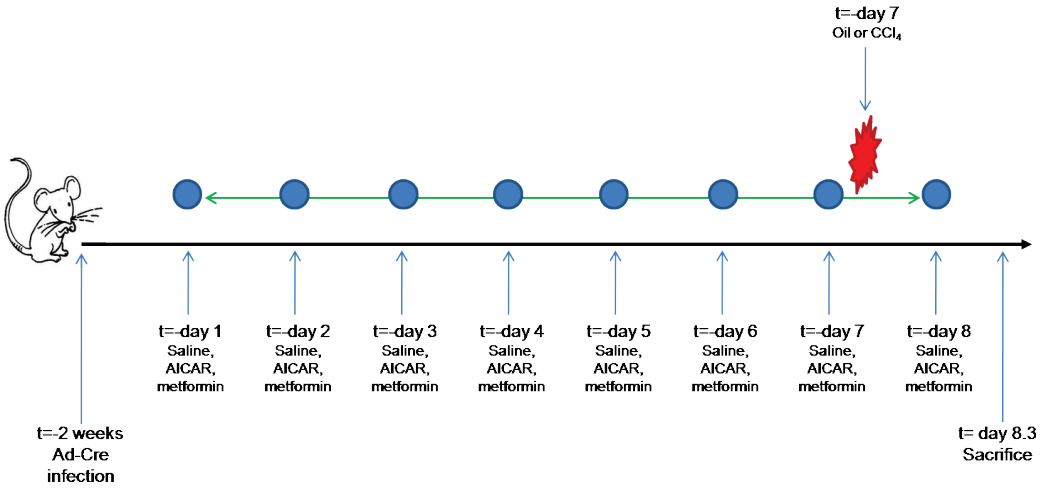


Figure 21. Schematic illustration of the pre-treatment experiment. Mice either wild type or LKB1 L/L are given once daily injections of saline, metformin, or AICAR for one week before being challenged with CCl_4.

In wild-type oil-treated mice this treatment regimen did not alter basal levels of CYP2E1 expression (Figure 22). There was no increase in L/L livers of CYP2E1 enzyme after daily treatment with metformin or AICAR. After CCl_4 intoxication, +/+ livers display a striking decrease in CYP2E1, again due to the suicide substrate effect of CCl_4 on the enzyme. Saline treatment did not alter the previously seen phenotype of basal suppression of phosphorylated p53 in L/L livers, while metformin and

AICAR both increased phosphorylated p53 in L/L oil-treated livers; approaching that seen in +/+ oil-treated livers. The basis for this increase in p53 phosphorylation is unknown. Upon intoxication with CCl_4, there is a slight increase in phosphorylated p53 in metformin-treated +/+ animals, suggesting that there may be a synergistic effect of combined treatments, even if there are no changes in CYP2E1 protein abundance at this timepoint.

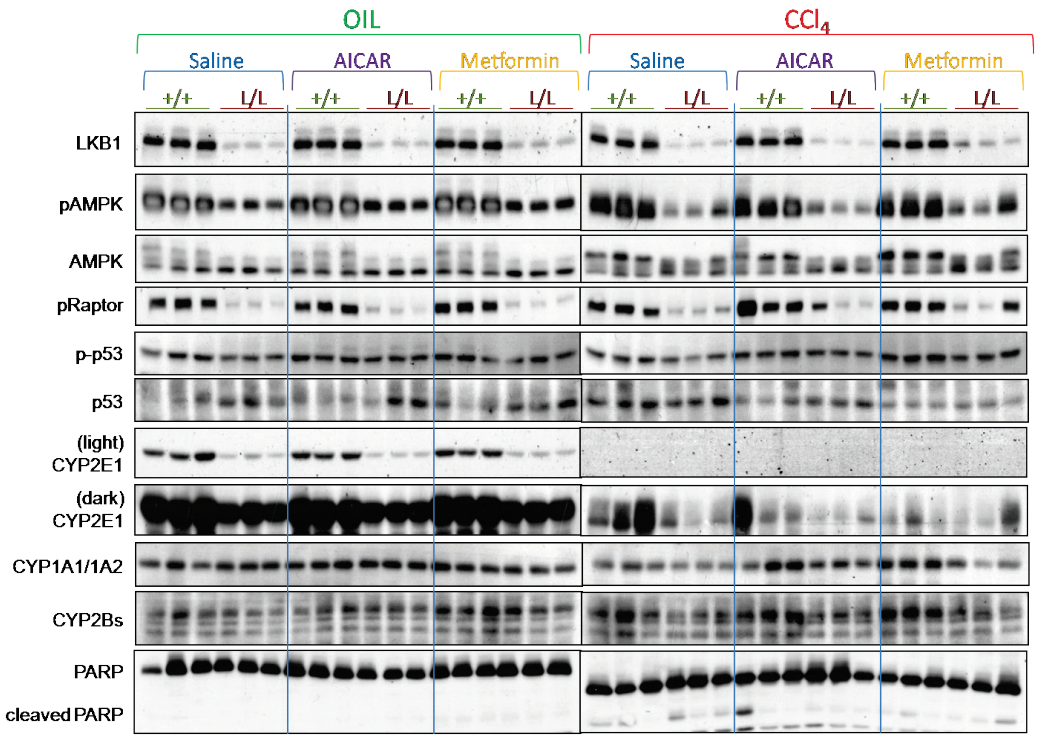


Figure 22. Results from the pre-treatment experiment. Total liver lysates from mice of the indicated genotype and treatment. Daily doses of metformin or AICAR do not increase basal CYP2E1 expression or significantly alter expression of CYP1A1/1A1 or CYP2B enzymes. After CCl_4 intoxication, there is a slight increase in phosphorylated p53 in metformin treated animals, suggesting that metformin and CCl_4 synergize to promote the damage response. Both metformin and AICAR treatment stabilize CYP1A/1A2 protein levels in +/+, but not L/L, animals following CCl_4 treatment.

Metformin pretreatment caused a very slight increase in the amount of CYP2B enzymes, perhaps due to CAR activation as mentioned in previous work. Interestingly, the genotypic differences in CYP2B expression become most apparent after intoxication with CCl_4. Wild-type CCl_4-treated mice have an increased amount of CYP2Bs while L/L livers lack the induction of these enzymes. Other groups have shown that CAR knockout mice are slightly resistant to CCl_4-mediated toxicity, and CCl_4 administration in mice causes an up-regulation of CYP2B10 and CYP3A11, two other CYP enzymes with the capability to metabolize CCl_4 when administered at very high doses [108, 109]. It appears that at this dose, 1500mg/kg CCl_4, expression of CAR target genes are being induced which is dependent on an intact LKB1-AMPK pathway.

Intriguingly, the expression of CYP1A1/1A2 changes dramatically in a genotype-specific manner after CCl_4 treatment. Oil-treated animals display a steady-state CYP1A1/1A2 protein expression that is not perturbed by loss of hepatic LKB1-AMPK signaling or treatment with AMPK activators. Cyp1a1/1a2 enzymes are not CAR target genes and have not been found to be influenced by AMPK expression. Cyp1a2 has response elements for both the aryl hydrocarbon receptor (AhR) and the pregnane X receptor (PXR) while Cyp1a1 has a response element for AhR alone [76]. *In vivo* administration of CCl_4 is known to inhibit induction of both Cyp1a1/1a2 isozyme mRNAs and proteins [110]. Indeed, in both +/+ and L/L livers CCl4 treatment decreases CYP1A1/1A2. Unexpectedly, AICAR and metformin pretreatment protect against this inhibition.

Using this particular protocol it is impossible to determine if CYP2E1 expression was transiently increased after every injection of

metformin or AICAR. It may be that a transient increase in CYP2E1is coupled to increased degradation of the extra protein to maintain a steady-state level of enzyme. It is clear that at the endpoint chosen here there is no significant increase in CYP2E1 expression. Consistent with these findings, once daily injection of metformin or AICAR in rats and mice was also shown to not be sufficient to promote expression of CAR target Cyp2b10, despite its induction in an AMPK-dependent manner in hepatocytes [111]. These data suggest that the prolonged level of AMPK activation achievable with once-daily therapy is not sufficient to induce significant transcription. Perhaps more sustained or prolonged treatment with more potent AMPK-activating agents would increase these CYPs. It remains to be seen if this pretreatment regimen is capable of altering immediate hepatocyte response to CCl_4 intoxication. Useful assays to parse out such details include immunohistochemical staining for Ki67 and TUNEL, both of which would offer a wider insight into the hepatocyte behavior that may not be captured by western blot analysis. Given the increase observed in CYP2E1 protein at 3hr post-AICAR in mice (Figure 20), one might envision that exposure to a toxin within a narrow window after metformin or AICAR administration could alter sensitivity. Future studies would be needed to address this hypothesis.

Mechanistic insight into deregulation of xenobiotic metabolism by LKB1 loss

In an effort to understand the mechanism by which broad deregulation of xenobiotic metabolism occurs after LKB1 signaling disruption I used nuclear lysates prepared from +/+ and L/L livers.

Previous studies have identified HNF4α as a potential substrate for AMPK and bioinformatic analysis of the preferred AMPK substrate recognition motif suggests the sequence around serine 313 in the orphan nuclear receptor HNF4α is a good candidate for phosphorylation by AMPK, consistent with published *in vitro* kinase assays [112]. In fact, it has been shown that AICAR activation of AMPK results in diminished HNF4α protein levels and down-regulation of HNF4α target genes; many of which are involved in xenobiotic metabolism [112, 113].

If previous studies are correct, loss of AMPK would lead to less degradation of HNF4α leading to an increase in HNF4α target gene expression. In my work, nuclear extracts of +/+ and L/L livers show that on Ser^{313} is lost upon disruption of LKB1-AMPK signaling (Figure 23). After CCl_4 intoxication, there is a nearly global loss of phosphorylated HNF4α in wild-type livers, suggesting that transcriptional activation of HNF4α target genes is important during the detoxification process. Combining previous studies with my own work, this would indicate that HNF4α is active and should lead to transcriptional activation, not repression. Therefore, it is unlikely that HNF4α is responsible for the loss of xenobiotic metabolism genes observed in the LKB1-deficient livers, though it could play a role in the up-regulation of the Phase II enzymes.

Likewise, several groups have reported that mice with liver-specific knockout of β-catenin display deregulated xenobiotic metabolism genes similar to my results as both the conditional β-catenin and LKB1 knockout mice display nearly abolished expression of CYP2E1 [103, 104]. In the bioinformatic-based list of putative AMPK substrates, dishevelled 2, is a candidate substrate for phosphorylation by an AMPK-related kinase subfamily, the MARKs. This LKB1-MARK pathway-dependent

phosphorylation presumably turns off Wnt signaling through β-catenin; loss of this signaling should lead to increased transcription of β-catenin target genes. Indeed, in the LKB1-deficient livers there is a slight increase in nuclear β-catenin accumulation, suggesting that β-catenin is actively transcribing target genes. As is the case with HNF4α, it is therefore also unlikely that lack of β-catenin-based transcription is responsible for the deregulation of xenobiotic metabolism genes.

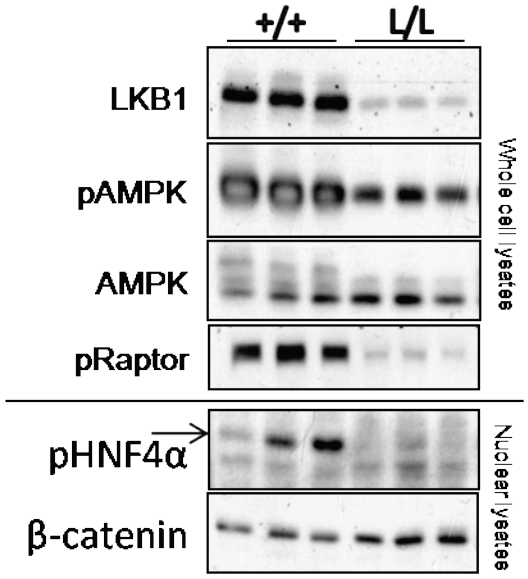


Figure 23. Possible mechanisms through which loss of LKB1 may deregulate the xenobiotic metabolism pathway. Total liver lysates indicating that loss of LKB1-AMPK signaling decreases the amount of phosphorylated, inactive HNF4α, which should enhance transcription of HNF4α target genes. Loss of LKB1-MARKs signaling increases the nuclear accumulation of β-catenin, which should enhance transcription of β-catenin target genes.

After combing through known and putative AMPK and AMPKR substrates gleaned from bioinformatic analysis by cross-referencing them in known literature to involvement in xenobiotic metabolism or the regulation thereof, I then used the microarray results to pick out CYP enzymes with significant expression changes upon genotype or treatment and subjected them to a transcription factor analysis. I hypothesized that there may be a DNA binding motif conserved among the CYP genes down-regulated versus the CYP genes up-regulated. Finding a conserved DNA sequence in the promoters of down-regulated CYP genes could provide information leading to identification of a transcription factor necessary for activation of these genes.

The program FIRE was used to identify de novo binding sites enriched in the CYP genes of interest. This program reliably finds DNA and RNA regulatory motifs by relying on the mutual information between sequence and gene expression measurements [114]. When the program was set to a high-stringency significance test, FIRE returned no significantly enriched sites. Upon lowering the stringency requirements, FIRE discovered the motif .TTTTATC. was enriched in CYP enzymes whose expression is induced after LKB1 loss (Table 4). This motif aligns best with a motif for the transcription factor hunchback. As this motif was found only in CYP genes up-regulated after LKB1 loss it was likely not involved in the decreased response of chemical intoxication. Based on this information, this motif and possible transcription factor were therefore not studied in depth.

Binding sites upstream of CYP genes	E-value	Alignment
hb: hunchback	5.8e-06	--TTTTATC- NTTTTTNYKS
TBP: TATA binding protein	5.7e-05	-GATAAAA------- NTATAAAWRNNNNNN
Evil	2.1e-04	-----TTTTATC-- NNTTATCTTRTCTN
Foxq1: HNF-3/forkhead-like protein 1	1.3e-03	GATAAAA---- WATAAACAATN
MEF2A: myocyte enhancer factor 2A	1.7e-03	--TTTTATC- CTATTTATAG

Binding sites upstream of X.M. genes	E-value	Alignment
MNB1A (Dof1)	1.9e-03	KGMTTKTGN -NCTTT---
PBF: prolamin box binding factor	2.5e-03	KGMTTKTGN -RCTTT---
Dof2	5.1e-03	KGMTTKTGN NGCTTT---
Dof3	7.3e-03	KGMTTKTGN NGCTTT---
Gfi: growth factor independent 1	7.6e-03	NCAMAAKCM--- --NMAATCWNNN

Table 4. De novo binding sites discovered by FIRE upstream xenobiotic metabolism genes. The motif .TTTTATC. was enriched in CYP genes whose expression was increased upon LKB1 loss. The motif .[AGT]CA[AC]AA[GT]C[AC]. was enriched in Phase I xenobiotic metabolism genes.

FIRE also discovered that the motif .[AGT]CA[AC]AA[GT]C[AC]. was enriched in 12 CYP genes as well as 4 flavin mixed-function oxidase genes, another component of phase 1 xenobiotic metabolism. From the microarray data, 5 of the 12 CYPs are down-regulated, 3 are up-regulated, and 4 do not change upon LKB1 loss without additional chemical treatment. This motif has some similarity with the known binding site of the Dof family of transcription factors; Dof1, 2, and 3 are involved in carbon metabolism in plants but have no mammalian orthologs [115]. Because the second motif was not conserved solely in CYPs down-regulated upon LKB1 loss I chose not to pursue it further.

To search for known transcription binding sites and factors, two publically available programs, TRANSFAC and MATCH, were employed. The TRANSFAC database gives information about eukaryotic transcription factors and their known genomic DNA binding sites [116]. MATCH allows the user to query genes of interest against the TRANSFAC database, searching for a wide variety of known transcription factor binding sites [117]. Binding site confidence was established based on mammalian conservation of sequence [118]. When a loose threshold was applied to the CYP gene set, Oct1 and C/EBP have conserved sites in mammals in ~20 of genes (Figure 24a). Oct1 conserved binding sites were found in 3 up-regulated CYPs, 5 unchanged CYPs, and 14 down-regulated CYPs. C/EBP sites were found in 4 up-regulated CYPs, 6 unchanged CYPs, and 9 down-regulated CYPs. After increasing the threshold to minimize the false-positive discovery rate MATCH discovered that myogenin and HNF4 have conserved sites in mammals in ~4 of the CYP genes (Figure 24b). Myogenin conserved binding sites were found

in 1 up-regulated CYP, 1 unchanged CYP, and 2 down-regulated CYPs. HNF4α conserved binding sites were found in 3 down-regulated CYPs.

Considering the results of the above, there apparently are no transcription factor binding sites, known or de novo, that appear in a significant fraction of the down-regulated CYP genes. Combining the de novo motif search with the TRANSFAC search indicates that transcription factor binding is likely not the mechanism by which LKB1 signaling is regulating transcription of a large fraction of CYP genes.

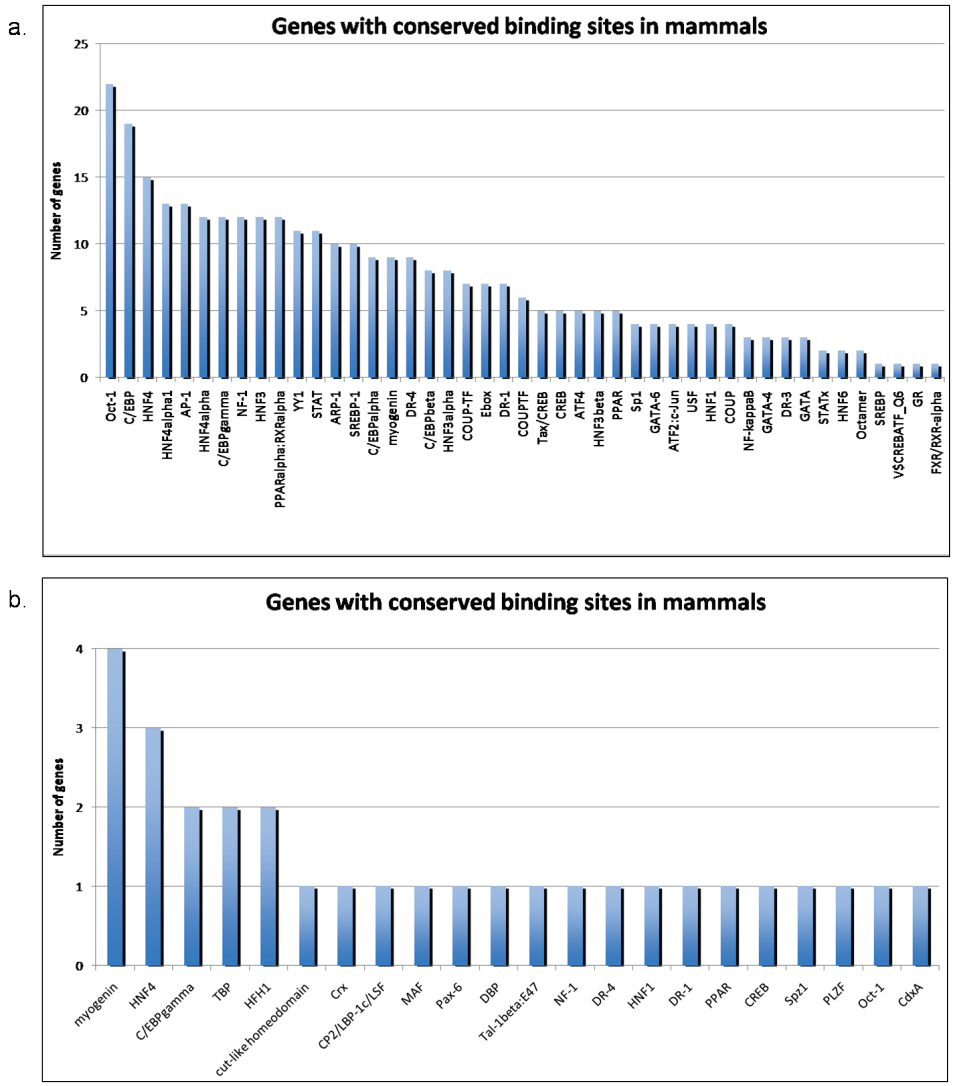


Figure 24. Transcription factor analysis. (a) Searching TRANSFAC for liver binding sites with a loose threshold reveals that the transcription factors Oct-1 and C/EBP have conserved sites in 20 of the deregulated CYP genes. Oct-1 sites were found in 3 up-regulated CYPs, 5 unchanged CYPs, and 14 down-regulated CYPs. C/EBP sites were found in 4 up-regulated CYPs, 6 unchanged CYPs, and 9 down-regulated CYPs. (b) Searching TRANSFAC for all mammalian binding sites with a strict threshold to minimize false-positives. Myogenin conserved binding sites were found in 1 up-regulated CYP, 1 unchanged CYP, and 2 down-regulated CYPs. HNF4α conserved binding sites were found in 3 down-regulated CYPs.

Discussion

Just as loss of LKB1-AMPK signaling decreases expression of many CYP enzymes, hyperactivation of AMPK signaling through pharmacological means transiently increases expression of many CYPs. If the above results hold true in the human liver there may be hitherto cryptic and detrimental consequences to AMPK activation. Metformin is one of the most widely prescribed anti-diabetic therapeutic for type 2 diabetes and lowers blood glucose levels through activation of the LKB1-AMPK pathway. In humans medical complications arising from diabetes and other concurrent diseases are often also treated by pharmacological intervention; important drug metabolism and drug-drug interactions could be altered for several hours following metformin treatment. Clinically this could have an important effect on hepatotoxicity, which is often major reason for drug withdrawal from the mass market, and frequently is not discovered in animal models or small clinical trials.

From the results of the FIRE and TRANSFAC analysis, I posit that it is improbable that the mechanism of down-regulation stems from lack of a DNA-binding transcriptional activator or, conversely, increased binding of a repressor in the promoter regions of the CYP genes involved in xenobiotic metabolism. It remains possible that a transcriptional coactivator or repressor, without DNA binding capabilities, may be involved in down-regulation of so many CYP enzymes. This transcriptional coregulator would need to be a fairly omnipresent member of transcription factor complexes in order to deregulate so many Phase I and Phase II xenobiotic metabolism genes that do not share common response elements. One possibility which could mediate the effects of

LKB1/AMPK on CAR-dependent regulation of Cyp2b10 and Cyp2c37 as well as perhaps some other NR-dependent activation of Cyp2e1 and Cyp2c50 would be the general coactivator histone acetyltransferase p300. p300 is a previously reported substrate of AMPK in the liver and the candidate AMPK site is found adjacent to the LxxXL interaction motif for nuclear receptors, making it likely that the presence of this phosphorylation event could alter NR-dependent transcription. Despite this interesting possibility, many additional reagents would be required to test this hypothesis *in vivo.* Further work in this area would be needed to complete the mechanism of LKB1-mediated expression of xenobiotic metabolism genes. Regardless of the lack of mechanistic insight, the biological outcome of xenobiotic metabolism deregulation remains an open-ended question.

CHAPTER 4: Loss of LKB1 is hepatoprotective against repeated and prolonged carcinogenic insult

LKB1 loss attenuates fibrosis

While I have shown that LKB1 loss in the liver protects against an acute, single dose of the carcinogens DEN and CCl_4 it may be that repeatedly challenging the L/L livers will close the gap between the two genotypes. The first stage in the long transition between normal healthy liver and a cancerous one is fibrosis (Figure 25a). Fibrosis is the excessive accumulation of extracellular matrix, deposited by activated hepatic stellate cells, resulting from an imbalance between fibrogenesis and fibrolysis. Fibrosis occurs in most types of chronic liver disease in humans and is thought to be reversible as long as the insult ceases [119].

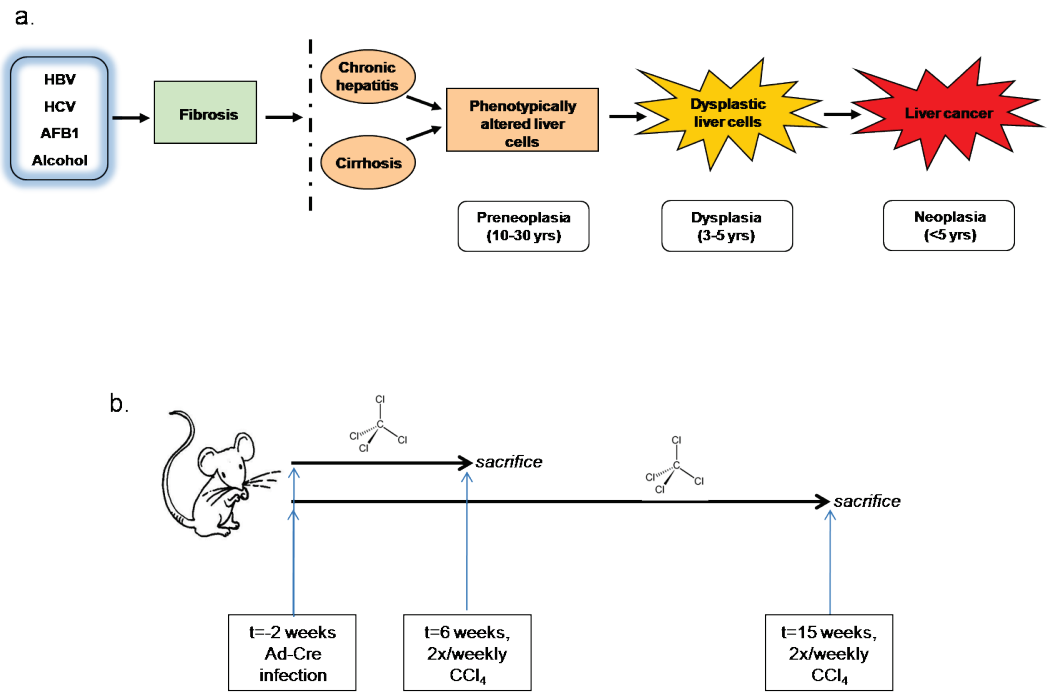


Figure 25. Modeling liver cancer. (a) Progression of liver disease into liver cancer in humans is linked to unremitting liver damage, leading to fibrosis. Continuing insult leads to cirrhosis and eventually carcinoma of the liver; the course of disease progression typically takes decades. (b) Mouse models of fibrosis, cirrhosis, and liver cancer often rely on chemical insult, in this case carbon tetrachloride, closely repeated over a period of time. Mice were given a tail-vein injection of Ad-Cre to mediate deletion of hepatic *Lkb1* and allowed to recover before beginning CCl_4 treatment. Mice were sacrificed at the indicated timepoints.

To assess the potential differences between +/+ and L/L livers during chronic injury I chose to use only CCl_4 as a hepatointoxicant as its metabolism occurs preferentially through CYP2E1 with very little contribution from other CYP enzymes. Unlike a single acute dose of CCl_4 chronic liver damage with this chemical also induces liver zone III

necrosis and hepatocyte apoptosis. Hepatic secretion of chemokines such as TGF-β1 and PDGF leads to hepatic stellate cell activation and tissue fibrosis. By repeatedly administering high doses of CCl_4 to achieve chronic damage the liver will enter a pathological state of fibrosis (Figure 25b) that in many respects mimics the human disease state [120]. With repetitive dosing CCl_4 can be used to induce bridging hepatic fibrosis after approximately 4 weeks of treatment, advanced micronodular cirrhosis after ~ 12 weeks and eventually leading to cholangiocarcinoma. Some mouse strains, such as C57BL/6, display greater resistance to CCl_4 than others, such as FVB, and as a result disease progression may take longer. Further dosing leads to cholangiocarcinoma (CCA), a lethal malignancy of biliary epithelium [121].

Mice treated for 5 weeks with twice-weekly CCl_4 injections have no obvious abnormalities in internal organs other than the liver upon gross visible examination (Figure 26). Oil-treated (vehicle) livers of both genotypes are dark red in color with no visible lesions. CCl_4 treatment of both +/+ and L/L livers leads to a slight lightening of liver color from dark red to reddish-brown. Interestingly, +/+ livers display externally visible nodules of hepatocyte regeneration throughout the surface area of the liver (marked by arrows) while L/L livers do not display such nodules on the surface.

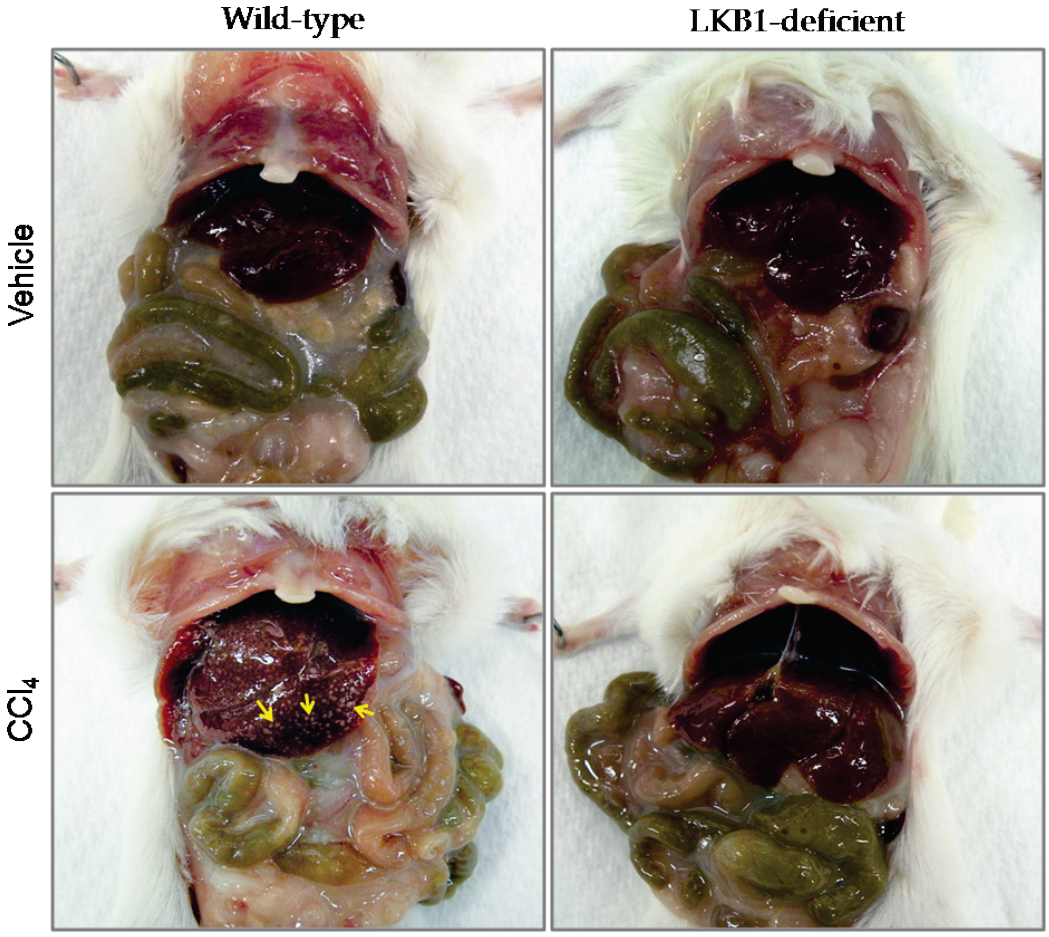


Figure 26. Gross physical examination of mice treated for 6 weeks with 2x/weekly CCl_4. Wild-type and LKB1-deficient livers treated with oil appear visually normal, and are dark red-drown in color. Wild-type CCl_4-treated livers appear lighter in color and have white nodules of regenerating perilobular hepatocytes; LKB1-deficient livers do not have such nodules.

During liver injury, the normally quiescent hepatic stellate cells become activated to myofibroblast-like cells characterized by *de novo* expression of α smooth muscle actin (αSMA). In +/+ oil-treated livers there is no activation of stellate cells as seen by αSMA protein but αSMA

is robustly expressed upon 6 weeks of CCl_4 treatment. In contrast, L/L livers do not show activation of stellate cells under any circumstances (Figure 27a). Activated hepatic stellate cells are also myofibroblastic-like in their ability to contribute to wound-healing. After liver injury activated stellate cells deposit large amounts of extracellular matrix, in particular collagen type I and II, which gradually replaces the basal membrane-like structures present along the sinusoids and in between hepatocyte plates.

This collagen deposition can be viewed in liver sections by using the histological stain Sirius red which preferentially stains only collagen fibers within a sample. Collagen in untreated livers is restricted to the basal lamina of blood vessels and bile ducts. Without promotion of fibrosis by CCl_4 there is no activation of stellate cells and no liver scarring as seen by Sirius red staining (Figure 27b). Consistent with stellate cell activation in +/+ CCl_4-treated livers there is an increase in fibrotic banding and perisinusoidal fibrosis. There is a no significant increase in collagen deposition in L/L CCl_4-treated livers.

Given these results it is likely that loss of the CYP enzymes is biologically relevant to the formation of fibrosis following chronic liver damage. Mice lacking hepatic LKB1 do not activate stellate cells in response to chemical intoxication; it is possible that therefore these livers will be resistant to carcinogenesis compared to wild-type mice following prolonged CCl_4 treatment.

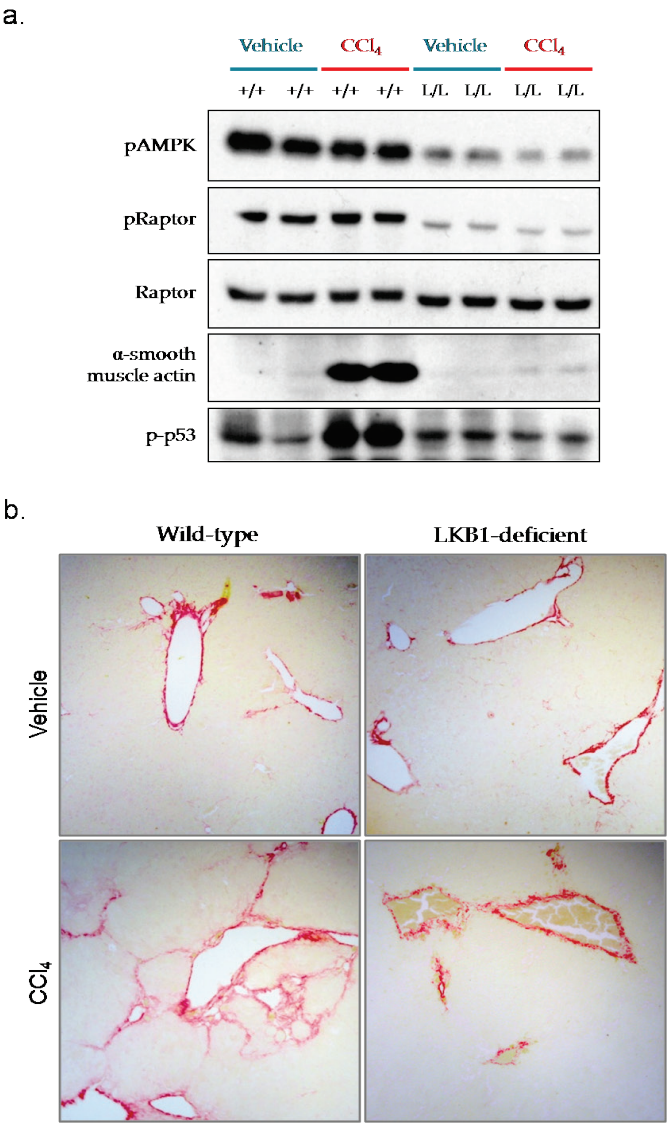


Figure 27. Activation of hepatic stellate cells. (a) Activation of stellate cells is seen by the marker α smooth muscle actin in total liver lysates from wild-type livers repeatedly treated with CCl_4 but not LKB1-deficient livers. Increased phosphorylated p53 is also seen in +/+ but not L/L livers upon 5 weeks of CCl_4 treatment. (b) Histology of livers from +/+ and L/L animals showing extended, branched fibrotic scarring, seen in red, is present in wild-type livers after 5 weeks of CCl_4 treatment. Scarring is not seen in LKB1-deficient livers.

AMPKα deletion alone does not attenuate fibrotic response

LKB1 is an upstream kinase to 14 kinases, all of which are closely related to AMPKα1 and AMPKα2. Because genetic deletion of both α alleles is embryonic lethal, I used $AMPK^{-/-}/AMPK^{flox/flox}$ mice (henceforth referred to as AMPK DKO) to address the hypothesis that AMPK is directly responsible for the loss of CYP enzyme expression. Mice were either wild-type for AMPKα1 and AMPKα2 or genetically null for the α1 and deleted for the α2 allele by adenovirus Cre-recombinase. The animals were then subjected to the same protocol as the previous experiment in LKB1-null livers.

In this experiment Cre-mediated deletion of the AMPKα2 allele is not complete and this reduced amount of functional protein retains partial ability to phosphorylate Raptor. It appears that this reduced level of AMPKα2 is sufficient to maintain wild-type levels of CYP2E1 protein (Figure 278a). Without a significant reduction in CYP2E1 enzyme levels in the AMPK DKO mice it was unlikely that CCl_4 metabolism would be depressed enough for genotype-specific differences in fibrotic response. Indeed, there is no difference in the marker of activated hepatic stellate cells αSMA in CCl_4-treated WT versus AMPK DKO mice. Furthermore, the livers of both genotypes appear to be equivalent in terms of the formation of regenerative nodules of hepatocytes (Figure 28b)

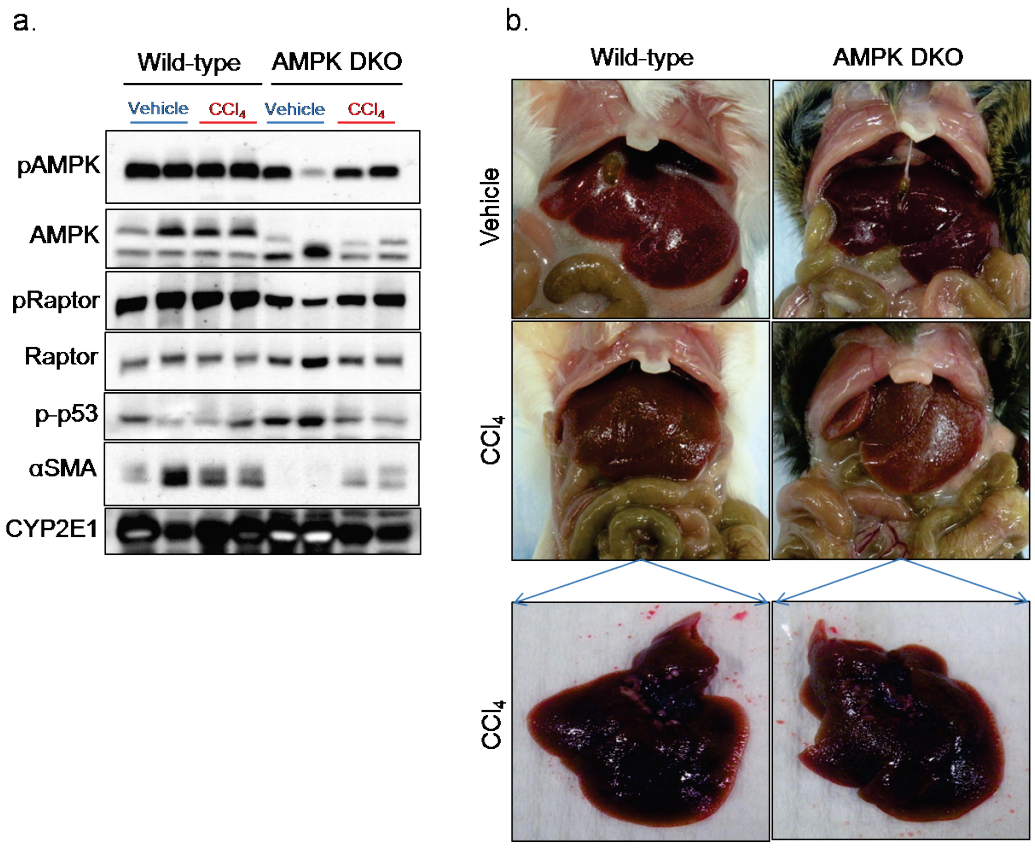


Figure 28. Reduction in AMPK-mediated signaling does not significantly alter fibrotic response. (a) Total liver lysates showing that activation of stellate cells is only mildly reduced in the AMPK DKO mice and there is no difference in CYP2E1 protein abundance. (b) Gross examination of livers in wild-type and AMPK DKO mice are similar visually in the number of regenerative nodules.

In this case the AMPK-mediated signaling is solely from the poorly-recombined AMPKα2 allele with no contribution from the AMPKα1 allele. A previous study by Salt *et al* had discovered that not only was the AMPKα2 subunit more dependent on AMP for allosteric activation than the AMPKα1 subunit but that a significant fraction of AMPKα2 was localized to

the nucleus while AMPKα1 was localized to the cytoplasm [122]. Because this difference in subcellular localization may result in differential gene regulation between the two subunits I tested each allele independently of the other to determine if the loss of CYP2E1 expression was due to a specific AMPKα subunit.

After nearly complete deletion of AMPKα2 there is an increase in AMPKα1 protein levels, suggesting there is compensation for AMPKα2 loss (Figure 29a). Similarly, there is a slight increase in AMPKα2 when AMPKα1 is lost. AMPK-mediated signaling is not abolished in either genotype as seen by phosphorylation of the downstream substrate Raptor. It is important to note here that the AMPKα1 mice are on a pure FVB background while the AMPKα2 mice are mixed FVB:C57BL/6. In each case, there are strain-matched wild-type controls in order to make the proper comparisons. As mentioned previously, C57BL/6 mice are resistant to CCl_4 intoxication compared to FVB mice and the strain variation in CYP2E1 abundance is likely the cause of this phenotype.

There is no suppression of CYP2E1 with loss of either AMPK allele but rather a slight but consistent increase in protein. This could be due to the compensation mentioned above, but may also be related to intact LKB1-AMPK related kinase signaling which may also have compensatory abilities upon AMPK loss. Therefore, deregulation of xenobiotic metabolism genes is dependent on complete suppression of the LKB1-AMPKR pathway created by LKB1 loss (Figure 29b).

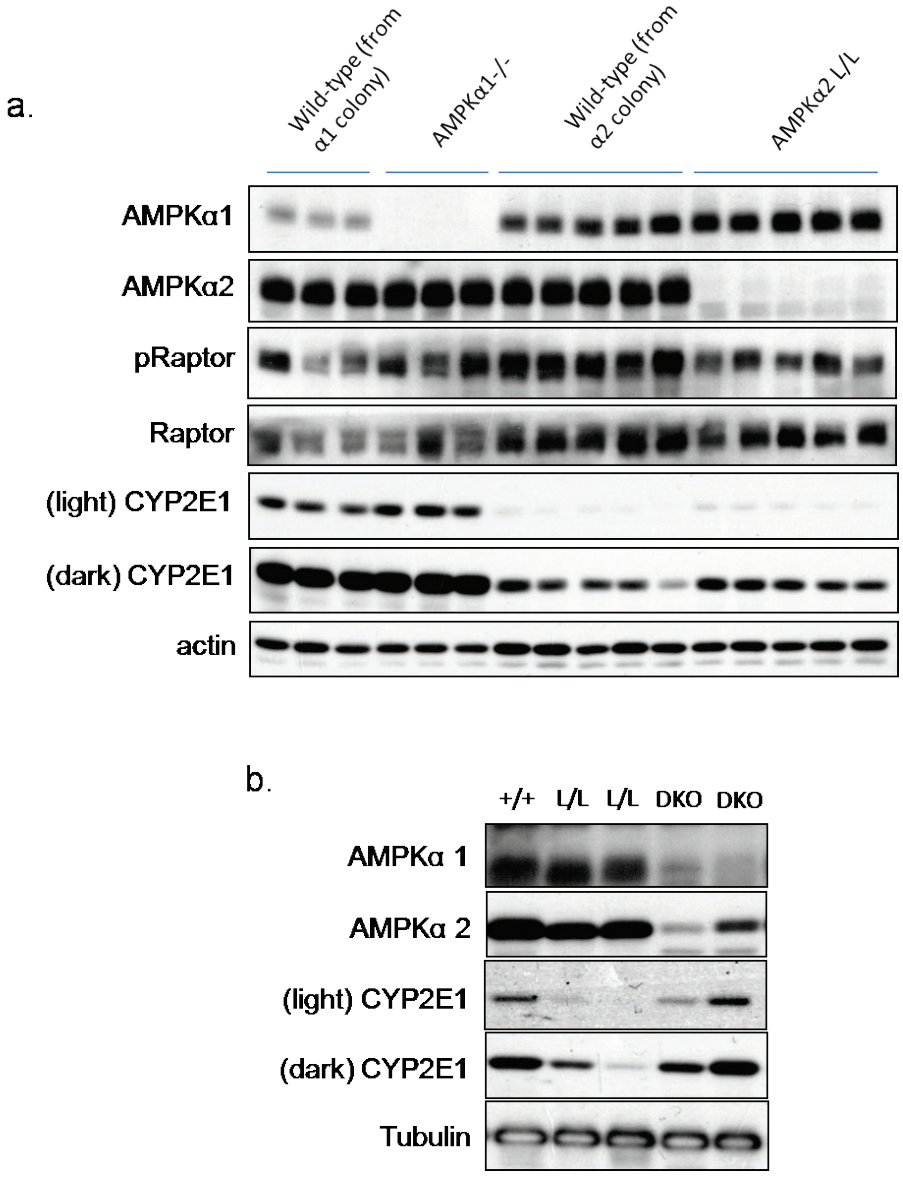


Figure 29. AMPK allele series. (a) Total liver lysates from animals deficient for either the AMPKα1 or the AMPKα2 allele were probed for their ability to express CYP2E1. Loss of either allele lead to a slight increase in CYP2E1 protein levels compared to the appropriate wild-type control liver. (b) Complete loss of LKB1 signaling is required to mediate deregulation of xenobiotic metabolism, likely due to compensation through AMPK-related kinases.

The circadian rhythm genes Cry1/Cry2 do not significantly influence CYP2E1 or fibrosis

The bioinformatic approach that identified Raptor as a novel substrate of AMPK also identified the circadian rhythm transcriptional repressor gene cryptochrome 1 (Cry1) as an AMPK substrate [123]. AMPK phosphorylation of Cry1 enhances its degradation and thus enables communication of nutrient status to the circadian clock in the liver. Circadian oscillation of gene expression, particularly xenobiotic metabolism genes, is an emerging field with exciting implications in cancer and drug metabolism [124]. Disruption of the circadian rhythm by chronic jet-lag exposure in mice increases the risk of multiple types of liver cancer after DEN exposure [125]. Several groups have shown diurnal regulation of CYP2E1 and constitutive Cry1 activity suppressed CYP2E1 expression in a human hepatocellular carcinoma cell line [126, 127]. There are slight fluctuations in CYP2E1 protein throughout the day and a peak in activated p53 at CT12 (6pm) (Figure 30).

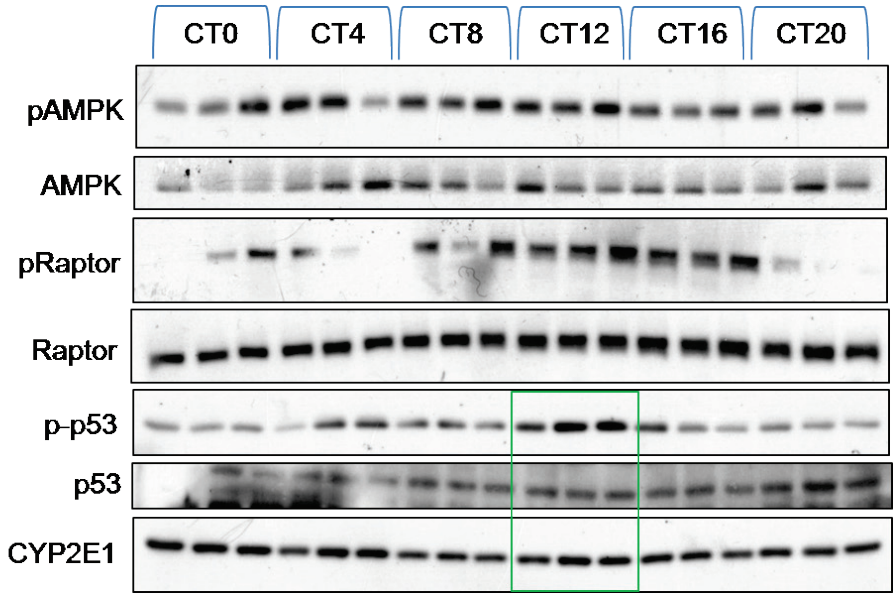


Figure 30. Circadian rhythm fluctuations in CYP2E1. AMPK activation changes throughout a 24-hour period. A peak of phosphorylated p53 abundance at circadian time 12 (6pm) coincides with a high CYP2E1 level (green box). 3 mice per timepoint were used.

Loss of LKB1-AMPK signaling should result in increased Cry1 stability and thus lowered CYP2E1 expression; conversely loss of Cry1 should increase CYP2E1 abundance. I hypothesized that mice lacking both alleles of cryptochrome, Cry1/Cry2 double knockout or Cry DKO, would display an enhanced fibrotic phenotype compared to wild-type control animals. Cry DKO mice did not display increased but rather very slightly decreased CYP2E1 by western blot analysis (Figure 31a). Upon completion of the 6-week fibrosis protocol, livers of +/+ and Cry DKO mice were examined for markers of activated hepatic stellate cells. Cry DKO mice treated with CCl_4 had decreased slightly αSMA expression compared to the wild-type controls (Figure 31b).

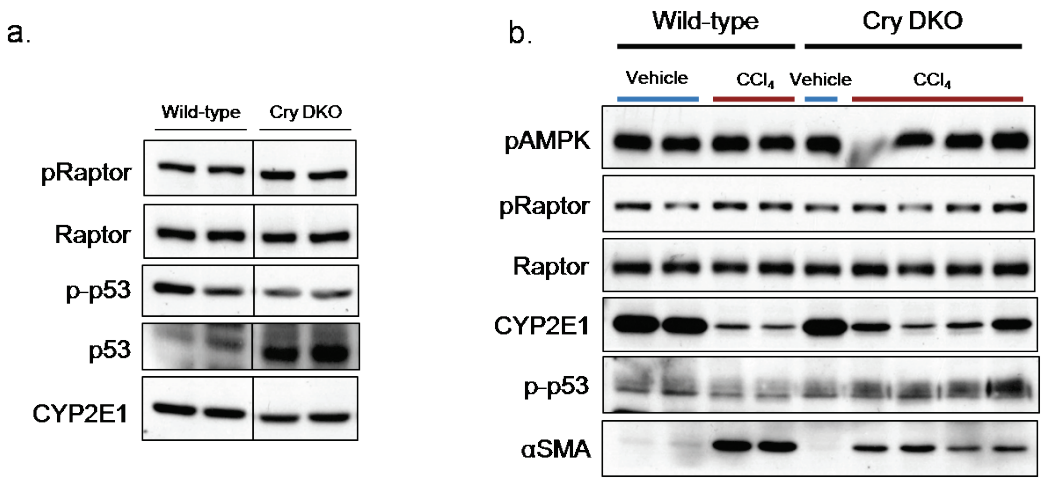


Figure 31. Cry double-knockout mice and CYP2E1. (a) Cry DKO mice have slightly decreased CYP2E1 protein abundance and likewise in phosphorylated p53. (b) Cry DKO mice still have intact but slightly attenuated fibrotic response after 6 weeks of CCl_4 treatment.

As there does not appear to be a significant difference in CYP2E1 enzyme level between the two genotypes it is unlikely that CCl_4 metabolism is radically altered. The causative link between Cry1/Cry2 and attenuated fibrotic response remains to be elucidated but it does not appear that AMPK-mediated circadian clock control is involved in the deregulation of xenobiotic metabolism seen in LKB1-deficient livers.

Mice lacking hepatic LKB1 are refractory to bile duct hyperplasia and early cholangiocarcinoma after prolonged exposure to CCl_4

By continuing the CCl_4 injection schedule past the 5 week window tested above to 16 weeks of treatment it is possible to provoke a hyperplastic or cancerous phenotype. Repeated CCl_4 intoxication causes an induction of bile duct epithelial cell proliferation which significantly coincides with increased fibrotic collagen deposition and changes in the physical structure of the liver such as formation of pseudolobules, nodularity, and architectural distortion. This increase in fibrotic extracellular matrix associated with chronic insult may participate in bile duct epithelial cell differentiation [128].

After completion of CCl_4 treatment for 16 weeks gross examination of oil-treated livers revealed no abnormalities of non-liver organs in the abdominal cavity but there were several small fatty cysts externally visible on livers from both genotypes (data not shown, and Figure 32). Wild-type livers treated with CCl_4 have larger fatty cysts than the vehicle-treated counterparts. In contrast, L/L livers treated with CCl_4 are remarkably visually similar to those treated with oil. Also noticeable upon visual examination is inward contraction of liver lobules in +/+ CCl_4-treated mice, caused by the contractile properties of activated hepatic stellate cells into myofibroblast-like cells [129].

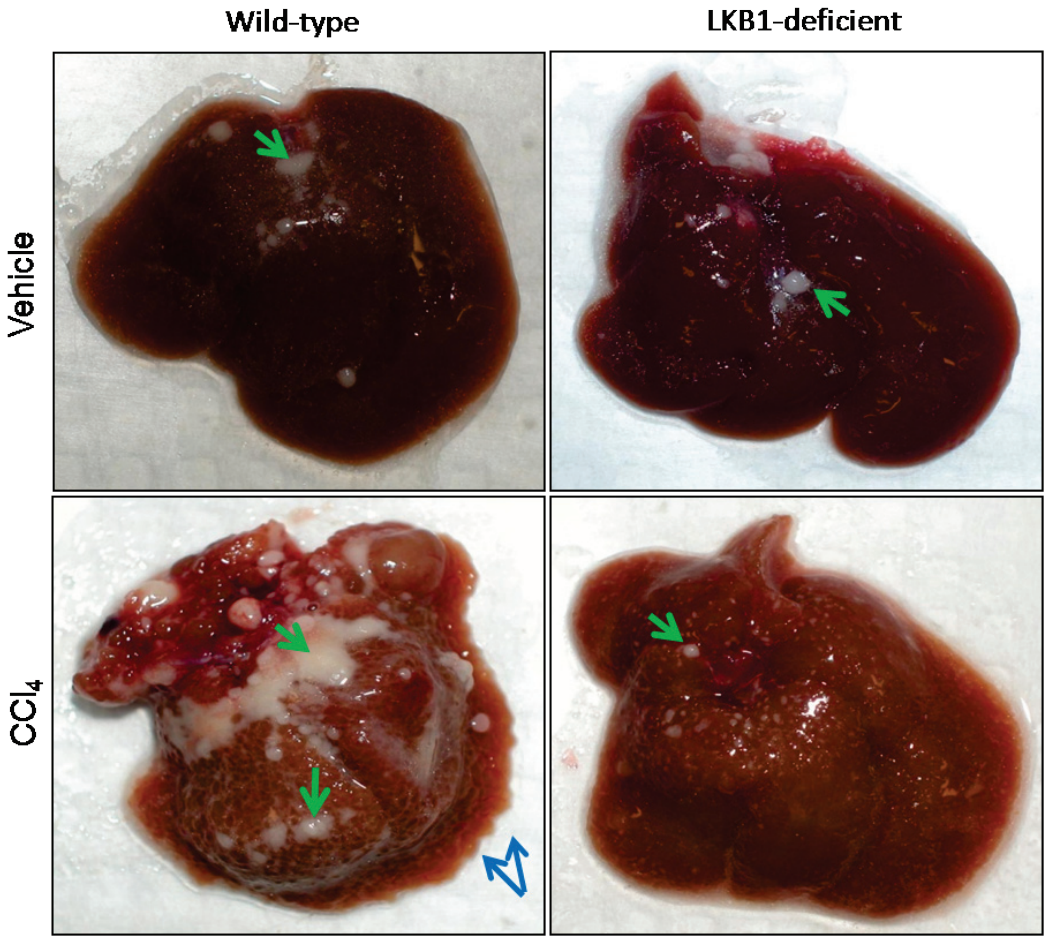


Figure 32. Gross examination of livers exposed to prolonged CCl4 intoxication. Wild-type and LKB1-deficient livers treated with vehicle are appear generally healthy with the addition of small fatty cysts (green arrows) primarily located around the bile duct. Wild-type animals treated with CCl_4 have large fatty cysts, bridging fibrosis, and contraction of liver lobules inward from the edge of Glisson's capsule (blue arrows). LKB1-deficient livers appear remarkably resistant to prolong CCl_4 intoxication.

In addition to liver damage most CCl_4-treated +/+ animals had moderate to severe lung necrosis (data not shown). It is likely that as chronic liver disease progressed in +/+ animals the capacity for

metabolism of CCl_4 decreased, leaving CCl_4 free to travel from the liver to the lungs via the bloodstream. Animals in which hepatic LKB1 is lost also showed signs of lung necrosis, though not as severe as wild-type animals. By using adenovirus infection to express Cre recombinase there is potential at high titer infections for viral "bleed-through" to the lungs; another organ highly susceptible to adenoviral infection. There may be a small population of cells in the lungs of $LKB1^{flox/flox}$ mice in which *Lkb1* has recombined and expression is lost. As the lung is another organ in which xenobiotic metabolism genes are important for cancer development it remains to be seen whether loss of LKB1 in the lungs alters expression of xenobiotic metabolism genes. This could explain the apparent attenuation of lung necrosis in $LKB1^{flox/flox}$ mice.

Biochemically, vehicle treated wild-type and LKB1-deficient livers show no signs of activated stellate cells as seen by lack of α smooth muscle actin (Figure 33a). Upon repeated exposure to CCl_4, wild-type livers accumulate α smooth muscle actin, while the LKB1-deficient livers display greatly decreased α smooth muscle actin. CYP2E1 protein levels in LKB1-deficient livers are still much lower than their wild-type counterparts, indicating that there is no other method of compensation even over a period of several months to increase CYP2E1 expression and that deregulation of xenobiotic metabolism may be a permanent effect of loss of LKB1 signaling.

I scored livers for collagen:non-collagenous protein as seen by the double staining technique sirius red:fast green. As seen from the fibrosis experiment, wild-type livers treated with CCl_4 have increased fibrotic scarring compared to controls than LKB1-deficient CCl_4-treated livers (Figure 33b). Similarly, wild-type CCl_4-treated livers have a significantly

higher proliferative index than vehicle-treated livers as seen by the marker of proliferation Ki67. Both of these biological changes in wild-type CCl_4 treated livers were quantified and are statistically significant (Figure 34). There is an increase in proliferation in LKB1-deficient livers but it is not statistically significant when compared to either genotype treated with vehicle.

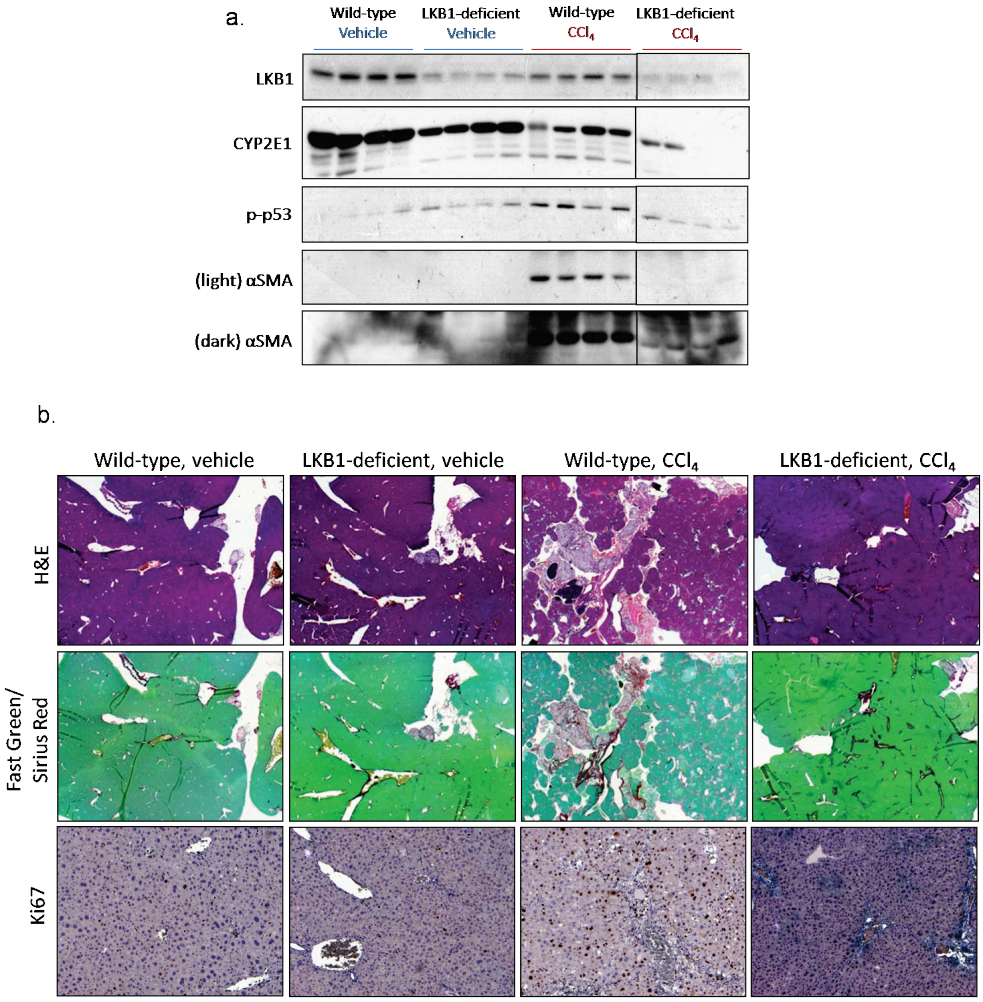


Figure 33. LKB1-deficient livers are refractory to prolonged hepatotoxic insult. (a) Total liver lysates from wild-type livers have activate hepatic stellate cells after prolonged treatment with CCl_4 but LKB1-deficient livers do not. CYP2E1 protein levels are consistently decreased with LKB1 loss. (b) Histology and immunohistochemistry of +/+ and L/L livers. Active hepatic stellate cells in wild-type CCl_4 treated livers deposit large amounts of collagen-containing extracellular matrix; the lack of active hepatic stellate cells in LKB1-deficient livers results in nearly abolished fibrotic scarring. Proliferation of liver cells is higher in wild-type CCl_4 treated livers than their LKB1-deficient counterparts.

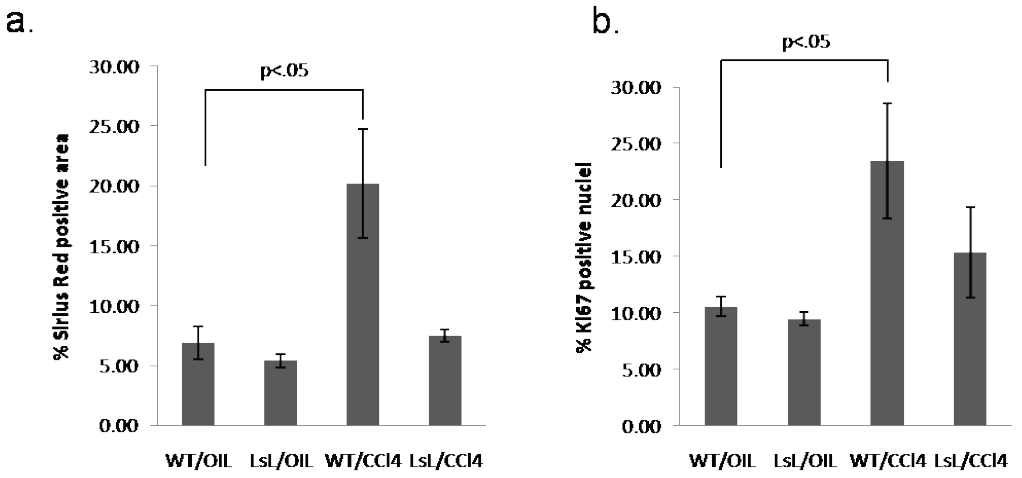


Figure 34. Quantitation of histology. (a) Wild-type CCl_4 treated livers have significantly greater fibrosis than other treatments as seen by the higher percentage of collagen to non-collagenous protein. (b) Wild-type CCl_4 treated livers have a higher proliferative index than other treatments as seen by Ki67 positivity. Statistical significance only where indicated.

Pathologically, prolonged treatment with high doses of CCl_4 is known to cause cholangiocarcinomas in mice [121]. In order to determine what types of lesions wild-type versus LKB1-deficient livers contained, hematoxylin and eosin-stained liver sections were sent to a trained pathologist (Figure 35). Wild-type and LKB1-deficient livers treated with vehicle were defined as having minimal hepatic inflammation, microscopic fatty cysts, and few necrotic hepatocytes. Wild-type livers treated with CCl_4 were defined as having chronic active necrotizing and fibrosing hepatitis while the LKB1-deficient counterparts were defined as having chronic active fibrosing hepatitis. Marked bile duct hyperplasia occurred in +/+ CCl_4 treated livers; moderate biliary hyperplasia was observed in L/L livers. In a few wild-type, but not LKB1-deficient, CCl_4

treated mice there were multiple foci of atypical bile duct hyperplasia and early carcinoma lesions (Figure 36).

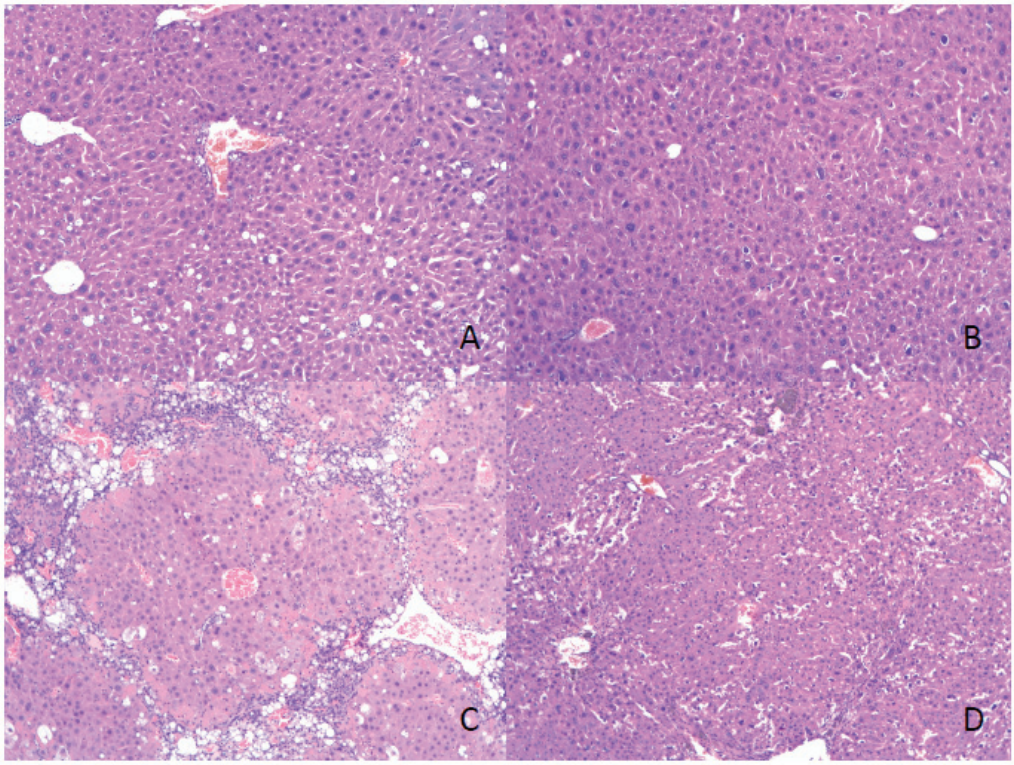


Figure 35. Hepatic lesions in wild-type and LKB1-deficient mice treated with vehicle (A, B) or CCl_4 (C, D) for 16 weeks (H&E sections, 10X). In general, there was minimal, multifocal hepatic inflammation centered around rare necrotic hepatocytes or microscopic fatty cysts in +/+ (A) and L/L (B) livers. Livers of CCl_4-treated +/+ mice (C) exhibited moderate to marked periportal hepatocyte lipid vacuolar degeneration and necrosis as well as marked bile duct hyperplasia, bridging fibrosis (portal-portal and portal-central) and nodularity with separation into pseudolobules and architectural distortion. Portal space and fibrosis showed variable degrees of mixed inflammation composed of lymphocytes, plasma cells, macrophages, and neutrophils. CCl_4-treated L/L mice (D) showed similar bridging fibrosis and inflammation with moderate biliary hyperplasia and rare degenerating or necrotic hepatocytes.

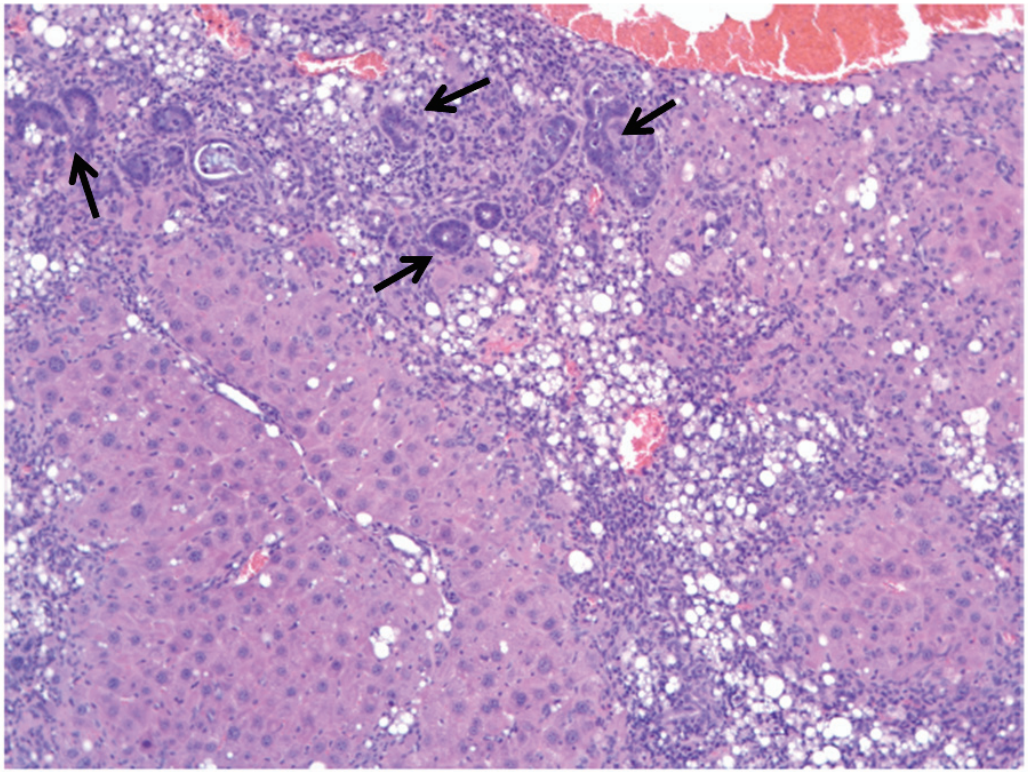


Figure 36. Evidence of cholangiocarcinoma precursors in a few wild-type CCl_4 treated livers showed multiple foci of atypical bile duct hyperplasia and early carcinoma lesions marked by arrows. (H&E, 20X)

Discussion

Wild-type animals subjected to repeated CCl_4 intoxication show all the hallmarks of fibrosis, hyperplasia, and early cholangiocarcinoma lesions. Expression of CYP2E1, the cytochrome P450 enzyme responsible for metabolizing the inactive CCl4 into two free radical metabolites, is nearly abolished after deletion *Lkb1* in the mouse liver. Without this conversion from intrinsically harmless CCl_4 to cellular macromolecule-destroying free radicals there is essentially no damage

created in LKB1-deficient livers. Other chemical carcinogen-based methods of promoting liver cancer, such as hepatocellular carcinoma promotion in young male pups injected with DEN, would likely also be compromised upon hepatic LKB1 loss [130].

It remains to be seen if combinatorial loss of LKB1 signaling pathways with activation of oncogenes can promote tumor formation in the liver. Oncogenes with previously studied involvement in the progression of hepatocellular carcinoma such as *c-myc*, *CCND1* (cyclinD1), *CTNNB1* (β-catenin), or *c-met* oncogenes, as well as mutations in components of the Ras/PI3 kinase pathways could be useful in studying HCC formation in model systems that is not based on xenobiotic metabolism [131].

Chapter 5: Conclusions and Perspectives

Loss of hepatic LKB1-AMPK signaling coupled with prolonged treatment with the hepatointoxicant CCl_4 results in a dramatic decrease in fibrosis, hyperplasia, and formation of neoplastic lesions in the liver. This marks the first instance that LKB1 *loss* functions to suppress tumor formation in any tissue studied thus far. Presumably, lack of CYP2E1 expression in livers deficient for LKB1 is the underlying cause of the different biological outcome in this model of tumorigenesis versus other models studied previously.

Based on this study, one question that remains to be addressed is how three different mouse models of human cancer: lung, skin, and now liver; could have such different biological outcomes. LKB1 mutations are common in human non small cell lung carcinomas, head and neck squamous cell carcinomas, and pancreatic cancer; all of which are significantly associated with tobacco smoking [132]. Polycyclic aromatic hydrocarbons (PAHs) are potent carcinogens in mice and humans and are found in cigarette smoke and other environmental pollutants such as automobile exhaust. 7,12-Dimethylbenz(a)anthracene (DMBA) is a classical PAH with carcinogenic effects in most species. As mentioned earlier, mice lacking LKB1 expression in the skin are highly sensitive to DMBA treatment. PAHs are metabolized primarily through CYP1A1 and CYP1B1; intriguingly Cyp1a1 mRNA is up-regulated upon LKB1 loss in the liver (data not shown) [133, 134]. There may be a connection between increased Cyp1a1 expression and rapid tumor onset in chemically-induced tumors. Conversely in the cholangiocarcinoma mouse model system promoted by CCl_4 exposure; loss of CYP2E1

expression, as the primary enzyme responsible for metabolizing CCl_4 to toxic free radical intermediates, is markedly protective. This suggests that modulation of the LKB1-AMPK pathway may attenuate some chemically-induced cancers and potentiate others.

In addition to varying cancer risks based on tissue type and chemical exposure, LKB1-AMPK pathway activation may change metabolism of many medically relevant drugs and alter drug-drug interactions. Clearance of drugs from the body may be reduced upon CYP down-regulation, leading to a potential for drug overdose. CYP down-regulation could also phenocopy CYP enzyme inhibition, which can occur when two or more drugs compete for the same catalytic CYP. This leads to decreased metabolism of one or more drugs due to competition for active binding sites. Often this then leads to an increase in serum concentration of the less competitive drug, increasing the risk of toxicity. Some drugs that require biotransformation to be active will display decreased efficacy upon CYP inhibition [135]. In humans, CYP deregulation after metformin treatment could have a serious impact on the ability to co-administer many drugs.

Another question that remains is if restoring expression of CYP2E1 protein through infection with a viral vector carrying *Cyp2e1* under the control of a strong promoter would restore the LKB1-deficient liver's response without need for any other alterations in xenobiotic metabolism gene expression. It is possible that the increased expression of many Phase II genes in L/L livers will still cause an attenuated fibrotic/hyperplastic phenotype by increasing the elimination rate of toxic intermediates. Furthermore, loss of LKB1-AMPK signaling may influence expression of cytokines in hepatocytes, leading to alterations in

differentiation and proliferation in other cells types in the liver such as hepatic stellate cells and biliary epithelial cells; perhaps creating a liver wherein the hepatocytes cannot signal properly to liver support cells or to the immune system.

Thus I have presented a body of work in which I describe the deregulation of xenobiotic metabolism in livers that have defective LKB1-AMPK signaling. The ultimate biological endpoint is an attenuation in hepatic fibrosis, biliary duct hyperplasia, and cholangiocarcinoma precursor lesions when livers lacking hepatic LKB1 are treated with CCl_4. Therefore, loss of LKB1 is remarkably hepatoprotective against chemical-based liver insult.

Materials and Methods

Microarray, GO, and KEGG analysis

Total RNA was extracted using Trizol reagent (Invitrogen) and purity of the RNA was assessed by Agilent 2100 Bioanalyzer. 500 ng of RNA was reverse transcribed into cRNA and biotin-UTP labeled using the Illumina TotalPrep RNA Amplification Kit (Ambion). cRNA was quantified using an Agilent Bioanalyzer 2100 and hybridized to the Illumina mouseRefseq-8v2 Expression BeadChip using standard protocols (Illumina). Image data was converted into unnormalized Sample Probe Profiles using the Illumina BeadStudio software and analyzed on the VAMPIRE microarray analysis framework3. Stable variance models were constructed for each of the experimental conditions (n=2). Differentially expressed probes were identified using the unpaired VAMPIRE significance test with a 2-sided, Bonferroni-corrected threshold of Bonf = 0.05. The VAMPIRE statistical test is a Bayesian statistical method that computes a model-based estimate of noise at each level of gene expression. This estimate was then used to assess the significance of apparent differences in gene expression between 2 experimental conditions. Lists of altered genes generated by VAMPIRE were mapped to pathways using the VAMPIRE tool GOby to determine whether any KEGG categories were overrepresented using a Bonferroni error threshold of Bonf = 0.05. Heat map was constructed with cubic spline-normalized values using the CIMminer program at http://discover.nci.nih.gov/, a development of the Genomics and Bioinformatics Group, Laboratory of Molecular Pharmacology (LMP), Center for Cancer Research (CCR) National Cancer Institute (NCI).

Chemicals and adenovirus-Cre

Metformin, diethylnitrosamine, carbon tetrachloride, mineral oil, indocyanine green, sirius red, fast green FCF, hematoxylin, and phenobarbitol were purchased from Sigma. TCPOBOP was a kind gift from Dr. Ron Evans. AICAR was purchased from Toronto Research Chemicals (A611700). A .9% sterile saline solution was used where indicated. Adenovirus expressing Cre recombinase (pAd CMV CRE) was purchased from University of Iowa Gene Transfer Vector Core (Iowa City, IA).

Tissue isolation

Experimental animals were sacrificed by cervical dislocation and the liver was harvested immediately; snap-frozen in liquid nitrogen for biochemical assays and RNA isolation or fixed in 10% neutral buffered formalin for histological analysis. Frozen samples were homogenized on ice by a rotator-stator for 30 seconds in lysis buffer (20mM Tris pH 7.5, 150mM NaCl, 1mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM pyrophosphate, 50 mM NaF, 5mM β-glycero-phosphate, 50 nM calyculin A, 1 mM Na3VO4, 10 mM PMSF, 4 µg/ml leupeptin, 4 µg/ml pepstatin, 4 µg/ml aprotinin). Total protein was normalized using the Bradford method and 30-50ug of total protein lysates were resolved by SDS-PAGE.

Histology

For the senescence-associated β-galactosidase histology, liver sections were frozen on dry ice in O.C.T. compound (Tissue-Tek, #4583) and cryosectioned at 7µm intervals. Staining was performed using Senescence β-Galactosidase Staining Kit from Cell Signaling Technology (#9860).

For TUNEL staining, the ApopTag Peroxidase In Situ kit (S7100) from Millipore was used on paraffin-embedded sections.

For most histology, liver tissue was fixed for 24 hours in neutral buffered formalin and then the buffer was replaced with 70% ethanol until samples were processed and embedded in paraffin, and cut into 5µm sections. Microwave-based antigen retrieval was performed in 10mM sodium citrate pH 6.0. Blocking kits (SP-2001), Elite ABC reagent (PK-7100), peroxidase substrate kit (SK-4105), and appropriate secondary antibodies are from Vector Labs. The Ki67 antibody (RM-9106-S1) is from Thermo Scientific. Sirius red staining used a solution of .1% sirius red or .1% sirius red/.1% fast green FCF dissolved in saturated picric acid; after rehydration slides were incubated in this solution overnight at 4ºC.

Slides were viewed on a Zeiss microscope and imaged using a CRi Nuance system. Automated scoring of positively-staining nuclei (Ki67 and TUNEL) or sirius red positive areas were performed using CRi's inForm software v1.0.0.

H&E slides were scored by a trained pathologist (Dr. Mathias LeBlanc, Salk Institute).

Biochemical analysis

For western blots, the following antibodies were used at a 1:1000 dilution overnight at 4ºC. From Cell Signaling Technology: pAMPK Thr172 (#2535), total AMPK (#2603), pRaptor Ser792 (#2083), total Raptor (#2280), LKB1 (#3050), pp53 (#9284), p4E-BP1 (#2855), total 4E-PB1 (#9644), pChk1 (#2348). From Abcam: NQO1 (ab28947), Cytochrome P450 Reductase (ab13513), CYP1A1/1A2 (ab81816), CYP2E1 (ab28146), Cyp2b1/2b2 (ab22719), pHNF4α (ab78356). From Millipore: CYP2E1 (AB1252). From Novocastra: total p53 (NCL-p53-CM5p). From Rockland: p-Chk2 (600-401-280). From BD Pharmingen: p21 (556431). From R&D Systems: pATM (AF1655). From Sigma: β-actin (A5441). From DakoCytomation: smooth muscle actin (M0851). Appropriate secondary antibodies were purchased from Pierce. Abcam CYP2E1 and Sigma β-actin antibodies were developed using a homemade ECL solution; all other antibodies were probed with SuperSignal West Femto substrate kit from Pierce (#34095).

Hepatocyte isolation and cell lines

Hepatocytes were isolated from adult male C57BL/6 mice as described previously [136]. Hepatocytes were treated with 250μM TCPOBOP, 500μM phenobarbitol, 2mM AICAR, or 5mM metformin for the times indicated in the experiment.

Primary MEFs from wild-type and LBK1$^{lox/lox}$ mice were isolated as described previously [42]. MEFs were subjected to 10Gy of gamma irradiation, 120,000μJ UV irradiation, 5mM phenformin, 2mM AICAR, glucose-free DMEM, 500μM adriamycin, and 1mM A769662.

Transcription factor analysis

We employed two distinct approaches to predict putative transcription factor binding sites (TFBS) in the upstream sequences of Cyp genes. First, we applied a phylogenetic-footprinting approach using TRANSFAC matrices to find putative TFBS [116, 137]. Each mouse Cyp gene was used to gather orthologous upstream regions from 19 vertebrate species

via the UCSC Genome Browser Database and a multi-species promoter alignment was assembled with DIALIGN [118, 138]. Each upstream sequence was then searched against all non-redundant vertebrate TRANSFAC matrices using MATCH with the "minSUM" score cutoff, which aims to minimize the sum of both false positive and false negatives. All hits were then mapped to the alignment to determine conservation levels for each putative TFBS in mouse [117]. Three tiers of TFBS conservation were defined based on alignment at identical location in more than 35% of all tetrapods, more than 35% of mammals, or mouse and rat alone.
Second, we used a mutual information approach through the program FIRE to identify uncharacterized motifs (i.e. de novo) whose presence and absence across sequences is highly informative of Cyp genes [114].

Animals and treatments

All animal research was performed in full compliance of the US government's Animal Welfare Regulations, the NIH's *Guide for the Care and Use of Laboratory Animals,* and is Council on Accreditation of the Association for Assessment and Accreditation of Laboratory Animal Care-certified. All animals were housed in colony cages with 12h light/dark cycle in a temperature-controlled environment. Mice had free access to water and normal chow diet (12% fat/60% carbohydrate/28% protein, LabDiet 5001), unless otherwise specified.

Ad-Cre mediated deletion of LKB1 in $LBK1^{lox/lox}$ mice was done by tail vein injection of 1×10^9 PFUs/mouse in 8 week old males. Wild-type control mice also received 1×10^9 PFUs/mouse Ad-Cre. To induce cre-mediated deletion in Albumin-creERT2 mice, mice were intraperitoneally (IP) injected with 1mg/ mouse of tamoxifen dissolved in mineral oil for 5 consecutive days.

For the microarray experiment mice were given IP 1000mg/kg CCl_4 in mineral oil or the equivalent volume of mineral oil, or 500mg/kg AICAR in normal saline solution or the equivalent volume of saline. For the acute carcinogen treatment mice received a single IP injection of 500mg/kg DEN dissolved in normal saline solution, CCl_4 dissolved in mineral oil, or the equivalent volume of saline solution. For the fibrosis and pre-neoplasia experiments mice received twice-weekly IP injections of 1500mg/kg CCl_4

dissolved in mineral oil or equivalent volume of mineral oil for 5 weeks-16 weeks. For the neoplastic lesion experiment mice received twice-weekly IP injections of 1500mg/kg CCl_4 dissolved in mineral oil or equivalent volume of mineral oil for 8 weeks before the dose was lowered to 1200mg/kg for an 12 additional weeks of treatment (a total of 20 weeks). For the pretreatment experiment mice were given IP 500mg/kg AICAR, 250mg/kg metformin, or the equivalent volume of saline; followed by 1500mg/kg CCl_4 or mineral oil.

For the indocyanine green clearance experiment: Indocyanine green was administered via tail-vein injection at 20mg/kg in saline. Anesthesia prior to imaging was by gas inhalation of isoflurane until deep sedation had occurred. Images were taken with Caliper Life Science's IVIS Kinetic animal imager using an 800nm emission filter and quantification was performed using Living Image software.

qPCR analysis

mRNA from liver tissue and primary hepatocytes was isolated using RNAeasy (Qiagen) kit and reverse transcribed using *SSII* RT Reverse Transcriptase. qPCR reaction was carried out using Syber GreenER (Invitrogen). Primer pairs were picked from PrimerBank [139].

Statistical analysis

Comparisons were made using the unpaired Student's *t*-test. SEM +/- are represented as error bars. Statistical significance only where indicated.

Additional Acknowledgments

Microarray, GO, and KEGG analysis was performed by Ruth Yu. qPCR analysis was performed by Jacqueline Alvarez and Michael Downes. Transcription factor analysis was performed by Ana Rodrigues. Pathology scoring was done by Mathias Leblanc.

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