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Sara Charawi, Pierre-Alexandre Just, Mathilde Savall, Shirley Abitbol, Massiré Traore, et al.. LKB1 signaling is activated in CTNNB1 -mutated HCC and positively regulates β -catenin-dependent CTNNB1 -mutated HCC. *Journal of Pathology*, 2019, 247 (4), pp.435-443. 10.1002/path.5202 . hal-02365767

HAL Id: hal-02365767

<https://hal.science/hal-02365767>

Submitted on 15 Nov 2019

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LKB1 signaling is activated in CTNNB1-mutated HCC and positively regulates β -catenin-dependent CTNNB1-mutated HCC

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Abstract

Hepatocellular carcinomas (HCCs) are known to be highly heterogeneous. Within the extensive histopathological and molecular heterogeneity of HCC, tumors with mutations in CTNNB1, encoding β -catenin (CTNNB1-mutated HCC), constitute a very homogeneous group. We previously characterized a distinctive metabolic and histological phenotype for CTNNB1-mutated HCC. They were found to be well-differentiated, almost never steatotic, and often cholestatic, with a microtrabecular or acinar growth pattern. Here, we investigated whether LKB1, which controls energy metabolism, cell polarity, and cell growth, mediates the specific phenotype of CTNNB1-mutated HCC. The LKB1 protein was overexpressed in CTNNB1-mutated HCC and oncogenic activation of β -catenin in human HCC cells induced the post-transcriptional accumulation of the LKB1 protein encoded by the LKB1 (STK11) gene. Hierarchical clustering, based on the expression of a murine hepatic liver Lkb1 (Stk11) signature in a human public dataset, identified a HCC cluster, composed of almost all the CTNNB1-mutated HCC, that expresses a hepatic liver LKB1 program. This was confirmed by RT-qPCR of an independent cohort of CTNNB1-mutated HCC and the suppression of the LKB1-related profile upon β -catenin silencing of CTNNB1-mutated human hepatoma cell lines. Previous studies described an epistatic relationship between LKB1 and CTNNB1 in which LKB1 acts upstream of CTNNB1. Thus, we also analyzed the consequences of Lkb1 deletion on the zonation of hepatic metabolism, known to be the hallmark of β -catenin signaling in the liver. Lkb1 was required for the establishment of metabolic zonation in the mouse liver by positively modulating β -catenin signaling. We identified positive reciprocal cross talk between the canonical Wnt pathway and LKB1, both in normal liver physiology and during tumorigenesis that likely participates in the amplification of the β -catenin signaling by LKB1 and the distinctive phenotype of the CTNNB1-mutated HCC.

Keywords: hepatocellular carcinoma; liver; CTNNB1; LKB1

Introduction

Hepatocellular carcinoma (HCC) is the fifth cause of cancer-related death worldwide (GLOBOCAN 2012 v1.0 database, International Agency for Research on Cancer, Lyon, France, 2013. Available from: <http://globocan.iarc.fr>, accessed on 4 June 2018). It is a highly heterogeneous disease, with multiple risk factors and a large spectrum of histopathological features. Large-scale genomic and transcriptomic studies have classified HCC according to various molecular subclasses, often associated with clinical and phenotypic

traits [1 – 3]. Within HCC, tumors with activating mutations of the gene encoding β -catenin (CTNNB1) (18 – 40% of cases) constitute a very homogeneous subgroup [4]. Indeed, we and others have shown that CTNNB1-mutated HCCs are well-differentiated, often cholestatic, chromosomally stable, and associated with a better prognosis than other HCCs [3,4]. Furthermore, CTNNB1-mutated HCCs are almost never steatotic and usually display microtrabecular and/or acinar growth patterns. Tumor cells in pseudoglandular or acinar structures show a columnar apicobasal polarity (simple-type polarity) that differs from hepatic hepatocyte-type

polarity [4], which is required for the efficient removal of excreted bile [5]. *CTNNB1*-mutated HCC is also classified in the nonproliferative molecular subgroup of tumors. In contrast, non-*CTNNB1*-mutated HCCs display heterogeneous histopathological phenotypes, usually with a macrotrabecular growth pattern and poorer differentiation; steatosis is observed in approximately 30% of cases, and cholestasis is infrequent [1–3].

Aside from its oncogenic function in HCC when aberrantly activated, the Wnt/ β -catenin canonical pathway also plays a critical role in liver physiology as it is the master regulator of the establishment of hepatic metabolic zonation [6].

We previously sought candidate genes that could explain the distinctive phenotype of *CTNNB1*-mutated HCCs and identified liver kinase B1 (LKB1), encoded by *STK11*. *STK11* encodes a serine/threonine kinase that functions as a tumor-suppressor gene. Germline mutations of *STK11* are associated with Peutz–Jeghers syndrome (PJS), an autosomal-dominant disorder that is linked to an increased risk of cancer [7,8]. However, somatic *STK11* mutations are not commonly found in sporadic cancers, except lung cancer [9]. The active form of LKB1 is found in a heterotrimeric complex with STRAD and MO25, which regulate its stability, kinase activity, and subcellular localization [10]. LKB1 has been called a ‘master kinase’ due to its ability to phosphorylate at least 14 downstream proteins; the AMP-activated protein kinases (AMPK); and 12 proteins related to AMPK, called ARK for AMP-related kinase (ARK) [11]. LKB1 regulates multiple biological pathways, including those involved in cell polarity, cell metabolism, and cell growth and proliferation [7,8]. Strikingly, LKB1 is a cell-autonomous determinant of the apical polarity of epithelial cells and can, for example, polarize single, isolated intestinal epithelial cells [12]. It negatively controls cell proliferation via the AMPK/mTOR, *TP53*, and YAP cell growth signaling pathways [7,8]. Finally, LKB1 coordinates the metabolic response to energetic stress, activating catabolic processes, such as fatty acid oxidation, and inhibiting anabolic pathways, such as lipid synthesis [7]. Therefore, the morphologically specific signature of *CTNNB1*-mutated HCCs, namely, low proliferation, the lack of steatosis, and the acquisition of a simple type of polarity, may be consistent with the activation of LKB1 in such tumors. This would confer a counterintuitive oncogenic function to *STK11*, which is usually considered to be a tumor-suppressor gene. However, this oncogenic function of LKB1 has already been observed in other settings [13,14]. In addition, numerous studies described molecular cross talk between LKB1 and the Wnt/ β -catenin signaling pathway [15–22].

Here, we showed that LKB1 is overexpressed in *CTNNB1*-mutated HCC relative to nonmutated HCC and that aberrant activation of β -catenin signaling induces post-transcriptional accumulation of LKB1. Using an *Lkb1* transcriptomic signature that we recently established using a liver-specific deletion of *Lkb1* in embryonic liver [23], we confirmed that

CTNNB1-mutated HCC express an LKB1 program. In addition, we found that LKB1 is required for specific physiological functions of β -catenin in the liver and exerts a physiological role as a positive modulator of β -catenin-dependent metabolic zonation.

Methods

Tumor specimens

We selected 48 samples from the human HCC cohort of patients treated for liver cancer in Cochin Hospital (Paris, France), previously described by Audard *et al* [4], for which we had both frozen tumor and nontumor liver samples. Half of the tumors were *CTNNB1*-mutated, and half were not. All tumor samples were frozen after surgery in accordance with French laws and ethical guidelines. The frozen samples were used for western blotting analyses. We also selected a validation set of human HCCs to confirm the expression of the hepatic *Lkb1* signature in *CTNNB1*-mutated HCC for which the RNA was already available [4]. The validation set was composed of samples of 18 *CTNNB1*-mutated HCCs and 22 non-*CTNNB1*-mutated HCCs, together with six human normal liver samples. The mutational status of the *CTNNB1* gene in these samples has been described previously [4].

Animals

Mice with a specific deletion of *Lkb1* in embryonic liver, *Lkb1^{lox,lox};Alfp-Cre* mice called LKB1KO^{Livemb}, have already been described [23]. All animal procedures were carried out according to French legal regulations and approved by an ethical committee (CEEA34.CP.077.12). All mice were kept in well-controlled animal housing facilities with free access to tap water and food pellets.

Cell culture and transfection assay

HuH7, HepG2, and HuH6 cells were grown in Dubelcco’s modified Eagle’s medium (DMEM; Gibco, Life Technologies, Carlsbad, CA, USA) with 4.5 g/l glucose, supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin at 37 °C in 5% CO₂.

For siRNA transfections, cells were seeded in six-well plates (0.3 × 10⁶ cells per well) and reverse-transfected with 100 pmol siRNA in the presence of Lipofectamine RNAiMAX Reagent (Life Technologies) according to the procedure provided by the manufacturer. *CTNNB1* Stealth Selected RNAiTM siRNAs were obtained from Life Technologies (HSS102460, HSS102461, referred as siRNA1 and siRNA2 in Figure 1, respectively). The StealthTM RNAi negative control duplex (referred to as Scrambled) was used as a negative control for sequence-independent effects. Cells were collected 72 h after transfection. For experiments described in

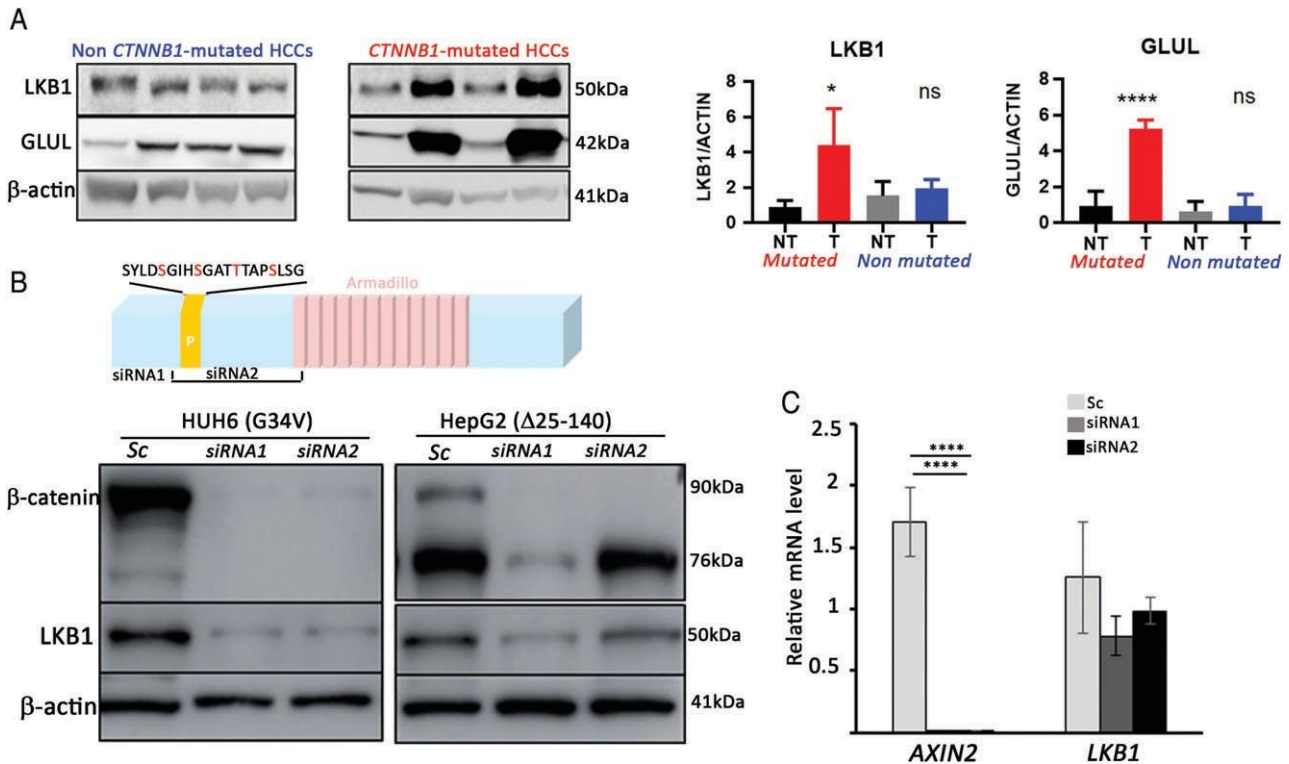


Figure 1. Oncogenic β -catenin drives LKB1 protein expression. (A) LKB1 accumulates in a large subset of *CTNNB1*-mutated HCCs. Western blotting analysis of LKB1, GLUL (a β -catenin target gene in the liver), and β -actin (loading control) in HCCs (T), selected based on their *CTNNB1* mutational status. NT indicates matched nontumor liver tissue for each HCC sample. Two independent representative samples are shown. Quantification of the western blots for GLUL and LKB1 is shown on the right. Statistical significance was evaluated using a two-sample unpaired Student *t*-test between KO and WT. **** $p < 0.001$. ** $p < 0.01$. * $p < 0.05$. $n = 24$ in each group. (B) Oncogenic β -catenin drives LKB1 protein expression in Huh6 and HepG2 cells. Huh6 and HepG2 cells were treated with scrambled siRNA (Sc) or various siRNAs against *CTNNB1* mRNA (siRNA1, siRNA2) and harvested 72 h after transfection. Western blotting analysis of β -catenin, LKB1, and β -actin (loading control). The diagram above the western blots depicts the localization of the siRNAs along the β -catenin coding sequence and points to the position of siRNA2 in the deleted *CTNNB1* sequence in HepG2 cells. (C) β -catenin drives LKB1 expression at the post-transcriptional level. RT-qPCR analysis of *AXIN2* and *LKB1* gene expression of HUH6 cells transfected with siRNA against *CTNNB1* or scrambled siRNA, as described in (B). Mean from three independent experiments. Error bars: standard deviations. Statistical significance was evaluated using a two-sample unpaired Student's *t*-test between KO and WT. **** $p < 0.001$.

Figure 2, we used the SMARTpool technology of Dharmacon to inactivate *CTNNB1* in *CTNNB1*-mutated (HUH6 and HepG2) and not mutated (HUH7) hepatoma cell lines. The siCTNNB1 (siCat) was ON-TARGET plus SMARTpool #L-003482-00-0005 and the scrambled control (Sc) #D-001810-01-05. A total of 20 pmol siRNA was used per well in six-well plates. Cells were collected 48 h after transfection.

RNA extraction and RT-PCR

Total RNA was extracted from mouse tissues and cell lines with Trizol Reagent (Life Technologies) according to the manufacturer's protocol. Reverse transcription was performed using 1 μ g total RNA and the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Life Technologies, Courtaboeuf, France) and random hexamers as primers. Quantitative PCR reactions were run using the Light Cycler 480 Sybr Green I Master Kit (Roche Diagnostics) and specific primers (Eurogentec, Eurogentec France SASU, Nantes, France) on a Light Cycler 480 thermocycler (Roche Diagnostics). Values were normalized to 18S ribosomal RNA

levels. For the mouse data, expression of the β -catenin target genes was expressed relative to that in the livers of WT animals ($n = 6$ animals) as an n -fold ratio. For the human data, gene expression in the tumor samples was compared to the mean level of the corresponding gene expression in normal liver tissues ($n = 6$), expressed as an n -fold ratio. For human hepatoma cell lines data, gene expression in the siRNA *CTNNB1*-treated samples was compared to the scrambled-treated samples ($n = 3$). The relative amount of RNA was calculated using the 2-delta CT method. Primer sequences are shown in supplementary material, Table S1.

Immunoblotting analyses

Total protein extracts from human and mouse liver were obtained from 20 to 200 mg frozen tissue that was bead-mill homogenized in lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM DTT, 0.1 mM 4-benzenesulfonyl fluoride, and 1% Triton X-100), supplemented with a mixture of protease and phosphatase inhibitors (Roche Diagnostics) in a 10 μ l/ μ g ratio using a TissueLyser disruption system

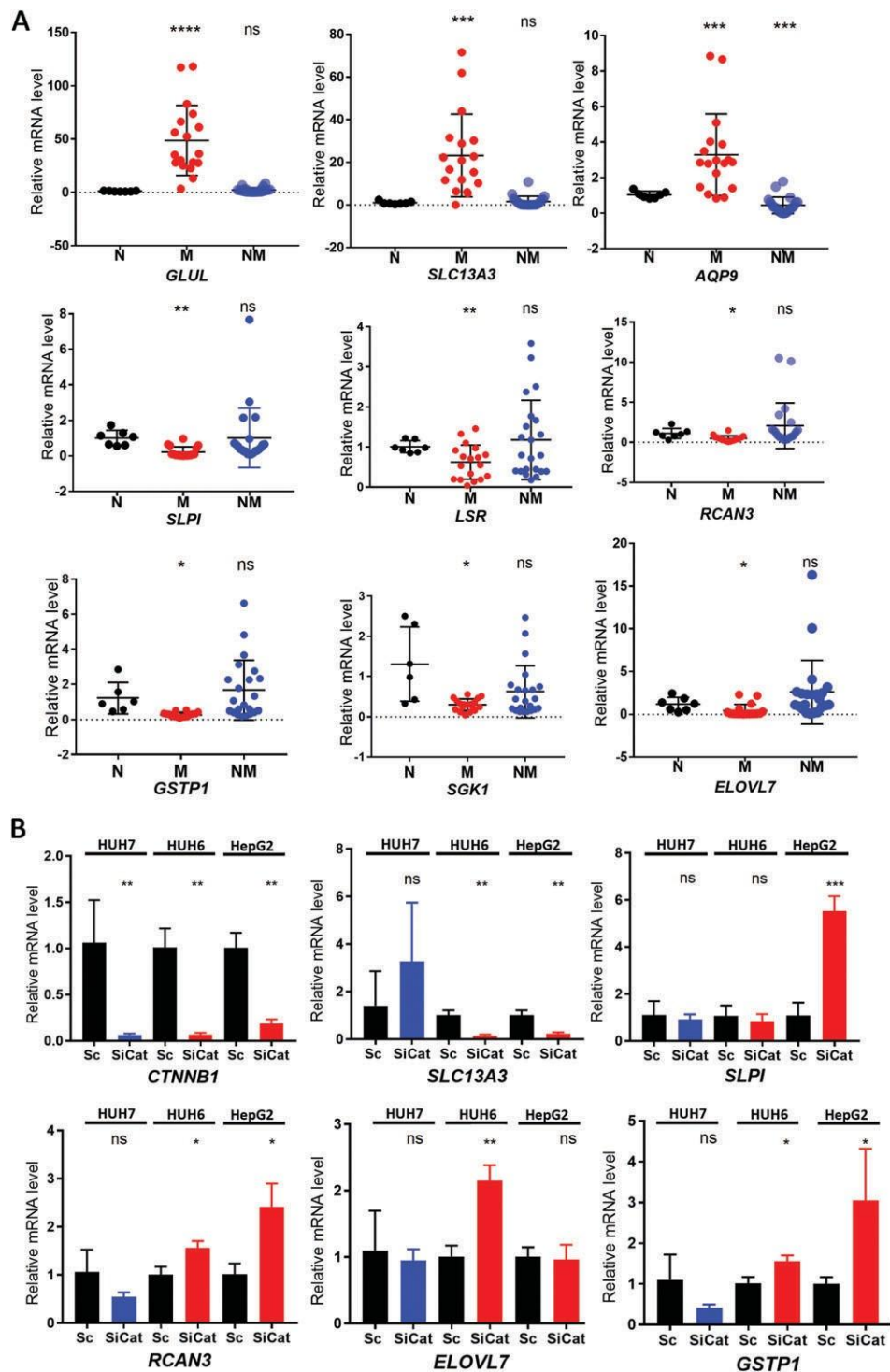


Figure 2. *CTNNB1*-mutated HCC express a hepatic *Lkb1* program. (A) *LKB1* expression profile in *CTNNB1*-mutated HCC. RT-qPCR was performed for the positively- (*GLUL*, *SLC13A3*, *AQP9*) and negatively- (*SLPI*, *LSR*, *RCAN3*, *GSTP1*, *SGK1*, *ELOVL7*) *LKB1*-regulated genes described in supplementary material, Figure S1 using mRNA isolated from normal human liver (N), *CTNNB1*-mutated HCC (M), and nonmutated HCC (NM). (B) *LKB1* expression profile in human hepatoma cell line after β -catenin silencing. β -Catenin silencing was performed using siRNA against *CTNNB1* (SiCat) and scrambled (Sc) as controls. RT-qPCR was performed for *CTNNB1* and the positive (*SLC13A3*) and negative (*SLPI*, *RCAN3*, *ELOVL7*, *GSTP1*) *LKB1*-related genes. HuH6 and HepG2 are *CTNNB1*-mutated hepatoma cell lines, while HuH7 is not mutated for *CTNNB1*. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

(Qiagen, Hilder, Germany). Samples were centrifuged at $13\,000 \times g$ for 10 min at 4°C , and the supernatants were collected and stored at -80°C until analysis. For human hepatoma cell lines, protein was extracted from the six-well plates using Laemmli buffer (Sigma-Aldrich,

Saint-Quentin Fallavier, France). Proteins were resolved by SDS – PAGE and then transferred to nitrocellulose, which was then blocked using 5% BSA or 5% milk. Blots were incubated with specific primary antibodies overnight at 4°C , washed, incubated with the

corresponding horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Ozyme, Saint-Quentin en Yvelines, France), and developed by enhanced chemiluminescence (Thermo Fisher Scientific, Waltham, MA, USA). Images were recorded using a 3.2-megapixel super CCD camera driven by a LAS 4000 mini device (GE Healthcare Europe GmbH, Velizy Villacoublay, France) and quantified using Multigauge software from Fujifilm (Fujifilm France, Asnières, France). Expression was standardized to the β -actin level and normalized to a nontumor sample that was set to 1. Primary antibodies were purchased from Cell Signaling Technology [LKB1: clone D60C5, dilution 1/1000; β -actin (8456, dilution 1/2000); GAPDH (97166, dilution 1/500); Gsk3 β (4337, dilution 1/1000), phosphoGsk3 β (9323 (S9), dilution 1/2000)] and BD Biosciences (BD, Transduction Laboratories, Rungis, France) (β -catenin (811054, dilution 1/2000) and glutamine synthase (GLUL) (610518, dilution 1/2000).

Immunohistochemistry and *in situ* hybridization

Mouse livers were cut into 3-mm-thick slices, fixed in 10% formalin for 12 h, and embedded in paraffin. For morphological analysis, sections were cut at 2- μ m thickness, dewaxed, rehydrated, and stained with hematoxylin and eosin. The protocols for glutamine synthetase immunohistochemistry and *in situ* hybridization have been described previously [6].

Human gene expression analysis

The data for GSE62232, which includes Affymetrix U133plus v2 array (GPL570) data for 81 HCC liver tumors samples, were downloaded in an already normalized matrix format via Gene Expression Omnibus. The *CTNNB1* mutations in this sample collection were reported in [22,23]. Hierarchical clustering was performed using Genesis clustering software as described in [24].

Statistical analysis

Data were analyzed using unpaired *t*-tests with Welch's correction in GraphPad Prism 7.04 (GraphPad Inc., San Diego, CA, USA).

Results

Activated LKB1 signaling occurs in *CTNNB1*-mutated HCC

We first analyzed LKB1 protein expression in a series of 48 human HCCs, selected based on their *CTNNB1* mutational status (24 with *CTNNB1* mutations and 24 without). Total proteins were extracted from cryoconserved tumor samples and matched nontumor liver samples for each patient. The very high expression of GLUL, a well-known β -catenin target in the

liver [25], confirmed activation of the β -catenin pathway in the *CTNNB1*-mutated tumors. LKB1 protein levels were significantly higher in *CTNNB1*-mutated HCCs than in nontumor liver tissue, whereas those in non-*CTNNB1*-mutated HCCs were no different from those in nontumor liver tissue (Figure 1A).

We then investigated whether activated β -catenin was able to stimulate LKB1 protein expression in hepatoma cell lines. HuH6 and HepG2 are two hepatoma cell lines with gain-of-function mutation alterations of *CTNNB1* (a p. G32V mutation and p. 25_140 deletion, respectively, within the DNA sequence encoding the degradation domain of β -catenin) [26]. In HuH6, two different siRNAs against *CTNNB1* mRNA, but not a scrambled siRNA, decreased LKB1 protein expression in parallel with β -catenin inactivation (Figure 1B). This was also true for HepG2 cells with siRNA1, but not siRNA2, which is targeted against a sequence deleted in the HepG2 mutated allele, preventing its inactivation (Figure 1B). This result shows that activation of the mutant β -catenin was required for the control of LKB1 protein expression (Figure 1B). While, as expected, RT-qPCR analyses showed that the mRNA levels of the canonical β -catenin target gene *AXIN2* were strongly reduced after *CTNNB1* inactivation mediated by siRNA, the *LKB1* mRNA levels were not affected by β -catenin silencing, indicating that the decrease in LKB1 protein observed in Figure 1B was not associated with a decrease in *LKB1* mRNA level (Figure 1C). These results show that the aberrant activation of β -catenin signaling induced the accumulation of LKB1 protein in liver tumor cells, which was regulated post-transcriptionally.

We then explored whether *CTNNB1*-mutated HCC expressed an *LKB1* program. This was performed using the liver *Lkb1* signature of 253 genes that we recently obtained from the study of mice bearing a specific *Lkb1* deletion in embryonic liver hepatoblasts, *Lkb1^{lox,lox}*; *Alfp-Cre* mice, designated LKB1KO^{Livemb} [23]. We studied the human HCC public dataset (GSE62232, 81 samples), for which data on the genetic status of *CTNNB1* and the RNA expression profile were available (described in reference [24]). Unsupervised hierarchical clustering of the GSE62232 human HCC dataset, using the hepatic *Lkb1* signature, grouped almost all *CTNNB1*-mutated HCCs within a single cluster, showing that most *CTNNB1*-mutated HCCs share a hepatic *Lkb1* program (see supplementary material, Figure S1). The hepatic *Lkb1* signature was composed of genes up- and downregulated in the liver of KO versus WT animals and accounted for a program suppressed by *Lkb1* in mouse hepatocytes. Thus, most (19/27) *CTNNB1*-mutated HCCs displayed activated *LKB1* signaling (see supplementary material, Figure S1). We confirmed these results using an independent cohort of human HCCs, consisting of *CTNNB1*-mutated and non-*CTNNB1*-mutated tumors, by RT-qPCR. The expression of several *LKB1*-related genes was statistically enhanced (*GLUL*, *SLC13A3*, *AQP9*) or repressed (*SLPI*, *LSR*, *RCAN3*, *GSTP1*,

SGK1, *ELOVL7*) in *CTNNB1*-mutated HCCs relative to nonmutated HCCs (Figure 2A). We validated the cross talk between β -catenin and LKB1 by analyzing the *LKB1* gene expression profile in *CTNNB1*-mutated (HuH6, HepG2) and nonmutated (HuH7) HCC cell lines after β -catenin silencing using siRNA. Although some genes were discarded as they were not expressed in the hepatoma cell lines, the expression of the positive and negative *LKB1*-related genes were significantly repressed or enhanced, respectively, after β -catenin silencing in *CTNNB1*-mutated HCC cell lines but were not affected in the nonmutated HCC cell line (Figure 2B).

Overall, our data show that β -catenin signaling in *CTNNB1*-mutated HCCs induces a post-transcriptional accumulation of LKB1, which in turn activates an LKB1 program likely contributing to the specific histological appearance of *CTNNB1*-mutated HCCs.

LKB1 positively regulates β -catenin signaling in liver zonation

Cross talk between Wnt/ β -catenin and *LKB1* signaling has been described in several studies, with often conflicting results [15–22]. The first pioneering study showed *LKB1* to be upstream of β -catenin signaling and to be required for full activation of β -catenin signaling in *Xenopus* development [15]. We explored whether *LKB1* may control Wnt/ β -catenin signaling in the mouse liver in a physiological situation in which the canonical Wnt pathway is known to have a critical function, such as metabolic zonation [6]. We thus examined liver zonation in *Lkb1*-deficient mice (LKB1KO^{Livemb}) [23]. Immunohistochemistry and *in situ* hybridization for *Glul* (glutamine synthase) and *Arg1* (arginase 1), together with immunohistochemistry for Lect2 (Leukocyte cell-derived chemotaxin 2) and Hal (histidine ammonia-lyase), showed that *Lkb1* deletion strongly affected liver zonation in LKB1KO^{Livemb} mice, with almost complete loss of the perivenous expression of *Glul* and Lect2, while the periportally expressed gene *Arg1* and Hal protein were overexpressed (Figure 3A). Accordingly, analysis of a 16-gene signature of liver β -catenin target genes confirmed decreased Wnt/ β -catenin signaling in LKB1KO^{Livemb} mice relative to controls. This decrease in β -catenin activity was confirmed by RT-qPCR using a different group of animals (Figure 3B,C). These results show that *Lkb1* is a positive modulator of Wnt/ β -catenin signaling during liver development and is required for the full establishment of liver zonation.

Previous studies also reported that LKB1 controls the Wnt/ β -catenin pathway through two different kinases, GSK3 β and PAR1A (MARK3) [15–17]. We analyzed the phosphorylation status of GSK3 β (pS9-GSK3 β) in the livers of LKB1KO^{Livemb} mice by western blotting and observed higher levels of pS9-GSK3 β in the mutant mice than in controls (Figure 3D). We were unable to detect phosphorylation of Par1a (Mark3) or any other Mark (data not shown). The decrease in β -catenin

signaling activity in LKBKO^{Livemb} mice did not correlate with increased phosphorylation of GSK3 β on S9 (known to inhibit GSK3 β function), normally associated with increased β -catenin activity. These results suggest that *Lkb1* controls the canonical Wnt pathway in hepatocytes, independent of GSK3 β .

Discussion

Wnt/ β -catenin signaling regulates critical functions in liver physiology, and its aberrant activation is found in several liver diseases, including HCC [27]. HCCs generally display a high level of heterogeneity [28]. However, we previously showed that *CTNNB1*-mutated HCCs form a homogeneous group of tumors, with specific cell metabolism, polarity, and growth hallmarks [4]. Here, we investigated whether the activation of *LKB1* signaling could explain the specific phenotype of *CTNNB1*-mutated HCC as *LKB1* is considered to be a master regulator of cell growth, metabolism, survival, and polarity [7,8]. We showed that *CTNNB1*-mutated HCCs display significantly higher LKB1 protein expression than non-*CTNNB1*-mutated HCCs. *CTNNB1*-mutated HCCs expressed a hepatic *LKB1* signature that we recently identified in mouse *Lkb1*-deficient livers [23]. Reciprocal positive control of the canonical Wnt pathway by *Lkb1* during development was previously described [15]. This led us to investigate whether *Lkb1* controls liver metabolic zonation, a well-established function of β -catenin signaling in the liver [6]. Our data show that *Lkb1* is required for the full activation of β -catenin signaling in mouse liver and is thus a positive regulator of the establishment of metabolic zonation.

Cross talk between Wnt/ β -catenin and *LKB1* signaling in both directions (β -catenin toward *LKB1* and *LKB1* toward β -catenin) has been described in many studies. It appears that the dialog depends on the context, developmental or oncogenic, with either a positive or a negative impact [15–22]. The molecular mechanisms involved are yet to be determined for all contexts. Here, we showed that oncogenic β -catenin signaling induced the accumulation of LKB1 protein in *CTNNB1*-mutated HCC. The regulation of *LKB1* is complex and still not well characterized. It is controlled epigenetically, transcriptionally, and post-translationally through the modulation of protein stability [29]. Little is known concerning the mechanisms that regulate the stability of LKB1 protein: the ubiquitin ligase CHIP has been proposed to be involved [30], but this still needs to be confirmed. A recent study reported overexpression of *LKB1* in human HCC that predicts poor survival outcomes. However, this was at the mRNA level and, thus, does not coincide with our study in which we showed accumulation of the LKB1 protein in *CTNNB1*-mutated HCC [31]. For the inverse relationship (control of β -catenin signaling by *Lkb1*), we showed that deletion of *Lkb1* in mouse hepatocytes led to a marked decrease

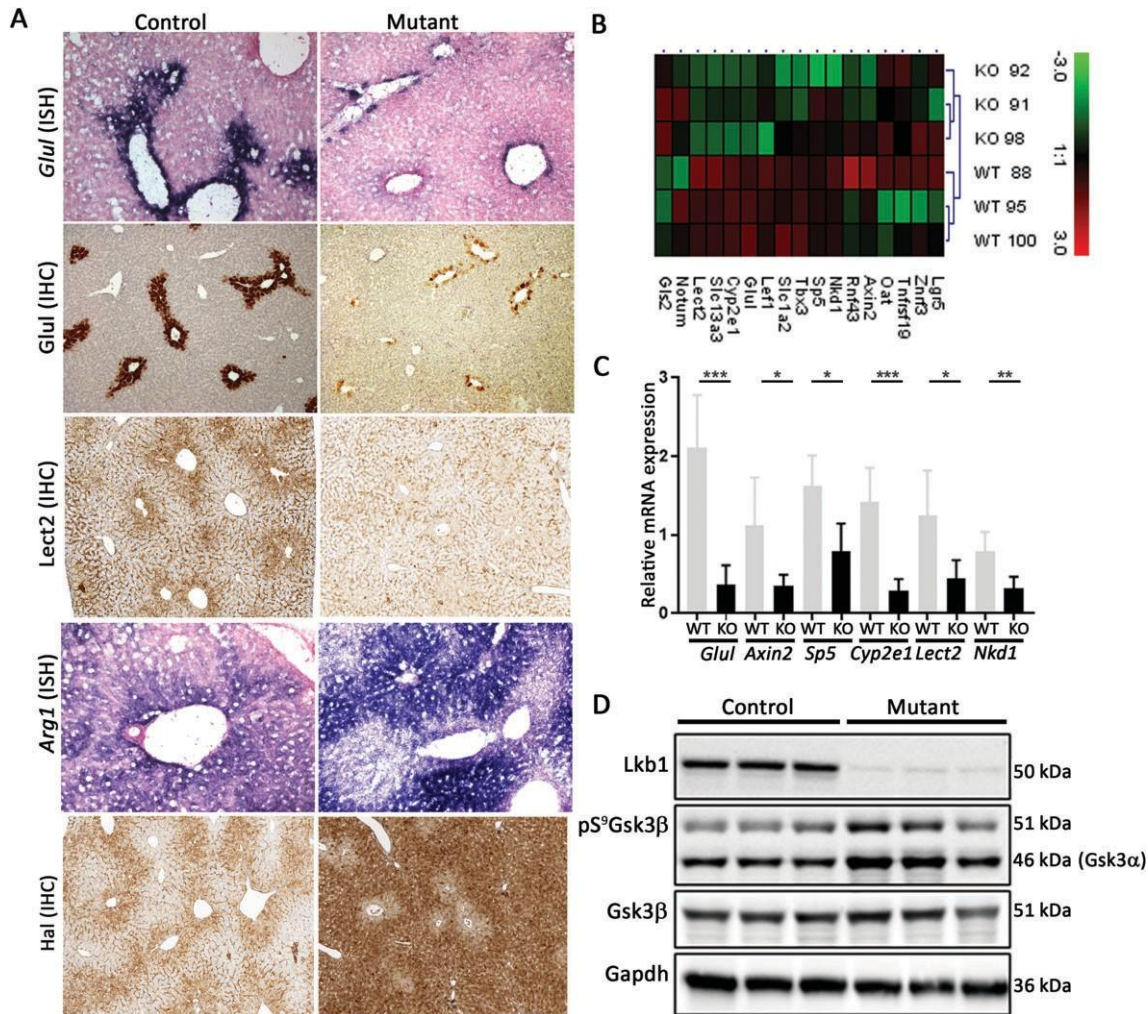


Figure 3. *Lkb1* positively regulates the Wnt canonical pathway and controls liver zonation. (A) Representative images for the expression of positively regulated β -catenin target genes, *Glul*, by immunohistochemistry and *in situ* hybridization and *Lect2* by immunohistochemistry together with negatively regulated β -catenin target genes, *Arg1*, by *in situ* hybridization and *Hal* by immunohistochemistry. LKB1KO^{livemb} mutant and control and mice were 3 weeks old. (B) Heat map of the liver β -catenin target gene signature from the gene expression profile of LKBKO^{livemb} mice [23]. (C) RT-qPCR analysis of various positively (*Axin2*, *Sp5*, *Glul*, *Lect2*, *Cyp2e1*, *Nkd1*) regulated β -catenin target genes in 14-day-old control and LKBKO^{livemb} mutant mice. $n = 4-6$ per group. Error bars: SEM. Statistical significance was evaluated using a two-sample unpaired Student's *t*-test between KO and WT. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (D) Western blotting analysis showing increased pS⁹Gsk3 β phosphorylation in mutant LKBKO^{livemb} mice relative to controls.

in liver zonation controlled by Wnt/ β -catenin. This was associated with increased phosphorylation of Gsk3 β (S9-Gsk3 β), leading to its inactivation. This result does not correlate with the decreased activation of β -catenin signaling we observed in the mutant mice and suggests that *Lkb1* controls β -catenin signaling independent of Gsk3 β . Overall, we identified positive reciprocal cross talk between β -catenin and *LKB1*, in which β -catenin activity induces LKB1 expression, leading to the activation of *LKB1* signaling, which in turn positively regulates β -catenin signaling. Such cross talk likely results in the amplification of β -catenin signaling by *LKB1* in *CTNNB1*-mutated HCC.

A positive role for *LKB1* signaling in *CTNNB1*-mutated HCC could suggest a synthetic lethal interaction between the Wnt pathway and LKB1. Although we have not been able to substantiate such an interaction (data not shown), a prosurvival role of LKB1

has already been described. Indeed, *Lkb1*-deficient mouse embryonic fibroblasts showed resistance to transformation by oncogenes, suggesting that the high energetic demands of malignant transformation may be incompatible with *Lkb1* loss [32]. Indeed, tumor cells must sense changes in their microenvironment to support unfettered cell proliferation, and the inability to respond to such environmental cues may lead to energetic stress and ultimately cell death. *LKB1* is an energetic sensor, and its function is required for the survival of tumor cells under energy stress [13]. Accordingly, *LKB1* is upregulated in human skin cancer cells and has been shown to be required for preventing apoptosis in several human cancer cell lines overexpressing AKT [33]. Indeed, the prosurvival role of *Lkb1* is not restricted to tumor cells but has also been described for quiescent hematopoietic stem cells [34-36]. Overall, these data show that *LKB1* may provide cancer cells with

the metabolic flexibility required to survive under conditions of nutrient stress, thus conferring a growth advantage. *LKB1* is likely to play this role in *CTNNB1*-mutated HCC. Furthermore, we recently showed that, in *CTNNB1*-mutated HCC, the tumor cells use fatty acids for energy, corroborating the lack of steatosis of those tumors [37]. This correlates with the positive role of *LKB1* on fatty acid oxidation in the liver [7].

In addition to the function of *LKB1* in metabolism, *LKB1* signaling also controls cell polarity, inducing apicobasal polarity in a cell-autonomous manner [12]. This could partially explain the cholestatic phenotype of some *CTNNB1*-mutated HCCs, mainly those with pseudoglandular structures [4]. The polarity present in acinar structures differs from the hepatic polarity required for the removal of bile excreted by hepatic cells [5]. It is possible that, in *CTNNB1*-mutated HCC, bile is secreted at the apical pole of tumor cells and accumulates in the lumen of acinar structures and cannot be removed from the tumor, leading to cholestasis. *LKB1* has been reported to be involved in the establishment of liver cell polarity [23,38]. However, the cholestasis observed in *CTNNB1*-mutated HCC is also linked to the positive effect of β -catenin signaling on bile acid metabolism that we previously described [39].

In *CTNNB1*-mutated HCC, *LKB1* may act as a tumor-suppressor gene, conferring a better prognosis, apicobasal polarity, and a well-differentiated growth pattern, but may also be required for tumor cell survival, acting more likely as an oncogene. Increased genomic instability is one of the various mechanisms by which *LKB1* loss leads to tumor development [40]. Accordingly, we recently showed that *Lkb1* inhibits liver cell proliferation and favors mitotic integrity [41] that could explain why *CTNNB1*-mutated HCCs belong to the chromosomally stable HCC subclass [28].

We have demonstrated reciprocal cross talk between *LKB1* and β -catenin, in which β -catenin enhances *LKB1* protein expression, favoring expression of the hepatic *Lkb1* program that enhances β -catenin signaling. In the context of *CTNNB1*-mutated HCCs, such cross talk results in a positive activation loop that amplifies the β -catenin signaling.

Acknowledgements

We are very thankful to Christelle Desbois-Mouthon for her help in management of human hepatoma cell lines. We also thank people of the team for their critical discussions and people at the Animal Facility of Cochin Institute who took care of the mice.

Author contributions statement

CP, PAJ, and SC conceived the study, carried out experiments and analyzed data. MS, SA, MT, NM, and RT carried out experiments. CC and BT characterized HCC at the genomic level. All authors were involved

in writing the paper and had final approval of the submitted version.

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