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Hnf4 α and Choline Metabolism Role in β -catenin Activated Liver Carcinogenesis

Chiara Sartor

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Chiara Sartor. Hnf4 α and Choline Metabolism Role in β -catenin Activated Liver Carcinogenesis. Molecular biology. Université Paris Sud - Paris XI, 2015. English. NNT: 2015PA11T046. tel-01236633

HAL Id: tel-01236633

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UNIVERSITÉ PARIS-SUD
ÉCOLE DOCTORALE 418 :
DE CANCÉROLOGIE
Laboratoire : Inserm U1016-Institut Cochin

THÈSE DE DOCTORAT
ASPECTS MOLÉCULAIRES ET CELLULAIRES DE LA BIOLOGIE

par
Chiara SARTOR

Hnf4 α and choline metabolism role in β -catenin activated
liver carcinogenesis

Date de soutenance : 24/09/2015

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ACKNOWLEDGEMENTS

I would like to thank my committee members, who have accepted to evaluate and discuss my work, in particular Agnès Ribeiro and Yu Wei for their comments and feedback on my manuscript.

I would like to express my special appreciation and gratitude to my advisor, Sabine. I would like to thank you for encouraging my work and for allowing me to grow as a scientist.

I would like to thank Christine for welcoming me in her team and all its members for the work environment they create.

A special thanks is for the people of our little “sub-group”. I want to thank Angélique and Cécile for being there when I arrived, and for teaching me a lot of secrets and tricks in practical lab life. Angélique, I am so happy that you have obtained your surely deserved CR1 position. Your scientific brilliance is a model. Cécile, your expertise and your enthusiastic way to approach new techniques always surprises me. Rozenn, I have to thank you mostly for being yourself. Don't stop and don't change. A special thanks also for being a wonderful cat-sitter. I'll write you a reference letter if you need it. Anaïs, you have just started your scientific experience, be focused and you will obtain wonderful results, I wish you the best. I would like to warmly thank Pascale and Nadia that are a precious help in the metabolic field. I enjoyed working with you, not only for the results obtained, but also, and maybe mostly, for the atmosphere during the experiments. You are as good in science as in real life.

Béa and JP, my consideration of your scientific capability is unquestionable, but I want to thank you in particular for other practical advices that you gave me. From the beginning you have been really welcoming with me and I am very grateful for this.

I would like to thank the “youngs”, Angé, Rozenn, Nadia, Anaïs, Marie, Julie, Coralie, Séverine, Vanessa, Manon, Sandrine and Antoine for being always there when there is a surprise to prepare, a birthday (or whatever) party to join. It is nice to spend happy moments with nice people at work and to find not only colleagues but also friends.

I would like to express my gratitude also to all the members of the team that have already left, in particular to Johny, Fred, Medhi, PAX, Laura, Ingrid, Aline. We had a great time together.

Anne-Marie, Jocelyne and Jean Christophe, I have a high consideration for your work, but I appreciate you in particular for your jokes and your direct (and not always politically correct) opinions. I enjoyed talking with you at lunch time about cats, books, trips and a lot of other topics. I really appreciate your attentions (i.e. fried vegetables during the PhD redaction, merci!!).

I would like to thank Carole's team, past and present members for being always available and nice with their neighbors. A special thank to Sara Zum, friend in real and lab life: your presence was often very important for not

going crazy, and also to Sabine, my favorite yoga mate, and Mariangela: keep calm and sii brava!

I would also like to thank Christophe Ferreira for being my reference during the year of my “mission d’expertise”. Your professionalism and competence taught me a lot.

I would especially like to thank people of Cochin and Tenon facilities. The histology and genomic facilities. The animal care members, in particular the ones of the “animalerie FAC”, Isabelle, Claire and JB. The PIPA’s members, especially Gilles, Franck and Carmen. The Limp members, Aurélie and Claire. I thank you all for your kindness and availability. All of you have been there to help me during my PhD experiments, demonstrating huge competence.

Je tiens particulièrement à remercier la Ligue contre le cancer pour avoir financé ma 4^{ème} année de thèse et pour avoir donc permis de conclure ces travaux.

A PhD is mostly a work experience. But it wouldn’t be the same without my wonderful “non-biological” family.

In primis gli amici storici Zum ed Erigo, dagli spriz e pomeriggi di studio a Padova siamo finiti a ritrovarci per un apéro post lab lungo Senna! Dopo questi anni in cui le nostre esperienze hanno coinciso, sarà un grosso cambiamento non avervi vicini. Aspetto solo che diventiate ricercatori con posto fisso per farmi assumere.. :) Un grazie speciale anche a Silvio, alla Giacomina, a Pietro Gori e Alice, presenze anche loro storiche e sempre piacevoli!

I Parigini DOC che si sono rivelati ottimi amici, nei momenti più festivi, quand’eravamo giovani erasmus, come nei momenti più buii: Fiask, Cigna, Eli Omodei, Matteo Cognome, Diana&Diana, Della Marina, Marchesini, Andrea Araldo, Sam, Anthony, Ag, Bizz, Danielina, François, Arianna, Lorenzo, Michele, Amedeo. Abbiamo condiviso tanti momenti, compleanni, feste a tema, cene, aperitivi, concerti, spettacoli, viaggi, matrimoni. Un grazie anche ai Parigini a breve termine: Omar e Paolo, Cattaneo, Chiaretta, Ciuffo, Campo e perché no anche un po’ Iacopo.

Grazie ragazzi, credo che la fortuna di avervi incontrato e di aver creato questo gruppo sia stata eccezionale.

I would like to express all my gratitude to my parents and my sister.

Il vostro sostegno è stato elemento cardine per arrivare al punto in cui sono oggi. Siete sempre stati un modello di vita e un motivo per impegnarmi. Grazie di tutto l’appoggio che mi avete sempre dato, vi voglio bene.

Un grazie anche alla mia sorella ad honorem Vale: nonostante la distanza, continui ad essere un’ottima amica.

Last but not the least, thanks to my little “kind of” family, Banti e Loli. Per esserci stati anche nei momenti più complicati e per continuare a sopportarmi quando vi rubo la coperta. <3

“Per aspera [sic itur] ad astra”

- L. A. Seneca - Hercules furens, II, 437

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LIST of ABBREVIATIONS:

A

α -SMA : α -smooth muscular actin
ACC : Acetyl-CoA carboxylase
ACh : acetylcholine
ACSS2 : acetyl-CoA synthetase enzyme
ADPKD : autosomal dominant polycystic kidney disease
ARPKD : autosomal recessive polycystic disease
AFP : alpha fetoprotein
ALB : albumin
ALD : alcohol liver disease
APC : adenomatous polyposis coli
ATP : adenosine triphosphate

B

BAs : bile acids
BHMT : betaine homocysteine methyltransferase
BMP : bone morphogenetic protein

C

CAR : Constitutive Androstane Receptor
CC : cholangiocarcinoma
CCK : cholecystokinin
ChoRE : glucose or carbohydrate responsive element
CHKA, CHKB : choline kinase α and β
ChREBP : carbohydrate responsive element binding protein
CHT : Choline transporter
CK1 : Casein kinase 1
CK19 : citokeratin 19
CTL : Choline transporter like
CT : computed tomography

CTNNB1 : catenin (cadherin-associated protein) beta 1
CTP (or Pcyt) : phosphocholine cytidyltransferase
CTP : cytosine-5'triphosphate
C/EBP α : CCAAT-enhancer-binding protein α

D

DAG : Diacylglycerol
DEN : N-diethylnitrosamine
DCP : des-carboxyprothrombin
DMBA : 7,12-dimethylbenz[a]anthracene
DNMT1 and 3 : DNA methyltransferase 1 and 3
DPM : Ductal plate malformation

E

ECM : extracellular matrix
EGF : epidermal growth factor (EGF)
EHBD : extrahepatic bile duct
EMT : epithelial-to-mesenchymal transition
ER : endoplasmic reticulum

F

FAP : familial adenomatous polyposis
FAS : fatty acids synthase
FBPase : fructose 2,6 biphosphatase
FDA : food and drug administration
 18 F DG : fluorine-18 fluorodeoxyglucose
FGF : fibroblast growth factor

FNH : focal nodular hyperplasia
FOXA1 : Forkhead box protein A1

G

GK : glucokinase
GLUT-1 : glucose transport 1
GS or GLUL : Glutamine synthetase
GSDs : glycogen storage diseases
GSK-3 : Glycogen synthase kinase 3

H

HAV/HBV/HCV : hepatitis A, B, C virus
HB : hepatoblastoma
HCA : hepatocellular adenomas
HCC : hepatocellular Carcinoma
HDL : high-density lipoprotein
HE : eosin hematoxylin
HGF : hepatocyte growth factor
HGFR : hepatocyte growth factor receptor
HNF 1 α /4 α /6 : hepatic nuclear factor 1alpha/4alpha/6
HHEX : Hematopoietically Expressed Homeobox
HIF : hypoxia-inducible factor 1
HRE : Hnf4 α Responsive Element

I

IL-6 : interleukin-6
INF : interferon
IHBD : intra hepatic bile duct
IHCC : intrahepatic cholangiocarcinoma
IHD1/2 : isocitrate dehydrogenase 1/2
IKK/NF κ B : IKK/nuclear factor-kappa B
IPH : idiopathic portal hypertension

J

JAK : Janus kinase

JNK : jun N-terminal kinase

L

LDHD : lactate dehydrogenase D
LRP 5/6 : Low-density lipoprotein receptor-related protein 5 and 6

M

MAPK : MAP kinase
MCD : methionine choline deficient
MDR2 : multi-drug resistance 2
MODY1/2 : Maturity Onset Diabetes of the Young 1 / 2
MRI : magnetic resonance imaging
MRS : magnetic resonance spectroscopy

N

NAFL : non-alcoholic fatty liver
NAS : National Academy of Sciences
NASH : non-alcoholic steatohepatitis
NSCLC : non-small cell lung cancer

O

OAT : ornithine aminotransferase
OCT/slca22a1 : Organic cation transporters
OSM : oncostatin M
OXPHOS : oxidative phosphorylation

P

PA : phosphatidic acid
PAS : Periodic Acid Schiff
PC : phosphatidylcholine
PCK1 : phosphoenolpyruvate carboxykinase 1
PCP : planar cell polarity
PC-PLC/D : PC-specific phospholipase C / D
PKD-1/2 : polycystin-1 and -2

PDX1 : Pancreatic and duodenal homeobox 1
PE : phosphatidylethanolamine
PEMT : phosphatidyl ethanolamine methyltransferase or Phosphatidylethanolamine N-methyltransferase
PEPCK : Phosphoenolpyruvate carboxykinase
PI3K : phosphoinositide 3-kinase
PKB : protein kinase B (AKT)
PKC : protein kinase C
PSA : prostate- specific antigen
PTEN: phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase

R

RAF : Radiofrequency ablation
ROS : reactive oxygen species

S

SAM : S-adenosyl-L-methionine
SAH : S-adenosyl-L-homocysteine
SMP30 : senescence marker protein
SNP : single nucleotide polymorphism

SOX7-9 : SRY-related HMG-box 7-9
SREB-1c : sterol regulatory element binding protein 1c
STAT : Signal Transducer and Activator of Transcription

T

TAA : thioacetamide
TACE : transarterial chemoembolization
TCA : tricarboxylic acid
TGF- β : transforming growth factor β
TLC : thin layer chromatography
TNF α : tumor necrosis factor alpha
TR β : thyroid hormone receptor β

V

VEGF : vascular endothelial growth factor
VLDL : very low density lipoprotein

W

WRE : Wnt Responsive Element

Abstract:

WNT/ β -catenin is a pillar during development and in adult physiology. In particular in the adult liver it is a double-edged sword: it is necessary to establish the metabolic zonation, requirement for having a functional organ, but it is also involved in the onset of 11-32% of hepatocellular carcinoma (HCC).

My thesis work has been based on the team previous results and it is focused on two main subjects: (1) the first aim was to decipher the role of Hnf4 α both in physiology and in HCC development and its relationship with WNT/ β -catenin signalling and (2) the second part explores the possible use of Fluoro-choline (FCh) positron emission tomography (PET) in the diagnosis of β -catenin-activated liver tumours.

In this study I used cohorts of patients having HCC, but also inducible and hepatospecific knock-out mice for adenomatous polyposis coli (APC) gene (thereafter called ApcKO mice). Apc is the most important negative regulator of β -catenin, and its hepatic loss leads to aberrant activation of β -catenin, disrupting liver zonation and initiating long-term liver cancers.

I generated also inducible hepatospecific Hnf4 α knock-out mice and I demonstrated an increased proliferation, lipids accumulation and disorganization in the portal triad architecture, together with a mild disruption of liver zonation. Then, looking at cancer onset, I demonstrated that Hnf4 α loss is not able *per se* to initiate liver cancer, and has no tumour suppressor role in β -catenin activated tumours onset and progression.

We performed a metabolic analysis of ApcKO livers, showing that β -catenin is able to deregulate lipids metabolism, in particular that of phospholipids derived from choline. In collaboration with clinicians, I studied human patients who underwent FCh/PET, showing that β -catenin-mutated tumours had an increased uptake of F-Choline whereas non-mutated β -catenin human HCC had not. Similar results were obtained with mice, either ApcKO β -catenin-activated HCC or β -catenin-independent mice HCC, obtained through a N-diethylnitrosamine (DEN) injection.

Choline in cells splits in two main pathways: it is both a methyl-group donor and a precursor for phospholipids production. I tested this through radiolabeled fluxes in *in vitro* experiments. In β -catenin activated hepatocytes and tumours there are more phospholipids and more methyl groups in DNA derived from choline than in control mice. Moreover in ApcKO DNA is hypermethylated, and it is dependent on choline supply from diet.

All these results together show the importance for β -catenin activated tumours to have a supply in choline, and so open a way not only in PET exploitation for having a precise diagnosis, but also in deciphering the importance of choline pathway, to possibly develop a targeted therapy.

Introduction

The liver

Liver structure

The adult liver is the second largest organ of the human body (the first is the skin) and it is also second in complexity (after the brain). Its architecture is critical to be functional (Yu et al., 2010), and reflects the wide range of functions that the liver has to fulfill in the body.

Different cell types compose the liver: the hepatocytes are the most abundant ones (70-80%). They are the parenchymal cells that manage lipids, glucides and aminoacids metabolism, bile production, liver regeneration after damage and drug detoxification. This is possible thanks to the cell organelles organization: around 15% of their volume is endoplasmic reticulum (ER), and there is a high amount of peroxisomes, lysosomes and mitochondria. Other parenchymal cells are the cholangiocytes that derive from the same precursor, the hepatoblast, and are the epithelial cells of the bile ducts (as explained in more details in next section). Non-parenchymal cells include Kupffer cells, the liver macrophages, having a role in immune response, stellate cells (involved in liver regeneration, fibrosis and cancer, due to their function of vitamin A storage), and different endothelial cells including sinusoidal endothelial cells (those limiting the sinusoids).

Each cell type has a proper role, but also the communication between hepatocytes and non-parenchymal cells is essential for normal liver function (Duncan, 2003).

The liver epithelium is a unique case among the vertebrate epithelia, as it is very different from the classical monolayer. The monolayer is generally composed by cells with a single basal surface at the opposite site of a single luminal surface, whereas the lateral surfaces are dedicated to cell-cell contact sides. In the liver bile ducts show this classical columnar epithelium organization. On the other hand, the hepatic epithelium is organised in plates one or two cells thick, with multiple luminal and basal domains, positioned in a non-perpendicular way to the lateral surfaces. The presence of multiple

surfaces and moreover the properties of the extracellular matrix (ECM) of the liver which is less dense and crosslinked than usual (due to the absence of laminin and nidogen) are able to maximise the molecules bidirectional exchanges between cells and blood (Musch, 2014).

The hepatocytes constitute the liver parenchyma and they do accommodate a huge macromolecules flow linked to both endocrine and exocrine functions (further detailed in the next section) thanks to the liver position in the body, its connection with the blood supply and the characteristic polarity of the hepatocytes.

The liver unit is the lobule; the lobule is organised along an axis that goes from the portal space to the central vein. In the portal space is located the so-called portal triad, constituted of a bile duct, the hepatic artery branches and the portal vein, which run in parallel. The blood arrives from the hepatic artery (oxygen-rich) and from the portal vein (nutrients-rich) and flows within the lobule through the sinusoids (small capillaries in the spaces between the hepatocytes). This gradient of oxygen, nutrients and hormones creates a specification in molecular functions at the two sides, also called the liver zonation (Jungermann and Kietzmann, 1996). On the contrary the bile flows on the other sense, from the pericentral area to the periportal one (Figure 1).

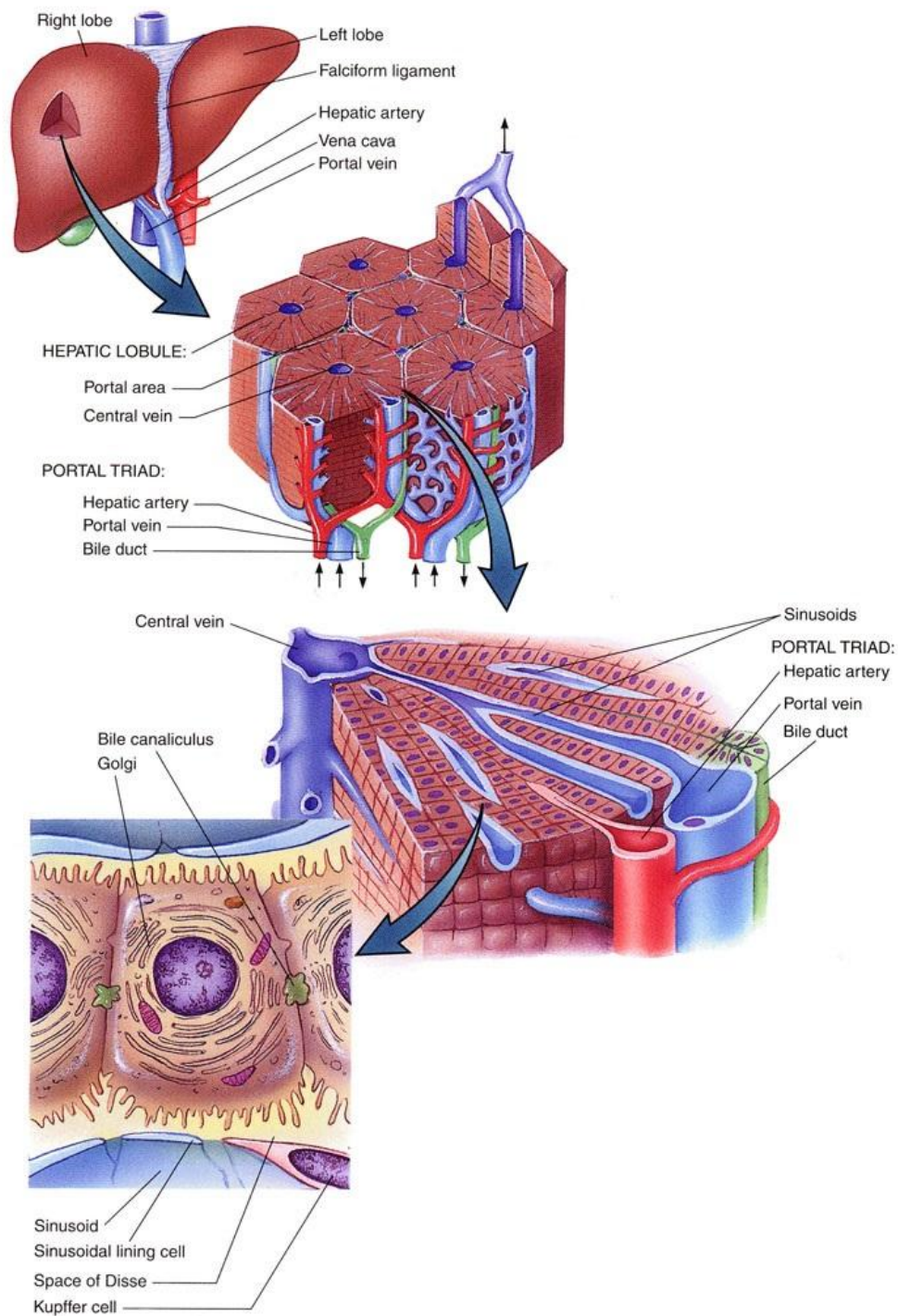


Figure 1. Liver structure.

The liver unit is the lobule with the central vein on one side and the portal triad (hepatic artery, bile duct and portal vein) on the other. Blood flows from the portal space to the central vein, whereas bile does the inverse journey. Hepatocytes are liver cells rich in organelles and polarised: at the apical side bile canaliculi collect bile and at the other side the space of Disse allows the contact of hepatocytes with blood.

Liver functions

The liver has a range of different roles. It is involved in the digestive process, a function strictly correlated with body metabolism control and also with the xenobiotics (drugs) detoxification. It is necessary for retinols activation, catabolism and excretion and during the embryonic life it is the site of hematopoiesis, producing red blood cells, and moreover various plasma proteins. Liver pathologies cause a variety of digestive and metabolic disorders (Yu et al., 2010).

Liver functions are both endocrine and exocrine:

1. The endocrine function is linked to the blood that flows from the portal space. The blood components are uptaken and processed (blood is conditioned through detoxification and serum factors secretion). This is possible thanks to the connections between the basal surface of hepatocytes and the blood flowing through the sinusoidal capillaries into the space of Disse.

Some examples of endocrine function, such as the carbohydrate and lipid metabolism will be described in the next sections.

2. The exocrine activity consists in bile production. The bile synthesized by the hepatocyte is recovered in canaliculi, which form a network connected by the canals of Hering to the bile ducts (at the hepatocytes apical side), to converge in the hepatic duct that brings them to be finally stored in the gallbladder.

(Duncan, 2003; Musch, 2014; Reglero-Real et al., 2014)

Carbohydrate metabolism

The liver is a key organ in blood glucose homeostasis. If glycemia is too high, insulin is secreted by the pancreatic β -cells and released in the blood circulation. Insulin is a peptide hormone able to stimulate hepatic glucose uptake and storage, while it inhibits hepatic glucose production. On the contrary if glycemia is low, glucagon is produced by pancreatic α -cells. It is another peptide hormone, and it is able to increase liver glycogenolysis and, when glycogen stored is depleted, gluconeogenesis, to finally raise glycemia. (Aronoff et al., 2004; Quesada et al., 2008).

In this context, the role of the liver is clearly fundamental to maintain the homeostasis of glucose level in blood. When insulin is secreted in portal blood, it arrives at the liver at the same time than glucose, which has been absorbed by intestine after a meal. Hepatic glucose uptake by the liver is stimulated by the portal insulin, and the liver sequesters 30-40% of the glucose passing through (Pagliassotti and Cherrington, 1992) and at the same time an important amount of the portal insulin (almost 70%) (Toffolo et al., 2006).

Insulin has a direct effect on the liver, it represses gluconeogenesis enzyme gene expression, and consequently hepatic glucose production (Duong et al., 2002; Ferrannini et al., 1999; Hall et al., 2000; O'Brien and Granner, 1996).

Hepatic Glucose uptake

In hepatocytes, glucose is uptaken mainly by the glucose transporter Glut-2 in a concentration-dependent manner. A hepato-specific Glut-2 deletion totally impairs liver glucose transport (Seyer et al., 2013). The rate-limiting enzyme for glucose metabolism is the glucokinase, whose expression is positively controlled by insulin (Girard et al., 1997, for review). Glucokinase is the enzyme responsible for the phosphorylation of glucose in glucose-6-phosphate. This phosphorylation not only activates glucose for further transformations, but it will also decrease intracellular glucose concentration, promoting further entry. Mice overexpressing glucokinase or mice carrying

additional glucokinase copies in their genome have an increased rate of glucose uptake, and glycolysis and glycogen synthesis are higher than in control mice (Girard et al., 1997, for review). Consequently the transgenic mice glycemia is decreased, and therefore glucokinase was first identified as a possible target to treat diabetes. Moreover, identified polymorphisms on the glucokinase gene are linked to Mature Onset Diabetes of the Young 2 (MODY-2) diabetes. (Ferre et al., 1996a, 1996b; Froguel et al., 1993; Stoffel et al., 1992) (Figure 2).

Hepatic glucose production

During the fasting period, the liver is able to supply the glucose need of the whole body, first by breaking its glycogen stores and then by producing glucose *de novo* (gluconeogenesis). The two processes do not occur at the same time: in the early fasting (2-6h after a meal) glycogenolysis is predominant, whereas in prolonged fasting gluconeogenesis becomes fundamental (Rui, 2014). As demonstrated by Roden *et al.* in humans during the early fasting period, hepatic glycogen level decreases linearly (Roden et al., 2001).

Gluconeogenesis is a tightly regulated process: enzymes expression changes in relation to hormones. Insulin represses PEPCK, fructose-1,6-biphosphatase (FP2ase) and glucose-6-phosphatase (G6Pase) gene expression, whereas glucagon induces their expression. Another way to control the process is by regulating the availability of gluconeogenesis substrates, that could be either produced directly by the hepatocytes or by peripheral tissues and delivered by blood circulation (Figure 2) (Rui, 2014).

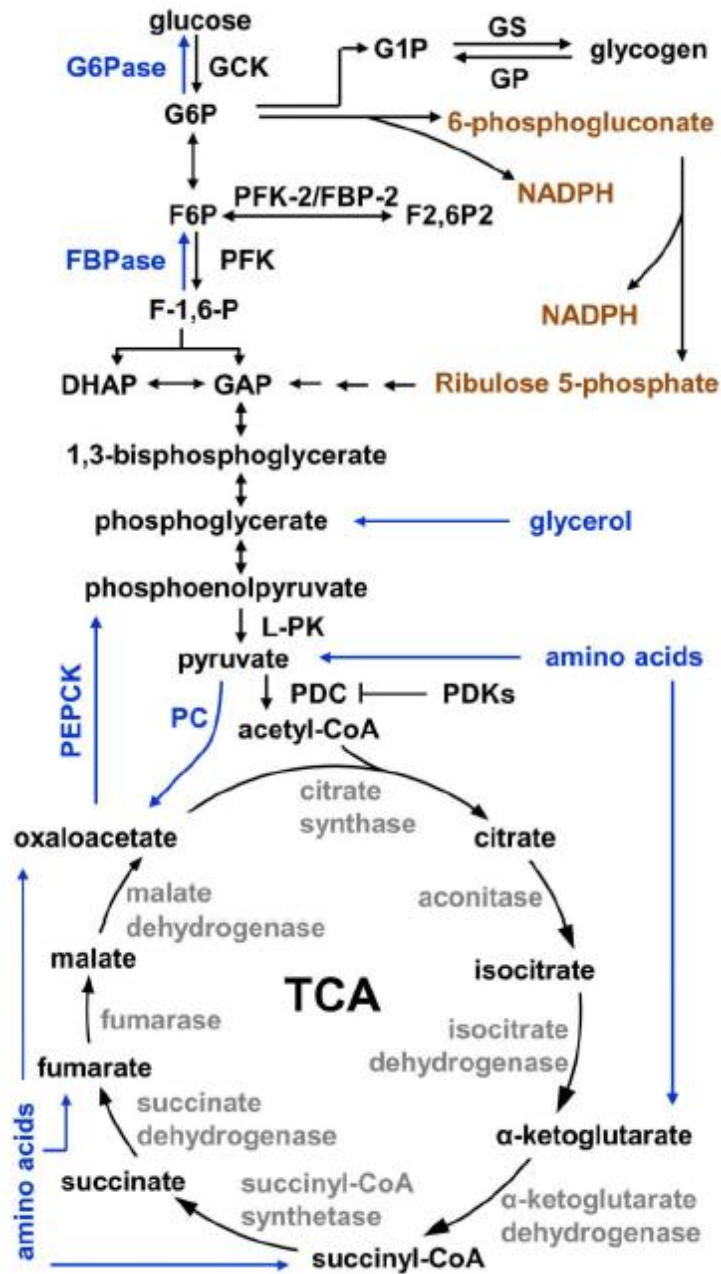


Figure 2. Glucose metabolism.

Intracellular glucose can be catabolised in order to provide energy supply through its oxidation in tricarboxylic acid (TCA) cycle or it can be stored as glycogen or fatty acids. The gluconeogenesis pathway is in blue, and the pentose phosphate pathway which generates NADPH (for lipogenesis and detoxification reactions) and nucleotides synthesis is in brown (Rui, 2014).

Lipid metabolism

In hepatocytes, fatty acids can be produced, stored in lipid droplets (as triglycerides), exported in lipoproteins, or oxidized for energy producing. Depending on the nutritional status, fatty acids can be produced, through lipogenesis, but they can also be obtained through the hydrolysis of the triglycerids stored in liver droplets or uptaken from blood circulation, in the liver mainly enter non-esterified fatty acids (Rui, 2014).

Lipogenesis

In the liver, glucose catabolism is not only a metabolic source for energy production, but mainly provides the citrate, through the TCA cycle. Once in the cytoplasm citrate will be converted into acetyl-CoA, precursor of the fatty acids synthesis (Figure 3). The cytoplasmic acetyl-CoA is then converted into malonyl-CoA by the acetyl-CoA carboxylase (ACC).

Malonyl-CoA and acetyl-CoA are then transformed into palmitic acid by the fatty acid synthase (FAS) (Wakil and Abu-Elheiga, 2009). The palmitic acid will be elongated into stearate then desaturated to produce oleyl-CoA, one of the most abundant fatty acid with palmitoyl-CoA (Gutiérrez-Juárez et al., 2006) (Figure 3).

Insulin stimulates the expression of two lipogenic enzymes: ACC and FAS, through the activation of sterol responsive element binding protein (SREB-1c), a transcription factor able to trigger the transcription of both glycolytic (as the glucokinase named before) and lipogenic genes (Foretz et al., 1999).

Therefore glucose is able to indirectly regulate lipogenic gene expression through insulin, but glucose also directly activates a transcription factor, the carbohydrate responsive element binding protein (ChREBP), that binds the carbohydrate responsive element (ChoRE) on the lipogenic genes, promoting their expression (Dentin et al., 2004, 2006).

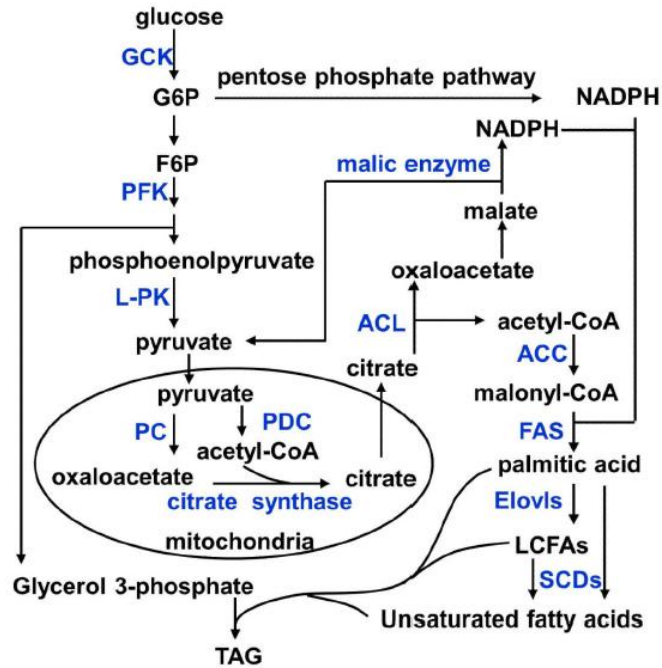
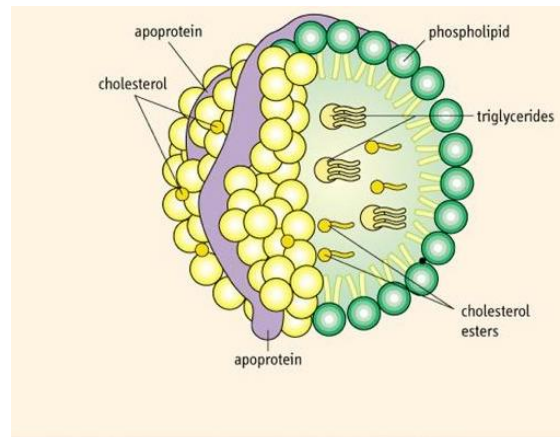


Figure 3. Lipogenic pathway.

Glucose intermediates are in black, enzymes in blue. Glucose supplies acetyl-CoA, which is essential for fatty acids formation (Rui, 2014).

VLDL production and secretion

The liver produces very low density lipoprotein (VLDL). Excessive fatty acids (that are not used as fuel though their oxidation) can be transformed, being esterified, and then collected within lipid droplets in the hepatocytes cytoplasm, to be further secreted as VLDLs in blood. In healthy conditions triglycerides cannot persist in hepatocytes but have to be secreted, otherwise their accumulation in cytoplasm will trigger steatosis and consequent pathologies. VLDLs are carriers in the blood circulation of triglycerides and cholesterol esters; the scaffold elements are a phospholipids membrane, apolipoproteins, and cholesterol (Figure 4) (Li and Vance, 2008; Noga and Vance, 2003; Stein and Shapiro, 1960).



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Figure 4. VLDL structure.

VLDLs are the triglycerides transporters in blood circulation, their membrane is composed of apolipoproteins, cholesterol and phospholipids (Li et al., 2005; Noga and Vance, 2003).

VLDLs are formed in two steps. In the first one a small dense VLDL precursor is generated. The essential component, apolipoprotein B, is translocated in the ER during its translation, and it starts to be conjugated with a small amount of triglycerides, phospholipids and cholesterol esters. If something in its structure is wrong, the apolipoprotein B and its conjugated elements are rapidly degraded. The second step consists in the VLDL precursor maturation, acquiring further triglycerides (Shelness and Sellers, 2001).

Fatty acid oxidation

In the liver there are two kinds of oxidation: β -oxidation and ω -oxidation. The first is localised in mitochondria and peroxisomes, the second in the ER. The **β -oxidation** site depends on the fatty acids chain length. Short (<C₈), medium (C₈-C₁₂) and long (C₁₄-C₂₀) are principally oxidised in mitochondria, and very long chain (>C₂₀) in peroxisomes; after the first degradation steps shortened chains are sent to mitochondria (Bartlett and Eaton, 2004; Reddy and Hashimoto, 2001). Fatty acids are first activated in acyl-CoA esters before being sent to mitochondria or peroxisomes. β -oxidation is a redundant process shortening at each step acyl-CoAs, with the loss of the two terminal carbons as acetyl-CoA subunits. In the liver, these acetyl-CoA can either be condensed in ketone bodies or enter in the TCA cycle (Laffel, 1999).

The **ω -oxidation** is able to use fatty acids chain from C₁₀ to C₂₆. It is an alternative pathway that is used in particular when the β -oxidation process is not functional. The first step consists in changing of the ω -methyl group of fatty acids in a ω -hydroxyl group. The second step is the dehydrogenation of this group into a dicarboxylic acid. The dicarboxylic acid is then sent to mitochondria or peroxisomes to be shortened (Sanders et al., 2006).

Bile production

The bile is mainly synthesized by hepatocytes. Bile is subsequently released and stored in gallbladder. This step needs to be coordinated with food arriving in the intestine, and is necessary because bile in gallbladder is concentrated (by removing water) to reach the functional concentration. Bile is then thrown in the duodenum where bile acids (BAs) are able to facilitate the digestion and absorption of dietary lipids and other lipophilic nutrients. This is a finely regulated process: in fact when in postprandial phase food enters in the duodenum, a peptide hormone, cholecystokinin (CCK), is produced and released. CCK stimulates the gallbladder contraction and so bile is released into the intestine. Bile acids are finally reabsorbed by passive diffusion and active transport in the terminal ileum and get back to the liver (Houten, 2006). The enterohepatic circulation, which is a continuous process in liver, allows this recycling.

Cholesterol is the biological precursor of bile acids, and bile primary biosynthesis takes place in the liver. Two different pathways exist: the classical (neutral) pathway and the alternative (acidic) one.

The **classical pathway** is a multi-step process and the seventeen enzymes involved are localised in cytoplasm and in different cellular organelles (microsomes, mitochondria and peroxisomes); it is responsible for the production of 75% of BAs in mice and 90% in human. It starts with the conversion of cholesterol in 7 α -hydroxycholesterol by cholesterol 7 α -hydroxylase (CYP7A1), also called cytochrome P450 7A1 (enzyme specifically expressed in the liver). The cholesterol hydroxylation by CYP7A1

is the rate-limiting step of the process, as in knock-out mice for this enzyme the amount of BAs produced is 75% less than in control ones (Schwarz et al., 1996).

The **alternative pathway** first step consists in a cholesterol oxidation by mitochondrial sterol 27-hydroxylase CYP27A1. Enzymes involved in this alternative pathway are not only specifically expressed in liver but also in other tissues.

Both BAs produced by the two parallel ways are called primary BAs. The composition of BAs pool is different among species. For instance in humans and rats the major components of BAs are the cholic acid and the chenodeoxycholic acid, whereas in mice they are the cholic acid and the muricholic acid. The primary BAs are conjugated in the liver with an aminoacid, which is taurine for rodents and glycine for humans. Thereafter, at the intestinal level, the conjugated BAs are transformed by the intestinal microbiota to give rise to secondary BAs (Figure 5) (Chiang, 1998, 2004; Goodwin et al., 2000; Russell, 2003).

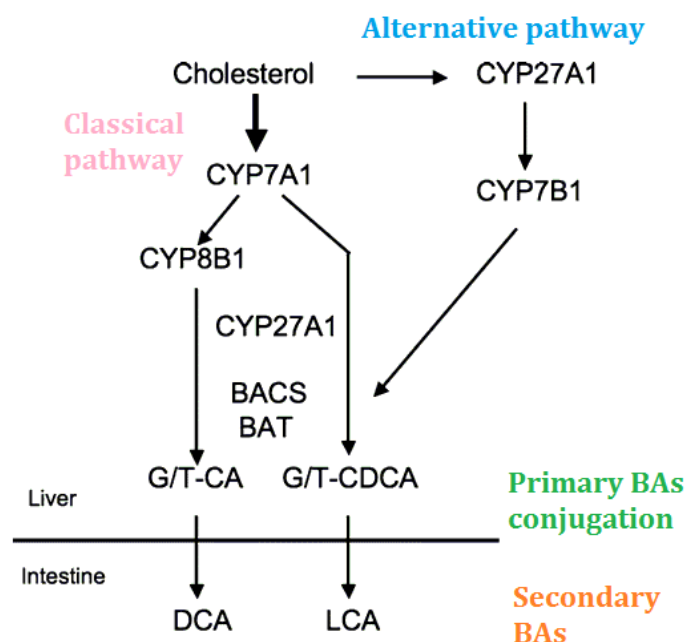


Figure 5. BAs synthesis.

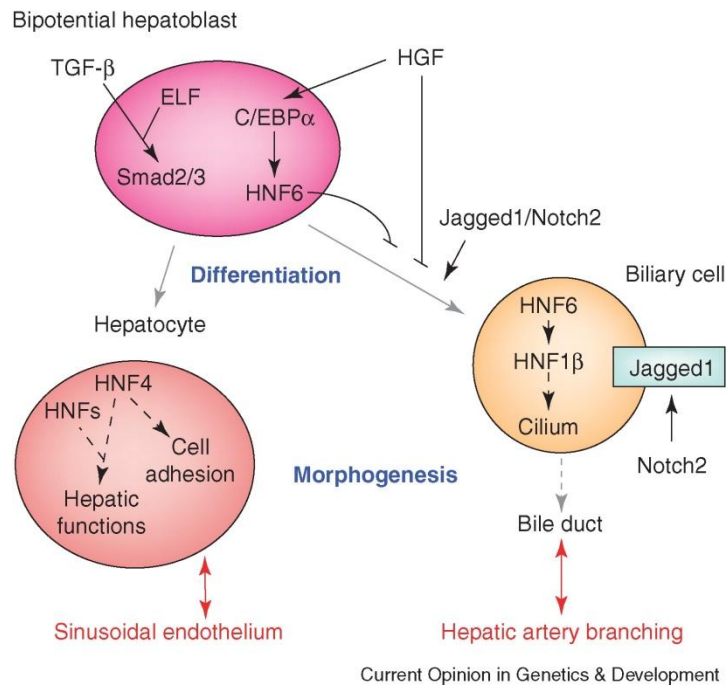
BAs can be produced by the classical or alternative pathway. Primary BAs are conjugated in liver and then modified by the intestinal microbiota to give rise to secondary BAs (adapted from Chiang, 1998).

Liver embryonic development

The foregut endoderm is the origin of liver tissue, appearing at 8.5 days post-coïtum (E8.5) in mice. At this stage, GATA4 and FoxA transcription factors are expressed in the foregut and are able to bind to the enhancer controlling albumin (*Alb*) gene transcription to promote its expression (Si-Tayeb et al., 2010). Shortly after that step, at E8.5 - E9, the liver bud formed. Specific genes such as *Alb*, alpha fetoprotein (AFP) and hepatic nuclear factor4 alpha (*Hnf4 α*) start to be expressed and the epithelial cuboidal cells become pseudostratified columnar cells, to form the liver diverticulum (Bort et al., 2006). A complex transcription factors network then controls the breakdown of the laminin-rich basal layer around endoderm to let the hepatoblasts delaminate and migrate to form the liver bud (Bort et al., 2006; Margagliotti et al., 2008). The liver bud then grows in a huge way and becomes the principal site of haematopoiesis, controlled by hepatoblast genes and paracrine signals from hepatic mesenchyme (Si-Tayeb et al., 2010).

The hepatoblast differentiation

The hepatoblasts are endodermal progenitors of the parenchymal lineages of the liver. They have an irregular shape, a big nuclear to cytoplasmic ratio and few organelles (in comparison to mature hepatocytes). They are bipotential as they are capable to give rise to both hepatocytes and cholangiocytes. The hepatoblasts start to migrate and associate with primitive endothelial cells that form the capillary-like structure between the migrating hepatic cords; the sinusoidal structures are also forming (Duncan, 2003). The differentiation of the hepatoblast is gradually determined by different transcription factors. This bipotential cell could become a cholangiocyte or a hepatocyte in relation to molecular pathways and transcription factors differentially activated (Figure 6).



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Figure 6. Bipotential hepatoblast

The fate of the bipotent hepatoblast is influenced by different transcription factors and signalling pathways, to differentiate into one or another cell type (Lemaigre and Zaret, 2004).

At the beginning, the hepatoblast expresses genes that characterise both adult hepatocytes (Hnf4 α and alb) and adult cholangiocytes (cytokeratin 19, CK19) and Afp, typical of embryonic liver. Hepatocyte growth factor (HGF) promotes the establishment of bipotency in the hepatoblast, through the induction of CCAAT-enhancer-binding protein α (C/EBP α) expression and consequently HNF6 (also called onecut-1), which avoids an early differentiation into biliary lineage. Indeed HNF6 knock-out mice present a premature and exaggerated biliary differentiation (Clotman et al., 2002; Lemaigre and Zaret, 2004; Si-Tayeb et al., 2010). Another involved pathway that collaborates with HGF is the transforming growth factor β (TGF- β) and its effectors, the smad2 and smad3 proteins. Mutants for the smads show hepatocytes organized in clusters, instead of cords (Lemaigre and Zaret, 2004; Weinstein et al., 2001). During differentiation TGF- β forms a gradient to help the commitment, being higher near the portal vein, where cholangiocytes form, and lower at the other side, where hepatocytes differentiate (Clotman and Lemaigre, 2006).

Commitment to the hepatocyte lineage

As anticipated, the TGF- β , WNT, FGF and NOTCH secreted factors form a gradient, being higher in the portal region. This gradient is necessary to stimulate the hepatoblast to differentiate; in the periportal area where these signalling pathways are higher, the hepatoblast will differentiate into a cholangiocyte. On the other side is not only the fact that hepatoblast is less exposed to these signals, but also the presence of HGF that will make the hepatoblast to adopt the hepatocytes fate (Suzuki et al., 2003). Another fundamental transcription factor for the hepatocytes lineage commitment is C/EBPa. In particular it is able to repress the expression of TGF- β receptor 2 in the hepatoblast, inducing its commitment into the hepatocyte lineage. On the contrary C/EBPb is able to induce its expression and so to stimulate the cholangiocyte fate. Both influences of C/EBPa and C/EBPb were proved also in *in vitro* experiments (Takayama et al., 2014). The liver during the embryonic life is the hematopoiesis site. The hematopoietic cells indeed migrates around E10 into the liver and are able to secrete interleukin-6-related cytokines. One of these is oncostatin M (OSM), which is able to induce the expression of differentiation markers, in particular markers of the hepatocyte fate (Ito et al., 2000; Kamiya et al., 1999; Santamaria et al., 2013). The hepatocytes fate is also controlled by another transcription factor, TBX3. The used of knock-out embryos indeed demonstrated that its loss allows the cholangiocyte fate, instead of the hepatocyte one. It acts both by allowing the expression of hepatocytes markers, C/EBPa and Hnf4 α , and in blocking the expression of Hnf6, necessary for the biliary lineage commitment (Suzuki et al., 2008).

To establish the hepatocytes adult phenotype, HNF4 α is one fundamental transcription factor involved and its roles will be addressed in a specific chapter.

Cholangiocyte and the bile ducts

The cholangiocytes are also called biliary epithelial cells and they constitute the bile ducts, responsible for carrying the bile. The cholangiocytes constitute

3-5% of total cells amount in the liver. The 3-dimensional structure formed by biliary cells is called the biliary tree, which develops in parallel with the hepatic artery network (Figure 7). It can be divided into intrahepatic biliary ducts (IHBDs) and extrahepatic biliary ducts (EHBDs). IHBDs are composed of bile canaliculi, canals of Hering and interlobular bile ducts. EHBDs are composed by the gallbladder, the common hepatic duct, the common bile duct and the cystic duct (Nakanuma et al., 1997).

From a physical point of view, the development of bile ducts begins from E13.5. Hepatoblasts around the mesenchyme near the portal vein first form a monolayer, which is called the ductal plate that then evolves in a bi-layer of cuboidal precursor cells expressing *CK19* (Figure 7). Around E17 the ductal plate remodels itself: focal dilatations start to form in the bi-layer and portal mesenchyme starts to surround it, whereas at the same time the remaining bi-layer regresses. The fate of remaining ductal cells was long discussed. For instance, Terada and Nakuma in 1995 hypothesised apoptosis mechanisms as a cause of elimination of the excess cells, even if Carpentier *et al.* showed that a part of ductal cells can be committed into periportal (but not pericentral) hepatocytes (Figure 7) (Carpentier et al., 2011; Si-Tayeb et al., 2010; Terada and Nakanuma, 1995). The other hepatoblasts differentiate into hepatocytes and organise in chords, with bile canaliculi at the apical surface (Lemaigre, 2003; Sergi et al., 2000).

The differentiation of IHBDs thus derives from liver precursors of the hepatic bud: it starts from the liver hilum and continues until the peripheral areas, to be after anastomosed with EHBDs. Extrahepatic biliary tract (gallbladder, hepatic, cystic and common bile ducts) and IHBDs developments are indeed distinct: IHBDs derive directly from intrahepatic progenitors emerging from the primitive gut, and only after become connected to allow bile flow from hepatocytes to gallbladder (Clotman et al., 2002; Lemaigre, 2010; Lemaigre and Zaret, 2004). Extrahepatic biliary tract originates from pancreatobiliary precursors coexpressing pancreatic and duodenal homeobox 1 (PDX1) and SRY-related HMG-box 7 (SOX7), as cells expressing only PDX1 differentiate into pancreatic precursors.

Looking from a molecular point of view, *HNF6* is a key transcription factor for cholangiocytes commitment from bipotential hepatoblast, as it is able to control *HNF1 β* transcription and so the cilium associated proteins expression. *HNF1 β* null mice show a dysplasia of big intra hepatic biliary ducts (IHBDs) and a lower amount of small IHBDs in comparison to controls; as an indirect consequence also interlobular arteries formation is lacking, because the biliary lineage differentiation and the artery branching are tightly linked (Clotman et al., 2002; Coffinier et al., 2002; Lemaigre and Zaret, 2004). For instance, an excess of artery branches correlates with an excess of bile ducts, and conversely a new proliferation of bile ducts due to external causes, is followed by new artery branches formation. Near the forming biliary epithelium the presence of a capillary network originated from the hepatic artery, called peribiliary plexus, is necessary to feed and oxygenate biliary cells. At a molecular level, the requested molecules for angiogenesis *in loco* are vascular endothelial growth factor (VEGF) and angiopoietin-1. VEGF is expressed in both cholangiocytes and hepatocytes whereas angiopoietin-1 is produced only by hepatocytes. At the beginning both cholangiocytes and α -smooth muscular actin (α -SMA) positive-cells (branch precursors) express the receptor for angiopoietin-1, but the cholangiocytes lose it while differentiating. The VEGF receptor is expressed in ductal plate cells and in portal endothelial precursors. A synergistic model has been hypothesised in which VEGF activates endothelial adjacent precursors and angiopoietin-1 is able to recruit myofibroblasts; the association between endothelial and fibroblastic cells forms the ducts. The final maturation of the hepatic artery will require the synergic action of VEGF and angiopoietin-1, thanks to the coexpression of both receptors in the same cells (Raynaud et al., 2011). Interestingly *HNF6* knock-out results in the absence of gallbladder and EHBDs are structurally different: normally each lobule is drained by hepatic ducts and all ducts converge into the cystic duct, which enter into the duodenum; in mutants, hepatic ducts are substituted by a big structure that connect liver to duodenum (Clotman et al., 2002).

The biliary cells are also characterised by the Notch2/Jagged1 pathway. Heterozygous mutants for both Jagged1 and Notch2 are lacking IHBDs in

mice, while in humans mutations in Jagged1 gene are responsible for the Alagille syndrome. One main aspect of this syndrome is a jaundice developed by the babies affected, due to a similar paucity in bile ducts (Lemaigre and Zaret, 2004; McCright et al., 2002). As previously anticipated, the localisation of IHBDs is linked to different signals: TGF- β gradient, whose presence is maximal in the portal area, and Notch2/Jagged1 signalling. One characteristic transcription factor for nascent IHBDs is SOX9 (Si-Tayeb et al., 2010).

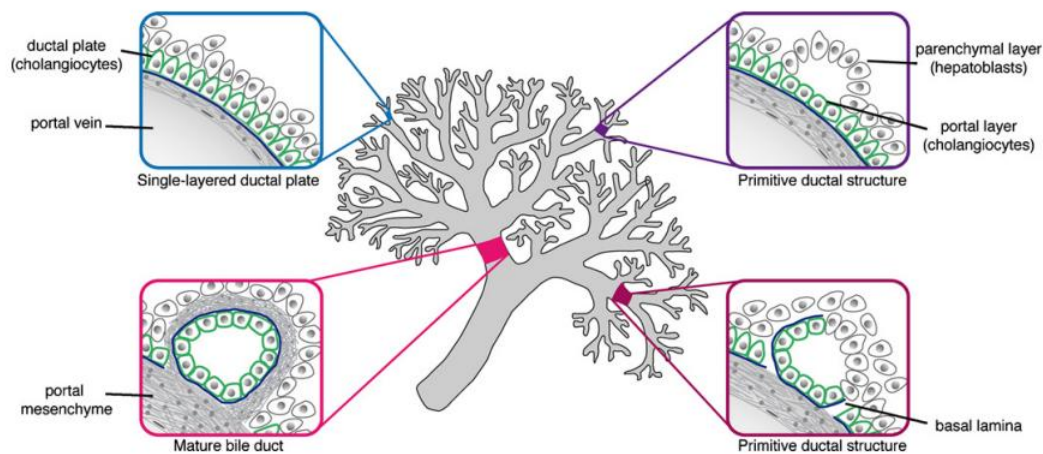


Figure 7. From ductal plate to bile ducts.

The ductal plate forms near the portal space and evolves forming PP hepatocytes and cholangiocytes, to finally create a duct able to transport the bile (Raynaud et al., 2011).

Liver pathologies

Cholangiocytes-linked

1. **Ductopenia** is a pathological condition caused mostly by Alagille syndrome, due to mutations in Jagged1 gene, modeled in mice by the invalidation of Jagged1 and Notch2. No other ductopenia is associated to bile ducts development regulators. For instance mutants for *SOX9* gene have a mild and transient paucity of bile ducts (but show other problems like campomelic dysplasia and autosomal sex reversal) (Lemaigre and Zaret, 2004; McCright et al., 2002).
2. **Ciliopathies** are pathologies in which bile ducts are present but defective and consist in hepatorenal fibrocystic syndromes. An example is autosomal dominant polycystic kidney disease (ADPKD). The formation of renal cysts is parallel with hepatic biliary cysts. The biliary cysts are characterised by cholangiocytes hyperproliferation, linked to increased sensitivity to insulin-like growth factor-1 and estrogen, which stimulates VEGF production. The cilia are normally localised at the apical side of cells and are involved in osmo-, mechano- and chemosensing, while in ADPKD cilia are smaller or absent. Having non functional cilia is tightly linked with dysgenesis of bile ducts. The two genes associated with this pathology are polycystin-1 and -2 (*PKD-1/2*). Another example is autosomal recessive polycystic disease (ARPKD) in which multifocal bile ducts dilatations are present in liver, associated to polyductin or fibrocystin (*PKHD1*) mutations, which is a transmembranous protein located also in cilia (Lemaigre, 2011; Raynaud et al., 2011).
3. **Ductal plate malformations (DPMs)** are lesions present in different pathologies: the previously mentioned ARPKD, the later described embryonic atresia, and congenital hepatic fibrosis. DPMs consist in the persistence of the embryonic biliary structures, due to mutations in genes controlling the ductal plate remodelling (in particular the phase of ductal plate regression). As underlined by their association with ARPKD, the

DPMs are also a kind of ciliopathy, meaning that in this case the cause of the persistent embryonic biliary structures is the cilium defective role (Bezerra, 2011; Desmet, 1992; Lemaigre, 2011).

4. **EHBDS pathologies**: extrahepatic biliary atresia is a rare disease, more frequent in neonates. There are both embryonic and postnatal forms (the most common one) are characterised. The pathogenesis of this disease consists in cycles of inflammatory and sclerosis processes that could provoke biliary cirrhosis (with the consequent occlusion of bile ducts). The aetiology is still not clear and many hypotheses have been made, like viral infection, autoimmunity and exposure to toxins and others. Choledochal cysts are another type of EHBDS pathologies, in particular in a context of congenital malformations. In this case, one or more biliary tract segments have cystic dilations, which could be focal lesions or a dilation of the entire common bile duct. The causes are unknown, but familial cases suggest that they could have a genetic component (Bezerra, 2011; Lemaigre, 2011; Raynaud et al., 2011).

5. **Biliary cirrhosis** is characterised by fibrous scar accumulation starting from peribiliary area and progressing until the portal area in the liver. Not only biliary cells are involved in the disease but also the portal fibroblasts. The biliary cirrhosis could be primary (an immunodeficiency disorder more likely to affect women than men, ratio 10:1), primary sclerosing (fibrosis inflammation able to destroy biliary ducts), biliary atresia (as explained in subsection 4) and cystic fibrosis hepatopathy (Hirschfield and Gershwin, 2013).

6. **Cholangiocarcinoma (CC)** is a cancer spreading from the cholangiocytes. It is classified as intra or extrahepatic, depending whether either the intra or extrahepatic tree branch is involved. The 50% of CCs originates from liver hilum, 42% from EHBDS and 8% from IHBDs. CC has a poor outcome due to the lack of both early diagnosis tools and treatments. The only therapy is surgery, even if often CC is diagnosed too late for this medical

approach. As the general cancer onset, CC is a multi step process and it is characterised by an uncontrolled cell proliferation, high invasion potential and so a high metastasis rate. A small portion of CCs can develop in a normal liver, but for the major part the onset is in a context of chronic inflammation of bile ducts. Risk factors are for instance virus infection, metabolic disorders and cirrhosis (Khan et al., 2005; Lazaridis and Gores, 2005; Patel, 2002).

A carcinoma can develop also in the gallbladder, mostly a women pathology, and known risk factors are gallstones, infection, metabolic disorders, environmental factors (Medina and Kaempffer, 2001; Randi et al., 2006).

Hepatocyte- linked

1. **Steatohepatitis** can be caused by alcohol consumption, namely alcohol liver disease (ALD) or come after non-alcoholic fatty liver disease (NAFLD). In the first case the elevated alcohol consumption is able to trigger a steatosis. NAFLD disease is linked to metabolic disorder like obesity and insulin resistance that also trigger steatosis. Both pathologies do lead to lipid accumulation in hepatocytes, which cause an inflammation in liver tissue (oxidative stress and mitochondria dysfunction). This induces a fibrotic state of liver, which often degenerates in cirrhosis (Browning et al., 2004; Ekstedt et al., 2006; French et al., 1993; Kleiner et al., Nonalcoholic Steatohepatitis Clinical Research Network, 2005; Paradis et al., 2009; Zakhari and Li, 2007).
2. **Hepatitis**: the most common cause of hepatitis is a viral infection. The main ones involved are the hepatitis B and C (HBV and HCV) viruses. On the contrary the hepatitis A virus (HAV) can only cause acute hepatitis, but never chronicle diseases (Sarin and Kumar, 2011). HBV has been well characterised and it has been associated with HCC. Chronically infected people indeed have a higher HCC onset, but the vaccine is effective and it is able not only to prevent HBV infection, but also HCC onset (Tiollais et al.,

1985; Yang et al., 2002). HCV is the most common cause of liver pathologies. HCV infected patients have fibrotic livers, associated in 30% of the cases with cirrhosis onset and after several years of chronic inflammation, cirrhosis can progress and causes HCC development (Poynard et al., 2001). At present time, no functional vaccine is available to prevent HCV infection, so it is necessary to develop antiviral drugs to stop its infection in the liver. Interferon therapy was tested in clinical trials, alone or in combination with other antiviral substances, like ribavirin, telaprevir and boceprevir. Recently, a new antiviral drug has been developed, which is a RNA polymerase inhibitor, the sofosbuvir. Clinical trials have reported very encouraging results but, due to the huge cost of the therapy, accessibility for patients is still a problem (Cortez and Kottlilil, 2015; Sun et al., 2011).

Hepatitis can also develop in a context of liver inflammation that can originate from repetitive infections and/or toxic agents exposure (Czaja, 2001; Manns and Vogel, 2006; Vergani et al., 2002).

3. **Cirrhosis** is a culminant pathological state of the liver, characterised by hepatic architectural disorganization and loss of liver functionality. It occurs after cycles of reparation in case of chronic liver damages, involving inflammation, tissue remodelling and fibrosis. It can develop after steatohepatitis and viral or autoimmune hepatitis (Wynn, 2008).

4. **Liver reaction against substances**: liver injury can also be caused by chemical and drug absorption. The liver damages are often linked to drugs processing by cytochrome 450 system and require different processes like lipid peroxidation, covalent binding of proteins or production of reactive oxygen species (ROS) (Park et al., 2005).

Moreover metal accumulation can cause liver injury. For example, Wilson's disease consists in an inherited abnormal copper accumulation in liver parenchyma. Copper accumulation stimulates free and hydroxyl radicals formation, causing oxidative injury to the cell membrane and DNA, impacting also protein synthesis (Schilsky et al., 1989).

Hemochromatosis consists in an iron accumulation in a variety of organs, including the liver, and is provoked by increased iron absorption from diet. Iron overload in liver involves an oxidative stress, inflammation and a consequent fibrosis. Hemochromatotic livers develop cirrhosis and so present an increased risk of developing HCC (Fix and Kowdley, 2008).

Another example of accumulation that can cause liver problems is a heterogeneous group of disorders called glycogen storage diseases (GSDs), which consist in defective glycogen storage in the liver. GSDs are either genetic or acquired (by alkaloids intoxications) and can involve liver, muscle and brain; different enzymes deficiencies can be the cause of GSDs (Matern et al., 1999).

5. **Impaired blood flow**: the blood supply in the liver is guaranteed by portal vein for the 65-75% and by hepatic artery for 25-35%, while the oxygen is delivered in liver mostly by the hepatic artery, but also by portal blood. The blood supply has to be constant, so it is strictly regulated between the two sources. Pathologies involving an impaired blood flow could involve arterial as well as venous blood flow. For instance hepatic artery can suffer of thrombosis, which is generally a consequence of liver transplantation and aneurysms that are mostly extra- and not intra-hepatic aneurysms. On the other hand, the portal outflow can also be impaired, and this is mainly the case in cirrhosis, in which hypertension is due to fibrosis altering tissue elasticity and vein dilatation ability. Other pathologies present blood flow obstruction, even if their aetiology is different (Lautt and Greenway, 1987; Shah and Singla, 2011; TYGSTRUP et al., 1962). An interesting example is the idiopathic portal hypertension (IPH), a non-cirrhotic hypertension at the portal side. Aetiology is not known and the hypertension is due to the stenosis of the portal vein, whose lumen is dramatically reduced. Knowing that TGF- β signalling is involved in fibrotic process, the study of Kitao *et al.* focused on this signalling pathway. In particular a staining of nuclear phospho-Smad2 (one effector downstream TGF- β signalling) was found in fibrotic areas of patients. Furthermore the patients had a high circulating TGF- β level, demonstrating an implication

of this pathway in the IPH pathology (Figure 8) (Kitao et al., 2009; Nakanuma et al., 2001).

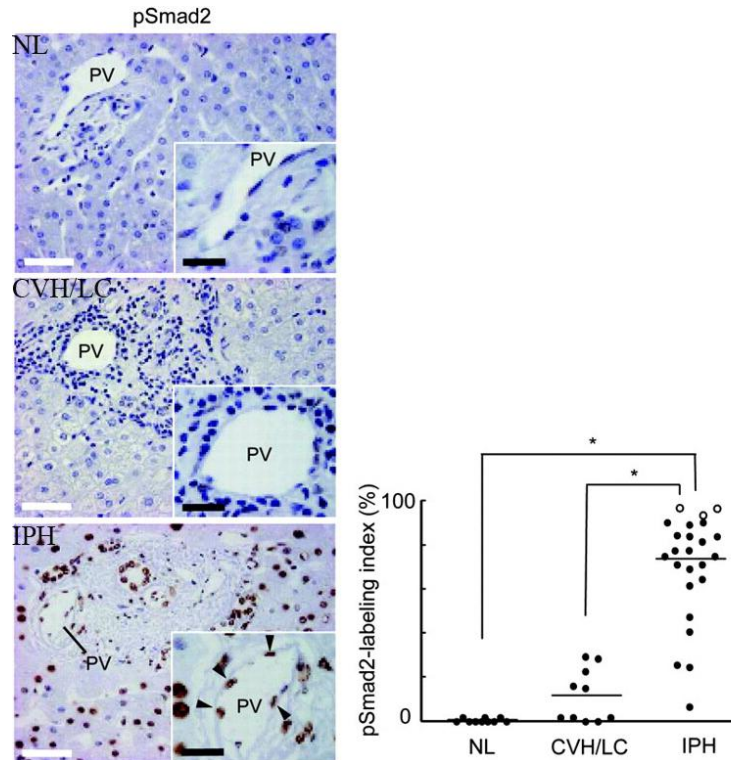


Figure 8. IPH phenotype and P-Smad2 staining.

The IPH consists in an increase portal tension due to the stenosis of the portal vein. These areas stain positive for phospho-Smad2 protein, showing an activation of TGF- β pathway, whereas normal liver (NL) and cirrhotic liver (CVH/LC) do not (Kitao et al., 2009).

6. **Neoplasies:** in liver different neoplasies may occur, being either benign or malignant.

The **benign** ones are characterised by hepatocytes proliferation, and the difficulty is the differential diagnosis between them and well-differentiated liver cancers (better described in next chapter, section hepatocellular carcinoma). The two most common benign neoplasies are focal nodular hyperplasia (FNH) and hepatocellular adenomas (HCA) (Bioulac-Sage et al., 2001, 2007). FNH generally onsets in women of 20-50 years old and a correlation between nodules growth and estrogens has

been described in literature (Scalori et al., 2002). Morphologically they have a fibrotic central core with malformed vascular structures. FNH is due to a response of the liver to an increased artery blood flow, and it has a low risk of side effects and to develop carcinoma (Cherqui et al., 1995; Wanless et al., 1985). HCA is a monoclonal, soft, well-demarcated tumour, with no or little fibrous capsule. Different subgroups exist, with specific molecular and phenotypic characteristics (Bioulac-Sage et al., 2007). The incidence is higher in women (85% of total patients affected) and in this case there is a correlation with the oral contraceptive intake (Bioulac-Sage et al., 2007, 2009; Edmondson et al., 1976; Vana et al., 1977). There are also other risk factors as androgen-anabolic steroids intake. The major risk in carrying a HCA is associated to the possibility of bleeding, and in some cases HCA have a risk to become malignant, in particular the ones associated to a mutation in β -catenin gene (Baum et al., 1973; Bioulac-Sage et al., 2010; Kerlin et al., 1983).

One **malignant** liver cancer is the hepatoblastoma (HB); it is the most common liver cancer in infants and young children (Perilongo and Shafford, 1999; Schnater et al., 2003). Generally it develops between 6 months and 3 years after birth, as only 10% of HBs appear in children of more than 4 years (Darbari et al., 2003). It is characterised by a high level of alpha fetoprotein (AFP) in the blood serum. Only 5% of HBs have a familial origin whereas the others are sporadic. There are two morphological types: pure epithelial HBs (56%) or mixed epithelial and mesenchymal ones (44%) (Okuda K, Tabor E, 1997). An implication of the Wnt/ β -catenin pathway has been demonstrated in a high number of HBs, as 50-90% have a mutated β -catenin gene, at the level of residues phosphorylated by GSK3 β , while 5-10% have a mutated Axin1 protein (Koch et al., 2004; Taniguchi et al., 2002).

The hepatocellular carcinoma (HCC) will be discussed more precisely in the next chapter.

WNT/ β -catenin pathway

The WNT signalling

WNT proteins belong to a family of lipid-modified glycoproteins; their length varies between 350-400 aminoacids. They are produced in cells and then secreted in extracellular space, thanks to a palmitoylation, a lipid modification, necessary to allow the secretion by targeting them to the cell membrane, and moreover for the binding (by a covalent attachment) to the receptor on the other cells surface (Logan and Nusse, 2004).

The WNT proteins are highly conserved in species (Nusse and Varmus, 2012) and their signalling pathways are conserved in various organisms from worms to mammals and play important roles in development, cellular proliferation, and differentiation (Piedra et al., 2001). WNT proteins have also a range of various functions, indeed different Wnt pathways have been described: the non canonical ones, both independent of β -catenin, and the WNT/ β -catenin pathway (Figure 9) (Rao and Kuhl, 2010).

In the organisms are present a variety of WNT proteins, but also of receptors that can modulate this signal in the cell.

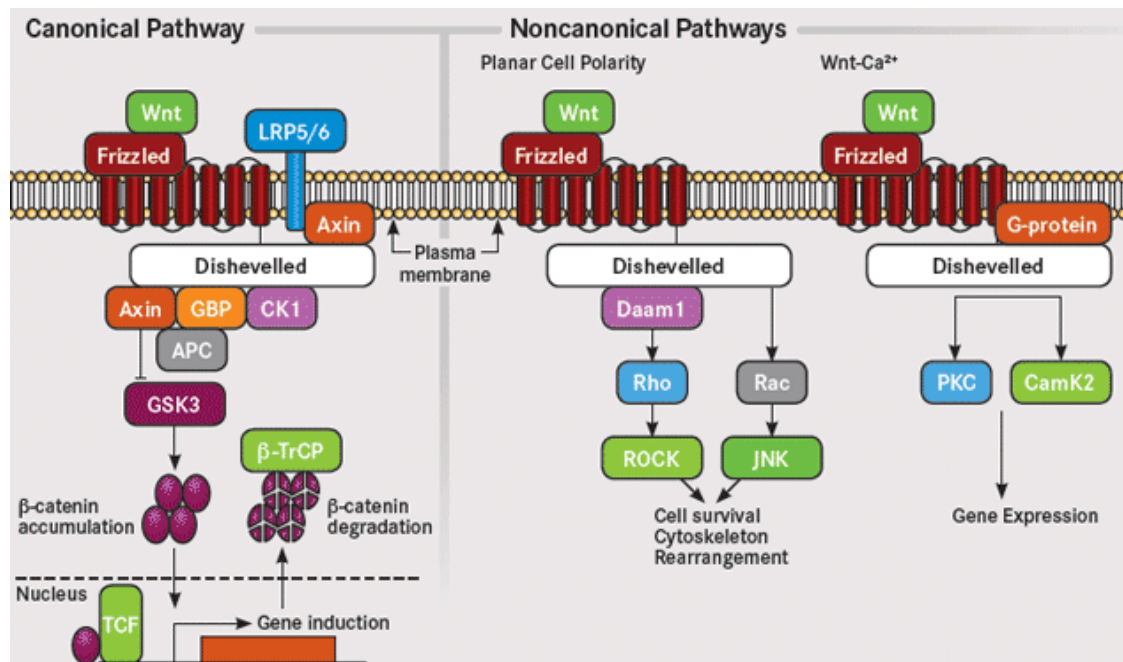


Figure 9. WNT canonical and non canonical pathways.

Three different WNT signalling pathways have been characterised. The canonical is the one involving β -catenin as effector (Hitt, 2013).

The non canonical pathways involve different intracellular mediators. First the WNT/Jun N-terminal kinase (JNK) signalling is able to finely regulate cell polarity. This planar cell polarity (PCP) pathway acts both in epithelia polarity and polarised cell movements, such as, respectively, wing hair development in *Drosophila* and egg gastrulation. Second, the WNT/calcium pathway has initially been characterised in *Danio rerio* and *Xenopus laevis* and is involved for instance in cardiac development and differentiation (Koyanagi et al., 2009). It has been demonstrated that WNT5a activates this pathway, based on heterotrimeric G proteins action. This process is very fast and the calcium released from the endoplasmic reticulum (ER) modulates the action of Ca^{2+} -dependent enzymes (Ma and Wang, 2006; Rao and Kuhl, 2010).

WNT/ β -catenin canonical pathway

The presented thesis work is focused on the canonical WNT pathway: it is based on the WNT-dependent accumulation of the β -catenin protein in the cytoplasm, its translocation in the nucleus and its action in synergy with LEF/TCF factors in activating gene expression.

More in details when WNT signal is not present at the membrane, β -catenin is first phosphorylated, then ubiquitinated to trigger it to proteosomal degradation, thanks to the destruction complex activity. Different proteins composed this complex: the tumour suppressors Axin and adenomatous polyposis coli (APC), and the kinases casein kinase 1 (CK1) and glycogen synthase kinase-3 (GSK-3). Axin acts as a scaffold protein recruiting APC and so permitting the phosphorylation of β -catenin in two steps: a priming phosphorylation of serine 45 (S45) residue by CK1, allowing subsequent phosphorylation on residues serines 33, 37 and threonin 41 (S33, S37, T41) by GSK-3. These phosphorylations generate a recognition site for the E3 ubiquitin ligase β -TrCP, needed for β -catenin ubiquitination and proteosomal degradation (MacDonald et al., 2009; Stamos and Weis, 2013).

On the contrary when WNT signals bind Frizzled receptors and low-density lipoprotein receptor-related protein 5 and 6 (LRP 5/6) co-receptors the destruction complex cannot be in place, because of its recruitment at the cell membrane, thanks to the interaction between Axin and Dishevelled (MacDonald et al., 2009). Unphosphorylated β -catenin is so free to enter in the nucleus where it acts as a transcription activator for inducing the expression of its target genes. β -catenin is not able to directly bind the DNA but a transcriptionally active complex is formed by its interaction with its coactivator factors, the LEF/TCF proteins, which have a DNA binding domain that let occupy the promoter/enhancer region of β -catenin positive target genes. In the absence of β -catenin, LEF/TCF proteins could otherwise interact with Groucho proteins, a family of non DNA-binding corepressors, able to stop transcription via a conserved intrinsic repression domain (Brantjes et al., 2002; Fisher and Caudy, 1998).

In different tissues, a role of WNT/ β -catenin has been demonstrated in cancer onset. In particular β -catenin is a proto-oncogene, so when an

activating mutation is present in the residues targets for the phosphorylations, or when a negative regulator has a loss of function mutation, β -catenin is aberrantly activated and becomes an oncogene responsible for example for hepatocellular carcinoma, colon cancer and ovarian cancer onset (Anastas and Moon, 2013).

β -catenin in cell adhesion

β -catenin is a 781 aminoacids protein (92 kDa), involved in cellular proliferation and cell fate, not only thanks to its control of transcription activation, but also because it is a structural protein. Indeed in cells there are three β -catenin pools: cytoplasmic, nuclear and membranous. β -catenin is mainly localised at the cell membrane because it is part of E-cadherin/catenin adhesion complexes (adherens junctions) which couple cadherins to the actin cytoskeleton. In this way it regulates cell to cell adhesion (Figure 10) (Piedra et al., 2001). β -catenin constitutive knock-out produces embryonic lethality, like E-cadherin mutants, but not for the same reason: E-cadherin knock-out is lethal for its protein structural function (lack of functional junctions), whereas β -catenin knock-out is lethal because it is defective in WNT canonical signalling. In these mice, plakoglobin or γ -catenin is able to established a compensatory mechanism to replace β -catenin at adherent junctions (Haegel et al., 1995).

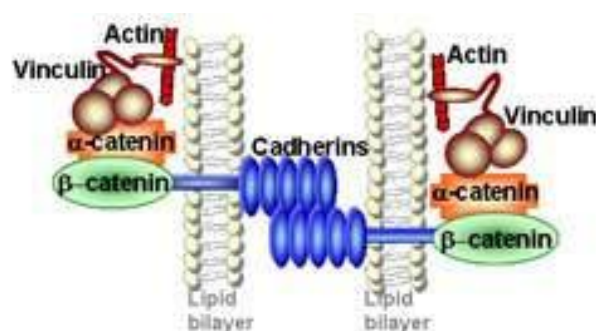


Figure 10. Adherens junction structure

The adherens junction are composed by cadherins extracellular domains linked one to the other in extracellular space and linked to β -catenin by their intracellular domain. The catenin α and β are the structural connection with vinculin and actin (cytoskeleton protein).

WNT/ β -catenin in development, maintenance and pathology

WNT/ β -catenin is one of the necessary pathways, like bone morphogenetic protein (BMP) or fibroblast growth factor (FGF), that play important roles during embryonic development in regulating several processes.

Indeed β -catenin constitutive knock-out produces embryonic lethality; around day 7 post coitum the mutants start to show a phenotype in the embryonic ectodermal cell layer. On the contrary embryos do not show an inefficient throphectoderm epithelium formation (like E-cadherin mutants), because as previously described plakoglobin or γ -catenin replaces β -catenin in its role in cellular adhesion (Haegel et al., 1995).

Later on, β -catenin plays a role in the gastrointestinal tissue specification and patterning. The embryonic visceral endoderm becomes first the embryonic gut tube and then evolves in the gastrointestinal tract (composed by stomach and intestine) and also in all the associated organs (like pancreas and liver) thanks to different patterning along its antero-posterior and dorso-ventral axis and specific cell differentiations. A fine regulation of WNT/ β -catenin signalling is necessary to the correct development of these different structures (Grigoryan et al., 2008). Once these structures are established WNT/ β -catenin pathway is also involved in some specifications. For instance in the intestine there are stem cell niches called crypts: in this compartment the self-renewing capacity of the intestine is ensured thanks to the asymmetric division that characterises stem cells, giving rise to a stem cell (for pool maintaining) but also the differentiated cell types that compose the villi (enterocytes, goblet cells) and the crypts (Paneth cells). In this case WNT is required for the self-renewal of the stem cells located in the proliferative compartment of the crypts and for Paneth cell differentiation (Andreu et al., 2005; Grigoryan et al., 2008).

An aberrant β -catenin activation in the intestine initiates more than 90% of colon cancers. Sporadic or hereditary mutations in the β -catenin negative regulator *APC* gene are the main genetic event leading to an aberrant β -catenin activation. The concept that WNT/ β -catenin pathway is involved in

early stages of cancer onset has been proven by genetic analysis of an hereditary disorder called the familial adenomatous polyposis (FAP): in this cancer predisposition syndrome, APC mutations lead to the development of a big amount of precancerous polyps that will give rise to colon cancer (Raskov et al., 2014).

WNT/ β -catenin in liver

Development and physiology

WNT/ β -catenin pathway shows contrasted roles in liver development. At the beginning of embryonic development, during the gastrulation, it is necessary to polarise the foregut endoderm along the antero-posterior axis. The presence of WNT is necessary in the posterior part, where it inhibits hematopoietically expressed homeobox (HHEX, a determinant for hepatic development). On the contrary FGF, mostly produced by the adjacent developing cardiac mesoderm, stimulates the anterior part to become the future liver. Here WNT has to be blocked by antagonists, the more important in the mouse liver is a secreted form of Frizzled receptor, Sfrp5. Later on, WNTs will cooperate with the other signals, BMP and FGF, to drive liver specification, expansion and differentiation (Si-Tayeb et al., 2010). In particular FGF is able to stimulate liver genes, through different pathways. The RAS/MAP kinase (MAPK) regulates hepatic gene expression, such as the albumin (*Alb*) gene, and phosphoinositide 3-kinase (PI3K) - AKT/protein kinase B (PKB) regulates liver cell growth (Bottcher and Niehrs, 2005; Calmont et al., 2006; Schlessinger, 2004).

At this stage β -catenin has a role of stimulating hepatic growth, in concert with different other signals, like proliferation regulators, cell survival and metabolic stress.

β -catenin is also essential in determining hepatoblast fate, as shown in our laboratory by Decaens *et al.* in 2008: through an APC inactivation in the hepatoblast, an aberrant β -catenin activation was obtained. Mice showed embryonic lethality (around E16.5 - E18.5) due to liver hypoplasia (liver

hepatoblasts are blocked in cell cycle) and more interesting they showed a lack of hepatocyte differentiation (as instance they lack *Alb* expression), having lower *Hnf4α* expression than control mice. Mice hepatoblasts from E14.5 started to organise into ducts and an increase of biliary lineage specific markers (cytokeratin-19 and -7) was found, confirmed also by microarray analysis. These results clearly depict that β -catenin overactivation in the hepatoblast commits this progenitor towards a biliary fate (Decaens et al., 2008).

Once the adult liver has formed, the WNT/ β -catenin pathway continues to have a fundamental role. Not all the hepatocytes are attempting the same molecular functions and their specialization derives from a different transcriptional program. Our team demonstrated that this is established and maintained by the presence of WNT signalling in the pericentral area of the lobule and the presence of the major β -catenin negative regulator, APC protein, at the periportal one (Benhamouche et al., 2006). More details will be elucidated in next section.

β -catenin engagement in the adult zonal liver

The liver lobules are structured along an axis from the portal side (portal vein, hepatic artery and bile ducts) to the central vein. The blood flow begins to the portal side: both from the hepatic artery, which is an oxygen-rich blood, and from the portal vein that is a full of nutrients (that derive from the intestine therefore from nutrients digestion) blood, and crosses the lobule through the sinusoids. Afterwards alongside the lobule, a gradient of oxygen, nutrients, secreted molecules and hormones is formed, which creates a specification in molecular functions of hepatocytes at the two sides. This is the so-called liver zonation, meaning that most of the hepatic molecular functions are not accomplished by all hepatocytes, but are organised at one or at the other side of the lobule; as anticipated in prior section, even if all hepatocytes have the potentiality to attempt to all functions (having the same genetic heritage), they are specialised in only periportal or pericentral functions (having a specific gene transcription). For instance the glucose and

energy metabolism, ammonia detoxification system and xenobiotic metabolism are localised functions.

Glucose metabolism is divided into gluconeogenesis and oxidative phosphorylation (OXPHOS) at the portal side and glycolysis at the central one, due to the presence of Phosphoenolpyruvate carboxykinase 1 (PCK1), a soluble enzyme that is the major regulation point for gluconeogenesis process and fructose 2,6 biphosphatase (FBPase). At the other side on the contrary we can find glucokinase (GK), lactate dehydrogenase D (LDHD) and enzymes involved in the tricarboxylic acid (TCA) cycle.

The **ammonia detoxification** mainly occurs through urea formation at the portal side, where arginase 1 (the last enzyme of the urea cycle) is present and active. The portal blood ammonia is mostly converted in urea within the periportal hepatocytes and only a residual part flows down to the pericentral hepatocytes, where it is used to produce glutamine, thanks to the presence of glutamine synthetase (GS) and ornitine aminotranferase (OAT).

The **drug metabolism** is another example of hepatocytes specialization, it occurs mostly in pericentral area, exploiting the presence of cytochrome 450, able to convert xenobiotics into excretable molecules; it is a double step process: a monooxygenation (in PC cells) followed by conjugation with glucuronic (in PC cells) or sulphuric acid (in PP ones).

The team found that β -catenin is activated in the pericentral area through WNT proteins and it controls the expression of PC genes involved in these metabolic processes, like GS, LDHD. On the contrary, it is repressed in periportal area, due to the high amount of its negative regulator APC, allowing the PP program to be expressed. When APC is deleted in the liver, it leads to a wholly pericentral liver. For these reasons APC has been called the “zonation keeper” of the liver (Figure 11) (Benhamouche et al., 2006; Torre et al., 2011).

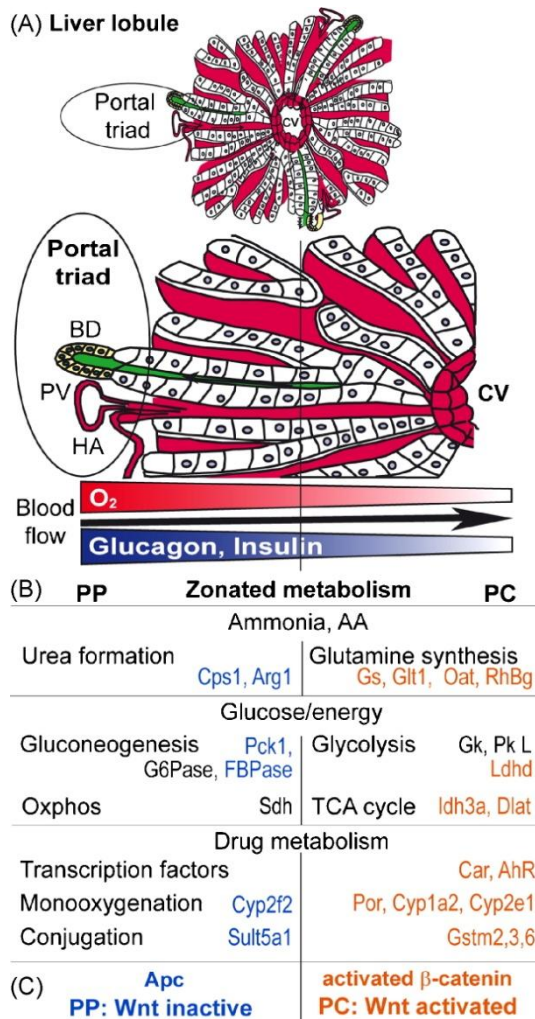


Figure 11. Liver lobule and molecular zonation

In the PC hepatocytes, the activated WNT/ β -catenin pathway allows the expression of particular enzymes involved in glycolysis, drug metabolism and GS synthesis. In the PP area, the absence of WNT/ β -catenin activity is necessary for PP genes to be expressed and exert PP functions (Torre et al., 2011).

Bile production and β -catenin

Few years ago Yeh *et al.* explored the role of β -catenin in bile production (Yeh et al., 2010). The study focused on β -catenin knockout mice and demonstrated that these mice in comparison to control ones had a higher seric and intrahepatic level of BAs. This was not associated with an increase of BAs rate-limiting enzymes, as on the contrary *CYP7A1* and *CYP27A1* expression were diminished, in comparison to control mice. The bile flow was found 50% slower in knock-out than in control mice and the hepatocytes had abnormal bile canaliculi. The model presented in this paper is that the loss of β -catenin affects actin cytoskeleton at the apical pole of the hepatocytes, generating defective canaliculi that are responsible for a secretory defect. This is the reason for bile intrahepatic accumulation (cholestasis) (Yeh et al., 2010).

The nuclear receptor Hnf4 α

HNF4 α is a key liver transcription factor for hepatocytes differentiation. It belongs to the superfamily of the nuclear receptors, even if, at present, the endogenous ligand remains unknown. It binds DNA as a homodimer, and it is expressed in liver, pancreas, kidney and intestine (Chen et al., 1994). In the liver, it is expressed at E9.0 for the first time, and it has been shown to bind the promoter of many hepatic genes (Odom et al., 2004). HNF4 α has different isoforms, derived from different promoters: P1 and P2. P2 is active in hepatoblast and gradually becomes inactive in adult hepatocytes, whereas P1 is hepatocyte-specific (Torres-Padilla et al., 2002). *HNF4 α* whole knock-out mice results in a phenotype of early embryonic lethality, as embryos are not able to undergo normal gastrulation. Using a liver conditional (Cre-Lox P strategy) and hepato-specific (Cre is under albumin promoter, so it is expressed only in the postpartum liver) knock-out, *HNF4 α* deletion has been shown to result in strong metabolic alterations and mice died around 6-8 weeks after birth with severe steatosis and hepatomegaly (Chen et al., 1994; Hayhurst et al., 2001). To overcome the lethality mice carrying a Cre recombinase fused with a triple mutant of human estrogen receptor were generated; the receptor becomes able to bind the Tamoxifen and not its natural ligand. Moreover Cre promoter is also hepato-specific, controlling in this way both the tissue specificity and the timing at which the inactivation is effective, through the Tamoxifen injection (Bonzo et al., 2012). These results will be described in the next section.

Hnf4 α liver functions

Hnf4 α in liver plays different roles. As anticipated in previous section, it is necessary to establish and maintain the **hepatocytes differentiation** state. Moreover in the liver it is an important actor of **lipid homeostasis**, as *HNF4 α* -null mice show hepatic steatosis, with the accumulation of lipid droplets in the cytoplasm of the hepatocytes. Furthermore the lipid serum

levels are altered: total cholesterol, triglycerides and high-density lipoprotein (HDL) cholesterol are lower than in control animals. On the other hand bile acid levels are higher, once again showing an impaired lipid metabolism and export in *HNF4α*-null livers, also demonstrated by a Western blot approach, testing proteins involved in lipid synthesis and secretion (Gonzalez, 2008; Hayhurst et al., 2001). Hnf4α is also necessary to **epithelial markers expression**. The knock-out mice demonstrated general liver architecture disorganization, correlated to the capacity of Hnf4α to regulate E-cadherin subcellular localization and cytokeratin 18 expression. In addition, Hnf4α is able to down-regulate mesenchymal genes such as N-cadherin, α-SMA, fibronectin and vimentin. All these data showed a clear role for Hnf4α to establish and maintain the epithelial phenotype, and to block the epithelial-to-mesenchymal transition (EMT) (Santangelo et al., 2011). Furthermore Hnf4α is involved in controlling **cell proliferation**. This is associated with an up-regulation of cyclins A2, B1, D1 and E2, cyclin-dependent kinase and c-Myc and PCNA in *HNF4α*-null mice in comparison to controls (Bonzo et al., 2012).

Hnf4α and β-catenin in liver

Hnf4α and β-catenin signalings are both involved in liver development and differentiation. It was therefore tempting to search for a link between both pathways. Cattin *et al.* have done this pioneering work in the intestine. They showed, using mice with Hnf4α-null intestines, that Hnf4α was able to interact with Tcf4 and to repress β-catenin target genes in this tissue (Cattin et al., 2009).

An interesting work of Stanulović *et al.* in 2007 explored this in the liver. By the use of hepatospecific *HNF4α*-null mice (through the use of a Cre under the control of albumin promoter) they demonstrate a control of Hnf4α on pericentral gene expression, in particular in GS, OAT or thyroid hormone receptor β (TRβ) (Stanulović et al., 2007). GS control was demonstrated by *in silico*, *in vitro* and *in vivo* approaches, and both chromatin occupancy of its

promoter by Hnf4 α and change in its expression were found. All three mRNAs in *HNF4 α* -null mice were no more only pericentral expressed (like in controls) but also in periportal areas, even if not at the same level as in pericentral ones. Finally when Hnf4 α is removed, phosphoenolpyruvate carboxykinase (PEPCK) expression is weaker than in controls. Interestingly they showed that Hnf4 α does not influence the zonation of PEPCK expression but its level. Knowing that WNT/ β -catenin pathway is the master regulator of the metabolic zonation in the liver, these findings let hypothesize a negative interaction between the two transcription factors: Hnf4 α and β -catenin/Tcf4 (Stanulović et al., 2007). Ning *et al.* in 2010 demonstrated the capacity of Hnf4 α to downregulate β -catenin transcriptional activity in cell culture, diminishing its nuclear translocation and so its target genes expression, and this would be mediated by an impact of Hnf4 α on E-cadherin expression, and a repressive action of Hnf4 α on Epithelial-Mesenchymal Transition (EMT) (Ning et al., 2010).

Another interesting study exploring this complex relationship is the one of Yang *et al.* in 2013. First *in vitro* they correlated the increasing β -catenin activation with a decreased Hnf4 α transcription and proposed a cascade in which WNT controls Snail and Slug, which themselves control Hnf4 α transcription by binding to its promoter. In addition they tested the inverse relationship and they describe the already known Hnf4 α capacity to limit β -catenin action through the direct binding between Hnf4 α and Tcf4. To elucidate the *in vivo* situation, a N-diethylnitrosamine (DEN) induced rat model of HCC was chosen and the results are that the more HCCs progress the more β -catenin protein accumulates preferentially in the nucleus and the less Hnf4 α is expressed, further illustrating the antagonism between the two transcription factors (Yang et al., 2013).

Hnf4 α and liver cancer

All its functions make Hnf4 α a good candidate to be a tumour suppressor gene, as it controls cell differentiation, proliferation, endothelial phenotype and also lipids metabolism, functions whose deregulation can lead to develop liver cancer. Experiments have been performed to test this hypothesis, in particular Ning *et al.* used rats injected with DEN; they described a correlation between hepatocarcinogenesis progression and the loss of Hnf4 α , showing that the progressive loss of Hnf4 α lets the EMT take place. If an adenovirus carrying Hnf4 α gene is injected in DEN treated rats, the state of cirrhosis (originally induced by DEN) was alleviated and E-cadherin expression restored (Ning et al., 2010). Another confirmation of Hnf4 α ability to reduce fibrosis is given by the study of Fan *et al.* in which rats fibrosis (induced by administration of thioacetamide (TAA)) was alleviated by adenovirus carrying Hnf4 α (Fan et al., 2013). This fits with previous results analysing male rats nodules and HCCs (so both preneoplastic and neoplastic lesions) in which Hnf4 α mRNA was found 46% of non-tumoural adjacent liver (Flodby et al., 1995). Also in mice tumours, induced by DEN or by 7,12-dimethylbenz[a]anthracene (DMBA) Hnf4 α expression was decreased (Kalkuhl et al., 1996). More recently Walesky *et al.* performed an interesting study, using conditional hepato-specific Hnf4 α knock-out mice: they first administrate DEN, and after 8 months Hnf4 α was invalidated thanks to Cre-LoxP strategy. The Hnf4 α deletion resulted in an increase of cell proliferation, and more importantly the HCC progression was accelerated, in comparison to Hnf4 α wild-type DEN treated mice HCCs progression (Walesky et al., 2013). Furthermore Yin *et al.* demonstrated by transfecting cell lines Hep3B and HepG2 with Hnf4 α a blockade of cell proliferation and a stimulation of apoptosis and cell cycle arrest. If cells with re-established Hnf4 α are inoculated in BALB/c nude mice the tumorigenicity is decreased in comparison to inoculation of cells without Hnf4 α . Finally also liver metastasis formation is blocked if after tumoral cell inoculation, an adenovirus carrying Hnf4 α is injected (Yin et al., 2008).

In human HCCs a cDNA array system performed by Xu *et al.* in 2001 found a plethora of genes down-regulated among which there was Hnf4 α (Xu et al.,

2001). The paper of Ning et al. in 2014 correlated the Hnf4 α decreased expression with the poor prognosis of patients (Ning et al., 2014).

In addition to these results an original study from Saha *et al.* demonstrated the effect of genetic alterations in isocitrate dehydrogenase1 and 2 (IDH1 and IDH2), the most common mutation occurring in 25% of intrahepatic cholangiocarcinoma (IHCC), on Hnf4 α . The enzymatic activity of mutants IDHs changes, generating an oncometabolite that silences Hnf4 α , impairing hepatocytes differentiation, driving together with K-ras the expansion of liver progenitor cells and so promoting biliary cancer (Saha et al., 2014).

Hnf4 α functions in other organs

Hnf4 α is also expressed in other organs: kidney, pancreas and intestine.

In the **kidney**, it cooperates with Hnf1 α to control the initiation and maturation of the proximal tubule, which is the duct system portion in the functional unit of the kidney (nephron) (Martovetsky et al., 2013).

In the **pancreas**, mutations in *HNF4 α* promoter region have been associated to maturity onset diabetes of the young 1 (MODY1), even if they are rare in comparison to other mutations associated to this pathology (for instance in *HNF1A* gene). MODY1 is a heritable form of type 2 diabetes, it is a monogenic pathology, characterised by autosomal dominant inheritance, onset age is often before 25 years of age, and pancreatic β -cells are dysfunctional (Ellard and Colclough, 2006; Love-Gregory and Permutt, 2007).

In the **intestine**, it has been associated with carcinoma, both in colon cancer cell lines in which a decrease of *HNF4 α* has been depicted, but also in human colon carcinoma biopsies where the pattern expression of *HNF4 α* is different from non tumoral tissue (Algamas-Dimantov et al., 2013; Tanaka et al., 2006).

Cancer

General introduction

Cancer is a malignant transformation that could happen in a multitude of different tissues, known as the cancer primary sites. Cancer is considered a group of diseases (more than 100 cancers have been classified), because molecular mechanisms of cancer onset, progression and spread in other tissues can change among different primary sites. It depends on genetic mutational events: activating a proto-oncogene or inactivating a tumour suppressor gene. In few cases mutations are germ-line ones, inherited from the parents, or, more frequently, are somatic. The accumulation of mutation is a multi-step process that underlying tumour development (Pecorino Lauren, 2012; Weinberg, 2007).

Different cancer hallmarks had been described by Hanahan and Weinberg in 2000, but the first cancer landscape has been recently revisited, adding emerging hallmarks, which mainly consist in avoiding immune destruction and deregulating cellular energetic. This latter aspect is a strategy that the cancer cells use to adapt their metabolism to their energetic and macromolecules needs (Figure 12) (Hanahan and Weinberg, 2000, 2011).

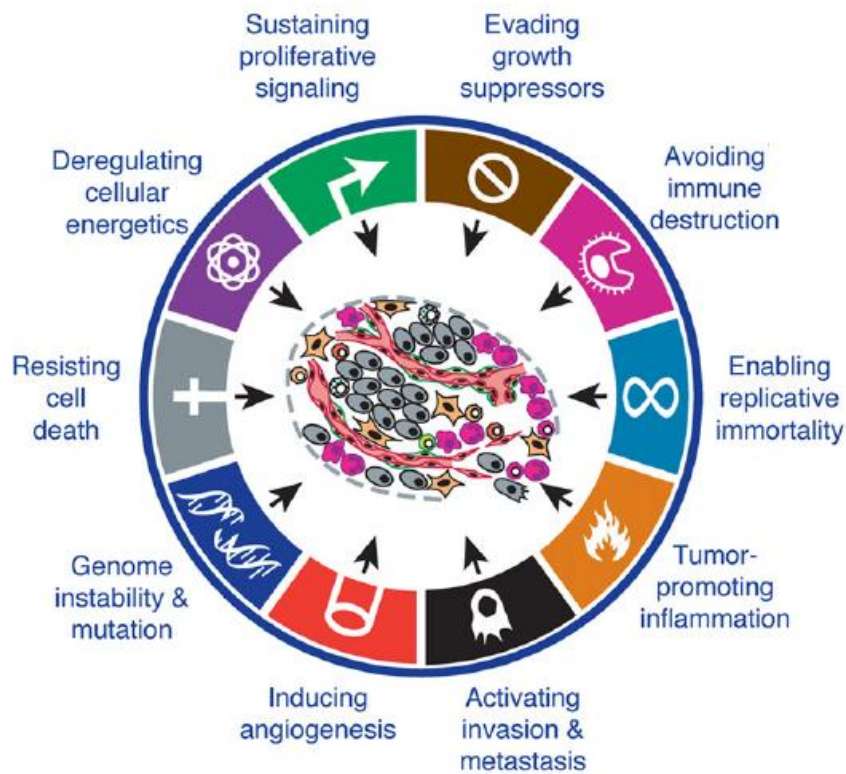


Figure 12. Cancer new hallmarks.

Cancer cells need changes to become oncogenic, involving different molecular processes fundamental for their growth (Hanahan and Weinberg, 2011).

Tumour metabolism

Even if it seems conflicting at first sight, tumours are both preparing for possible future starvation, by synthesizing and accumulating glycogen and lipids and at the same time they adapt their energetic needs to fuel the cancer cells.

Moreover solid tumours often experiment a hypoxic context, particularly in their central area, even if the tumour tries to compensate by increasing angiogenesis. Hypoxic tumours, due to their reduced oxygen concentration, have a nuclear stabilization of hypoxia-inducible factor 1 (HIF-1). HIF-1 is able to transcriptionally regulate different proteins, like glucose transporter 1 (GLUT-1) expression (to stimulate glucose uptake) and other hypoxia-responsive genes, to adapt the organ to the non-physiological situation, and in particular to favour a Warbourg effect (Amann and Hellerbrand, 2009).

Warburg effect

The Warburg effect is also called “aerobic glycolysis” and consists in a change in energetic metabolism to confer to the tumour an energetic advantage (Gallo et al., 2015; Xu et al., 2015). This property has been used in positron emission tomography (PET) studies. The PET technique is based on the injection to the patient of a tracer compound labelled with a positron emitting radionuclide, in this case the fluorodeoxyglucose (FDG). The radionuclide in the radiotracer decays, emitting positrons and the image is reconstructed to finally show the tracer distribution in the body of the patient. If a tumour experiments a Warburg effect, it will increase its fluorodeoxyglucose uptake and fixation, in comparison to the rest of the body. These tumours can be diagnosed by a simple FDG-PET clinical examination. In the presence of oxygen, differentiated tissues first metabolize glucose to pyruvate via glycolysis and then completely oxidize most of that pyruvate in the mitochondria to CO₂ during the process of oxidative phosphorylation. Oxygen is required as the final electron acceptor to completely oxidize the glucose, and so it is essential for this process. When oxygen concentration is limited, cells can redirect the pyruvate generated by glycolysis away from mitochondrial oxidative phosphorylation by generating lactate, and this is an anaerobic glycolysis process. This generation of lactate cycles NADH back to NAD⁺, but results in minimal ATP production, if compared with oxidative phosphorylation. In proliferating cells, ~10% of the glucose is also diverted into biosynthetic pathways upstream of pyruvate production (i.e. the pentose phosphate pathway): this allows the synthesis of macromolecules (nucleotides for example) required during a proliferation process. Warburg observed that cancer cells tend to convert most glucose to lactate regardless of whether oxygen is present. This so-called “aerobic glycolysis” is a property shared by normal proliferative tissues (Warburg et al., 1927) (Figure 13).

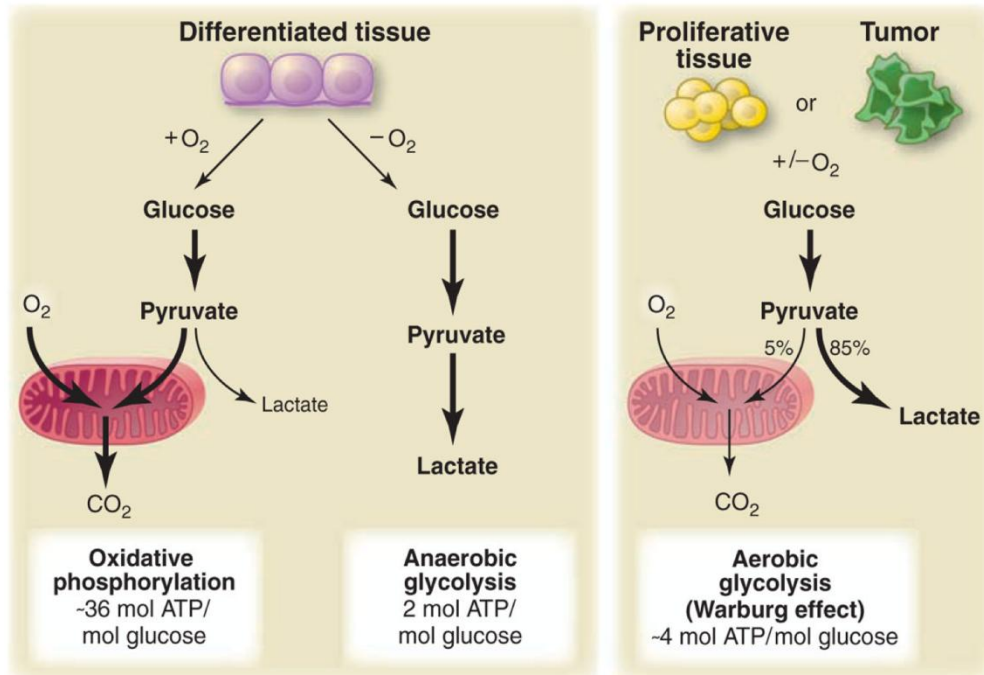


Figure 13. Two distinct glycolytic ways in differentiated versus proliferating/ tumoral tissues.

Mitochondria remain functional and some oxidative phosphorylation continues in both cancer cells and normal proliferating cells. Nevertheless, aerobic glycolysis is less efficient than oxidative phosphorylation for generating ATP, but more efficient for macromolecules synthesis (Vander Heiden et al., 2009).

Different and contradictory results exist in literature about the Warburg effect in the HCCs. Amann and Hellerbrand in 2009 described the increase of GLUT1 expression in a cohort of 152 HCCs, demonstrating at least the need of a higher glucose uptake in these tumours (Amann and Hellerbrand, 2009). In Beyoğlu and Idle work in 2013 metabolic changes were analysed in different pathologies and in HCCs a trend of switching versus glycolysis was found. Also thanks to a systematic comparison between biopsies of tumour and surrounding normal liver, the fold of glycolysis found was up to four-fold. (Beyoğlu and Idle, 2013).

But the glucose metabolism is not the only metabolic adaptation that the tumour can adopt. At least two other metabolic switches have been described: that of Acetyl-CoA and glutamine.

Other examples of metabolism adaptation

1. Acetyl-CoA, the “hub of metabolism”: (Figure 14)

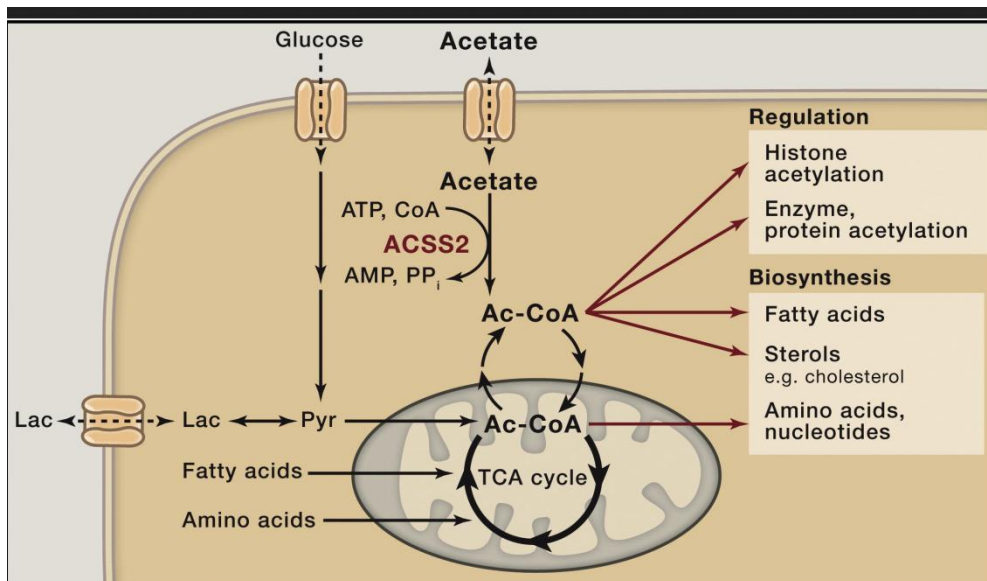


Figure 14. Acetate is the hub of metabolism.

Acetate is necessary to produce acetyl-CoA, to let pyruvate enters in the TCA cycle (Lyssiotis and Cantley, 2014).

Acetyl-CoA is the central node in carbon metabolism. It is a key substrate to bring pyruvate through the TCA cycle to produce fatty acids and sterol and high acetyl-CoA concentration stimulates histone acetylation. Recent studies underline the importance of having available acetyl-CoA in cancer cells to sustain their growth. In particular the central enzyme is the nucleocytosolic acetyl-CoA synthetase enzyme (ACSS2), able to produce acetyl-CoA from acetate. The loss of ACSS2 has been associated with different oncogene-induced liver tumourigenesis, like TAG (genetic modified mice, expressing the SV40 early region) or MYC overexpression associated with phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase (PTEN) loss. In both models, the absence of functional ACSS2 decreases both tumour onset and growth. Furthermore ACSS2 expression is higher in a lot of tumours and is correlated with an increased uptake of ^{11}C -Acetate in PET screening, demonstrating the physiological role of ACSS2 in human tumourigenesis (Comerford et al., 2014). On the other hand, in previous studies the routine

use of ^{11}C -Acetate in HCC diagnostic was already described as difficult because of the short half-life of the tracer, so it is possible only in laboratories having their own on-site cyclotron to constantly produce it (Talbot et al., 2006).

2. Glucose versus glutamine addiction:

An interesting study about two oncogene-induced liver tumours well depicted that each type of tumour has its own specific metabolism (Figure 15).

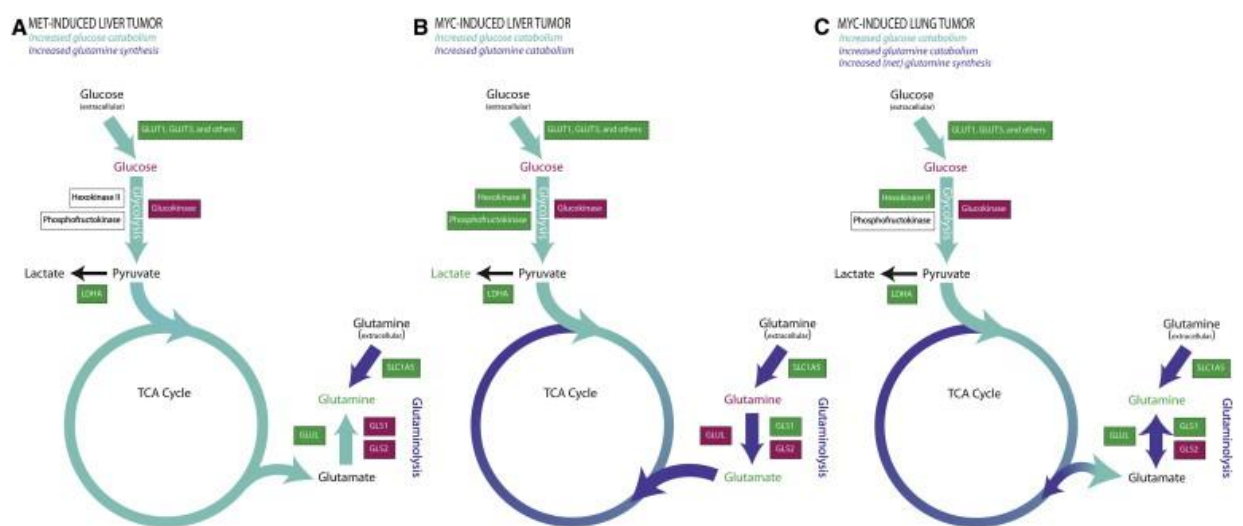


Figure 15. Specific metabolism for different type of tumours.

(A) Liver tumours induced by *MET*, (B) liver tumours induced by *MYC* and (C) lung tumours induce by *MYC* show different metabolism adaptations, even if in the same tissue (A and B), or induced by the same oncogene (B and C) (Davidson and Vander Heiden, 2012).

The metabolic phenotype of genetically-engineered liver tumours induced by *MET*, known also as hepatocyte growth factor receptor (HGFR), and of liver and lung tumours induced by *MYC* (which codes for a transcription factor) was studied. *MET* provokes liver tumours that are activated for different signalling pathways: PI3K/Akt, RAS/MAPK and WNT/ β -catenin. Target genes induced by *MYC* are involved in glucose uptake and metabolism, lactate excretion and glutamine catabolism. In both types of liver tumours it was

described an increase of glucose uptake (by ^{18}F FDG PET/CT), even if it was higher in *MYC* ones, which have also a major lactate production compared to *MET*-induced cancers. Another peculiarity is that in *MET*-induced cancers (not in *MYC*-induced) the GS expression is increased, situation congruent with the β -catenin activation in these tumours, and they are characterised by an increased glutamine anabolism. Conversely *MYC*-induced tumours showed an increase in both glucose and glutamine catabolism, in comparison to normal tissue, but also to *MET* tumours. In conclusion these experiments underlined that even in the same tissue, cancer progression can be driven by different metabolic adaptations, depending on the oncogene. Moreover in different tumours, driven by the same oncogene (liver and lung *MYC*-induced cancers were compared), tumour metabolism also differs (Davidson and Vander Heiden, 2012; Yuneva et al., 2012).

A last example of metabolism adaptation is linked to choline metabolism.

Choline physiological metabolism

Choline is a zwitterionic quaternary amine, whose plasma concentration has to be 10-50 μM (Inazu, 2014). Choline has been depicted quite recently as an essential nutrient: the recommended intake is about 550 mg/day and is commercially proposed as a dietary supplement. It has been added to the list of human vitamins, being recognised as a necessary component of our daily meals (Gossell-Williams and Benjamin, 2006; Mehedint and Zeisel, 2013). Moreover the level of daily choline has to be adapted if the patient is pregnant, demonstrating the importance of this substance in embryonic development (Zeisel and da Costa, 2009). If the choline intake is not sufficient, the liver develops fatty acids accumulation and consequently liver damages (Corbin and Zeisel, 2012).

Considering that choline principal source is food, choline needs first to be uptaken in cells. Due to the fact that it is a charged molecule, it cannot pass the plasma membrane freely, but needs transporters. Different families of specific transporters are known, but many other non-specific ones could be used for choline uptake:

1. OCT/SLC22A1-2: the organic cation transporters (OCT or SLC22A1-2) are polyspecific transporter of cations, which are low-affinity ones.
2. CTL/SLC44A1-5: the choline transporter-like proteins (CTL or SLC44A1-5) are intermediate-affinity transporters.
3. CHT/SLC5A7: the choline transporters (CHT or SLC5A7) are high-affinity shuttle proteins, able to specifically import choline in a Na⁺-dependent way. The CHTs are present in the cholinergic neurons and are strictly correlated with the production of acetylcholine (ACh) (Figure 16) (Inazu, 2014).

Protein name	Tissue distribution	K_m for choline	Sensitivity of HC-3 (K_i)	Sodium-dependency	Substrates
CHT1 (SLC5A7)	Brain, spinal cord	< 10 μ M	50–100 nM	Yes	Choline
CTL1 (SLC44A1)	Multiple tissues (brain, colon, etc)	10–50 μ M	10–100 μ M	No	Choline, organic cations
CTL2 (SLC44A2)	Tongue, muscle, kidney, lung, inner ear, neutrophil	Unknown	Unknown	Unknown	Choline
CTL3 (SLC44A3)	Kidney, colon	Unknown	Unknown	Unknown	Unknown
CTL4 (SLC44A4)	Intestine, kidney, stomach	Unknown	Unknown	Unknown	Unknown
CTL5 (SLC44A5)	Spinal cord	Unknown	Unknown	Unknown	Unknown
OCT1 (SLC22A1)	Liver, kidney, intestine, brain	300–400 μ M	Low (250 μ M)	No	Organic cations
OCT2 (SLC22A2)	Kidney, brain	100–500 μ M	Low (250 μ M)	No	Organic cations

CHT, high-affinity choline transporter; CTL, choline transporter-like protein; OCT, organic cation transporter.

Figure 16. Summary of know choline transporters.

CHT is the family with high affinity, CTL intermediate and OCT the low one. Their expression is different in the various organs (Inazu, 2014).

Once choline has entered into the cells it can have different fates. Mainly in neuronal cells, as already introduced, it is used in cholinergic synapses to produce ACh. At the synaptic level there is also a system of recycling: once ACh has been released by exocytosis and acts as neurotransmitter, there is a system for re-uptake to allow a reutilization (Kawashima and Fujii, 2000; Wessler and Kirkpatrick, 2008). It has been demonstrated that ACh can be produced not only in neurons, but also in other cells, like gallbladders', lymphocytes, some epithelial cells (Kawashima and Fujii, 2000; Wessler and Kirkpatrick, 2008).

Methyl group production is essential to control DNA methylation, one of the epigenetic tools to modulate gene expression, and alterations of these patterns are very frequent during developmental stages (Niculescu et al., 2006).

The enzymes responsible for catalyzing this methylation on DNA are the DNA methyltransferase 3 and 1 (DNMT3 and DNMT1), able to respectively establish the methylation pattern during the development (*de novo* methylation) and to maintain (actually to copy) it during later mitosis in the daughter cells (Blusztajn and Mellott, 2012; Chedin, 2011).

Choline as a Kennedy pathway substrate

The second scenario for choline fate in cells is the most important (around 95%) and it is a branch of the Kennedy pathway. The Kennedy pathway defines two mirroring cascades, working in parallel but differing by their substrates. The enzymes can be different or in some cases they overlap (Kent, 1995). One branch is based on ethanolamine, the second on choline. In both cases the first step is an ATP-dependent phosphorylation reaction, which activates the molecules (Gibellini and Smith, 2010; Kennedy and Weiss, 1956).

Choline kinase family proteins are responsible for this phosphorylation and are encoded by two different genes: the choline kinase α and β (*CHKA* and *CHKB*). In addition *CHKA* can produce two different isoforms: Chk α 1 and Chk α 2 (with 18 discriminating aminoacids). Choline kinase works as a dimer, homo- or hetero- according to the tissue, and its activity rate changes: higher in homodimer α/α , medium in heterodimer α/β and low in homodimer β/β (Aoyama et al., 2002; Gallego-Ortega et al., 2009). The different isoforms can be also activated in a specific manner by some signalling pathways (like Ras and Rho GTPase) and they play different roles in tumorigenesis, as further explained in next section (Gallego-Ortega et al., 2009). Furthermore Chk α can control cell proliferation, transformation and apoptosis. This was shown using a microarray analysis, of the cell line Hek293T, transfected with empty or *CHKA* carrying vector, and of xenografts realised with the same cells (Ramirez de Molina et al., 2008).

The next step of Kennedy pathway presumes the use of a cytosine-5'triphosphate (CTP) by the enzyme phosphocholine cytidyltransferase (CCT or Pcyt), to transform phosphocholine in cytidyldiphosphate-choline (CDP-choline). CDP-choline is transformed by choline/ethanolamine phosphotransferase (CEPT) or choline phosphotransferase (CPT) in phosphatidylcholine (PC). PC is an essential component of cell membranes, it is also an important molecule for triglycerides packaging and export in the VLDLs and for increasing solubilisation of bile salts (Corbin and Zeisel, 2012; Gibellini and Smith, 2010). The VLDLs are carriers in the blood circulation of triglycerides and cholesterol esters; they are composed of a phospholipidic membrane, of apoproteins and cholesterols. PC is necessary not only because is the main component of the VLDLs membrane, but also because it is the principal component of the membrane of the secretory pathway (from the ER, to the Golgi, to the plasma membrane) (Figure 4) (Li and Vance, 2008; Noga and Vance, 2003).

Moreover PC can be secreted in the bile, through the canalicular membrane of hepatocytes (at the apical side), by the mediation of the PC-specific flippase (a membrane transporter with catalytic activity), the multi-drug resistance 2 (MDR2) in mice, MRD3 (or ABCB4) in humans (Li et al., 2005).

The importance of PC functions in liver is demonstrated by the negative effects in case of a low choline diet in humans and also in rodents fed with a methionine choline deficient (MCD) diet. In rodents methionine deficiency is associated to choline deficiency in order to avoid methionine compensatory effects as methyl group donor. This model mimics the human pathology of non-alcoholic steatohepatitis (NASH), the most severe phase of NAFLD, presenting lipids accumulation in the parenchyma and a consequent liver injury, oxidative stress and inflammation.

PC can also be obtained in the liver from the phosphatidylethanolamine (PE), thanks to the action of phosphatidylethanolamine N-methyltransferase (PEMT) that is able to produce about 30% of the PC amount in liver. Indeed in PEMT knock-out mice the liver steatosis developed after a mild choline-deficient diet, demonstrating its important role in PC supply to hepatocytes (Li et al., 2005).

The different uses of phosphatidylcholine

PC can be transformed both in diacylglycerol (DAG) and in phosphatidic acid (PA), by two phospholipases, respectively by the PC-specific phospholipase C (PC-PLC) and the PC-specific phospholipase D (PC-PLD) (Exton, 1990). DAG is involved in biosynthesis and degradation of glycerolipids and moreover it regulates protein kinase C (PKC) activity (Bishop and Bell, 1988). Phosphatidic acid is a precursor of many lipids and it can act also as a signalling molecule, for instance in *Drosophila melanogaster* it mediates the phototransaction (conversion of the light in electrical signals) (Raghu et al., 2012).

Choline plays a role in different tumours

Choline pathway has been described as a hallmark of cancer metabolism adaptation, and the first evidence of an increasing turnover dates back to 1987 (Lacal et al., 1987).

In particular, the Magnetic Resonance Spectroscopy (MRS) progresses in cancer research (Griffiths et al., 1981), and the clinical need of non-invasive techniques to diagnose, stage and monitoring the tumours, incentivises the use of both MRS and PET imaging to study choline metabolism.

In different cancers, there is an increased use of choline pathway (Glunde et al., 2011). As previously described the major source of choline is the diet, even if a little portion could be recycled, and it is important to have functional transporters. Interestingly in the review of Glunde *et al.* transporters expression has been described in different cancer tissues. At instance in colon and lung cancers *CTL1* is increased, whereas in ovary tumours *CTL3* is induced. In lung cancers *OCT3* expression is found increased, whereas in breast cancers, it is the case for *OCT2* and *CHT1* (Iorio et al., 2010; Kouji et al., 2009; Wang et al., 2007; Yuan et al., 2006). The choline transport activity has been evaluated and seen higher than in adjacent normal tissue in breast, colon, prostate and liver tumours, and not systematically correlated with a higher transcription rate of the involved enzymes. In other studies only the import activity was tested *in vitro* using radiolabeled choline and in some

cases with the use of specific inhibitors of carriers, like in liver and prostate cancers, where the choline transport activity was found lifted up (Hara et al., 2006; Kuang et al., 2010; Müller et al., 2009).

Kennedy pathway has been dissected in several cancers (Glunde et al., 2011, for review). The activity of the first enzyme of the pathway, the choline kinase, can be measured by an enzymatic assay. In a multitude of tumour sites, like breast, bladder, colon, lung and ovary it has been found increased. In prostate cancers only Chk α expression was analysed and found increased (Hernando et al., 2009; Iorio et al., 2010; Nakagami et al., 1999; Ramírez de Molina et al., 2002a, 2002b, 2007). The choline kinase β activity has been shown to be not powerful enough to induce tumour growth (Gallego-Ortega et al., 2009). In last year, another advantageous approach has been to study the association between single nucleotide polymorphisms (SNP) of candidate genes and a multitude of pathologies. In this context an interesting population study found an association between SNPs in *CHKA* gene and HCCs. A particular SNP, rs3794186, was found associated with a high level of Afp in blood serum (superior to 200 ng/mL). No other association between SNPs and other tumour characteristics, like size and phenotype details, were significant (Park et al., 2015).

The next step of phosphocholine transformation in CDP-choline by CCT was examined in terms of gene expression and enzymatic activity. In colon and liver cancers, both were superior in tumoural tissue than in normal one (Dueck et al., 1996; Tessitore et al., 1999).

The amount and the activity of the phospholipases (PC-PLC and PC-PLD) were also analysed. In breast and colon cancers PLD was increased, whereas in ovary cancer PLC was increased. In breast, colon and ovary cancers, PLD activity was higher and furthermore PLC was found more activated in colon, liver and ovary cancers (Noh et al., 2000; Oshimoto et al., 2003; Saito et al., 2007; Uchida et al., 1997; Wu et al., 1997).

Even if at the beginning it seemed that choline was necessary in particular for its function in getting plasma membrane precursor, linking this need to

tumour proliferation, it appears more and more evident that the multiplicity of functions supported by choline supply could be involved in tumour onset and growth (Ramírez de Molina et al., 2004).

Choline-related cancer therapies

The most promising choline enzyme target for cancer therapy is choline kinase, due to the high number of cancers showing an increase of its transcription; in particular a pharmacological inhibition is conceivable.

A well known inhibitor is a compound based on hemicholinium-3, showing an anti-proliferative activity both in cell cultures and in mice xenografts (Sánchez-Martín et al., 2005).

MN58b is a first-generation drug able to block specifically choline kinase α isoform. It has been demonstrated that the use of MN58b has the same effects as the genetic inactivation of Chk α in cells that overexpress Chk α , both in cultures and xenografts (Gallego-Ortega et al., 2009; Lacal, 2001; Ramirez de Molina et al., 2008; Al-Saffar et al., 2006). The problem of using MN58b is related to the drug toxicity (different side effects) and moreover acquired drug resistance.

A second generation drug is TCD-717 (also called RSM-932A). TCD-717 is able to block Chk α , both in cell culture of HT-29 (human colon adenocarcinoma cells) and in mouse xenografts, of non-small cell lung cancer (NSCLC) H-460 and breast adenocarcinoma MDA-MB-231. TCD-717 is now in phase I of clinical trial, by TC Pharma®.

Both drugs (MN58b and TCD-717) are choline competitors at the level of choline binding site (de la Cueva et al., 2013; Lacal and Campos, 2015).

The hepatocellular carcinoma

The most common primary liver cancers (80-85%) are the hepatocellular carcinomas (HCCs) that are spawned from hepatic epithelial cells. The HCC is the third leading cause of cancer-related death worldwide (El-Serag and Rudolph, 2007; Llovet et al., 2003). It is the fifth and seventh most common cancer, for men and women respectively. The incidence is strongly associated to geographical position, the most elevated incidence rate is in Southeast Asia (where 75% of HCCs occur) and sub-Saharan Africa (20 per 100,000 people); a medium rate is present in Southern Europe and a low rate in Northern Europe and Northern and Southern America (<5 per 100,000 individuals) (El-Serag, 2012).

The incidence of liver cancer is increasing in Western countries due to the presence of well known risk factors: Hepatitis B and C virus infections, cirrhosis, metabolic diseases and alcohol consumption (Weledji et al., 2014). Even if for Hepatitis both the vaccine for HBV and therapies against HCV infection could act as a protection for liver cancer, for cirrhosis there is no available treatment (Camma et al., 2001; Yang et al., 2009).

HCC is dangerous not only because of the aggressiveness of the tumour, but mostly due to a lack of early and precise diagnosis and for being refractory to treatments (Knudsen et al., 2014).

Aetiologies

HCC is a cancer mainly associated with infection aetiologies, viruses (HBV and HCV), and aflatoxin-B1, NAFLD, and alcohol consumption. In general all liver pathologies that contribute to liver cirrhotic state will increase the probability of HCC onset (Badvie, 2000).

The presence in the liver of one of this factors triggers inflammation, and consequent liver injury, followed by cycles of necrosis and regeneration, through proliferation. After each cycle the liver structure is getting worse, with the formation of abnormal liver nodules and also the appearance of scar tissue (collagen) organized in septa, leading to cirrhosis formation. The

nodules can evolve into hyperplastic and then dysplastic ones acquiring genetic mutations to finally give rise to HCC (Figure 18) (Farazi and DePinho, 2006).

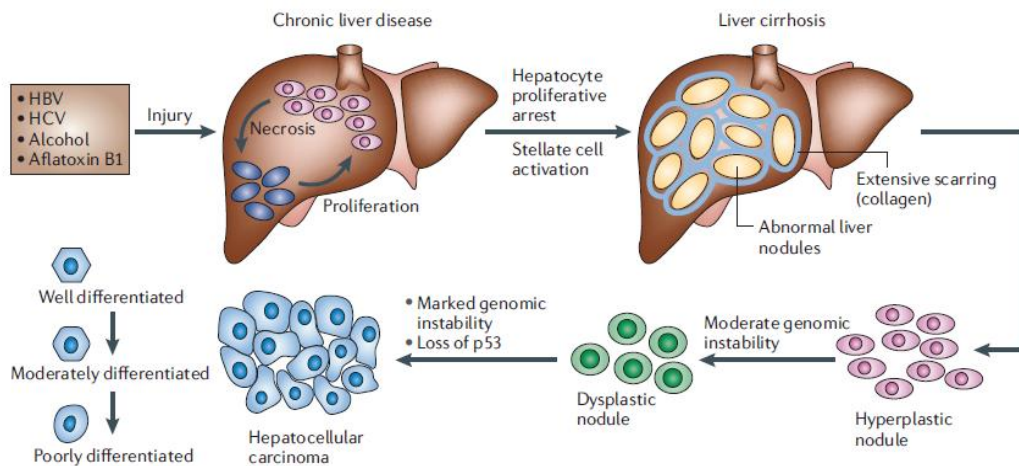


Figure 18. HCC histopathological progression.

Different factors can trigger hepatic injury cycles (destruction-regeneration), which foster the cirrhosis onset. Further step is the apparition of liver nodules, at the beginning hyperplastic, after dysplastic and they can finally evolve into HCC (Farazi and DePinho, 2006).

Like all cancers, HCC is a multi-step process, including genetic and epigenetic modifications, changing cells from normal to cancerous ones. Each aetiology factor can reach this final result of inflammation and necrosis/proliferation cycles or genomic instability through different mechanisms, provoking microdeletions and growth genes activation (HBV), in promoting cirrhosis onset (HCV), acting as mutagen (Aflatoxin-B1) or inducing oxidative stress and stimulating the production of inflammatory cytokines (alcohol) (Farazi and DePinho, 2006).

Therefore due to the fact that the early detection of the tumour is the best way to cure it, it is necessary to have a medical follow-up for people with at least one of these risk factors (Llovet et al., 2003).

Classification

Different teams, using genomic approaches, developed several classifications of HCCs. These studies converged on the identification of two main subgroups: the *CTNNB1*-wt and the *CTNNB1*-mut ones, which can both be modelised by mouse models (Figure 19) (Boyault et al., 2007; Guichard et al., 2012; Hoshida et al., 2009; Lee et al., 2004, 2006).

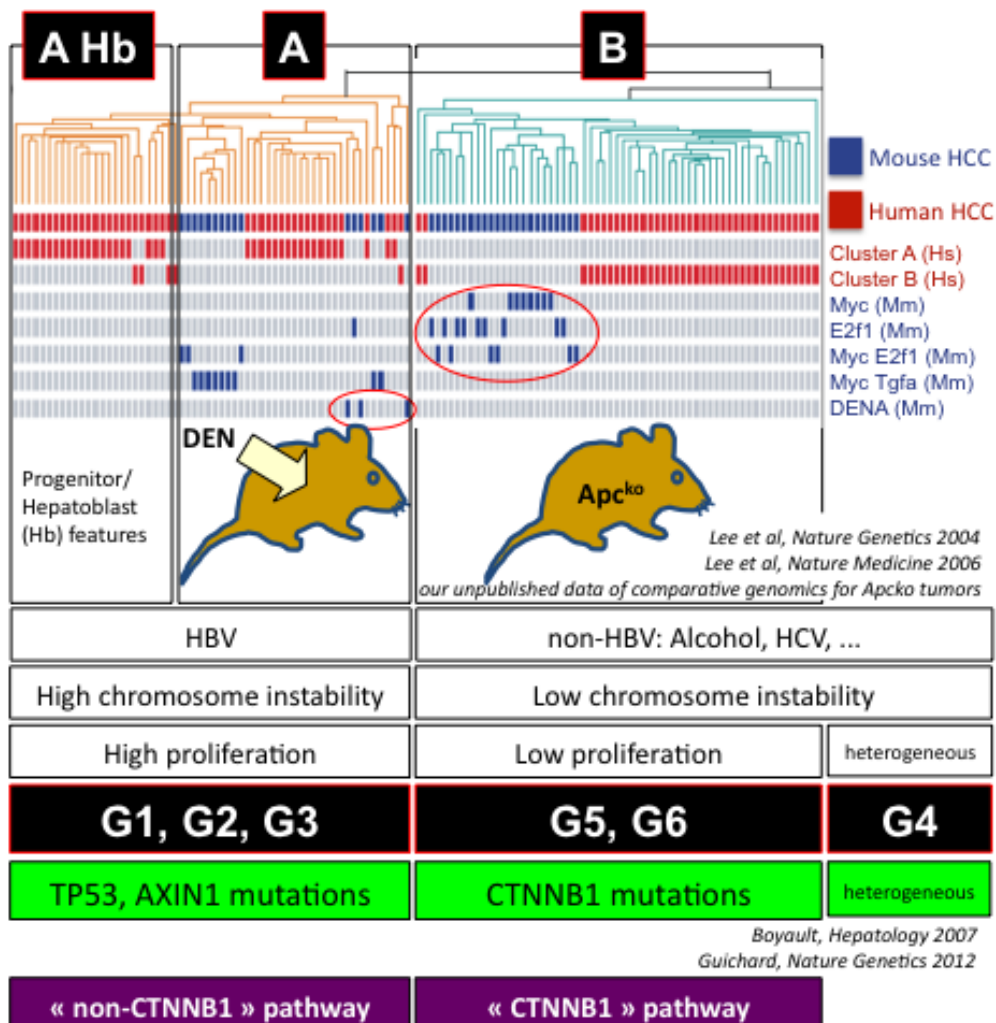


Figure 19. Classification of HCCs.

HCCs can be divided in two groups: one associated with β -catenin mutations (B) and the others (A and A Hb). Several mouse models, able to mimic these subgroups exist to explore human carcinogenesis process. (Lee et al., 2004). All the six transcriptomic signatures (G1-G6) described by Zucman-Rossi's group are represented (Boyault et al., 2007).

1. The **CTNNB1-wt** group is relatively heterogenous, and it is enriched with tumours from patients with HBV hepatitis. These tumours have high chromosomal instability and high proliferative rate. They are mainly associated with p53 and Axin1 mutations.

2. The **CTNNB1-mut** group is more homogenous, includes tumours with low proliferative rate, they are more stable chromosomically, but they are characterised by mutations in CTNNB1 gene, encoding β -catenin.

Recently, the possibility of whole genome sequencing (and so the possibility to explore more than 20,000 coding genes in few days) allows the characterization of the mutation landscape of different healthy tissues and tumours. In the case of liver tumours Guichard *et al.* in 2012 and Schulze *et al.* in 2015 did a huge HCCs genome characterization, through this technique. In Figure 20 it is possible to see the main deregulated pathways in HCCs. A candidate approach has also shown that the most common is the telomerase reactivation, which could be caused by TERT promoter mutation (54-60%), TERT amplification (5-6%) or insertion of HBV in TERT promoter (10-15%) (Nault and Zucman-Rossi, 2014a; Nault et al., 2013). TERT promoter mutations are frequently associated to CTNNB1 mutations, suggesting cooperation between the two molecular mechanisms, in cancer development. Other mutations have been described, for example mutations in *TP53* gene, altering the cell cycle, and in epigenetic regulators, both chromatin remodeling and histone methylation writers (Figure 20) (Nault and Zucman-Rossi, 2014b; Zucman-Rossi et al., 2015).

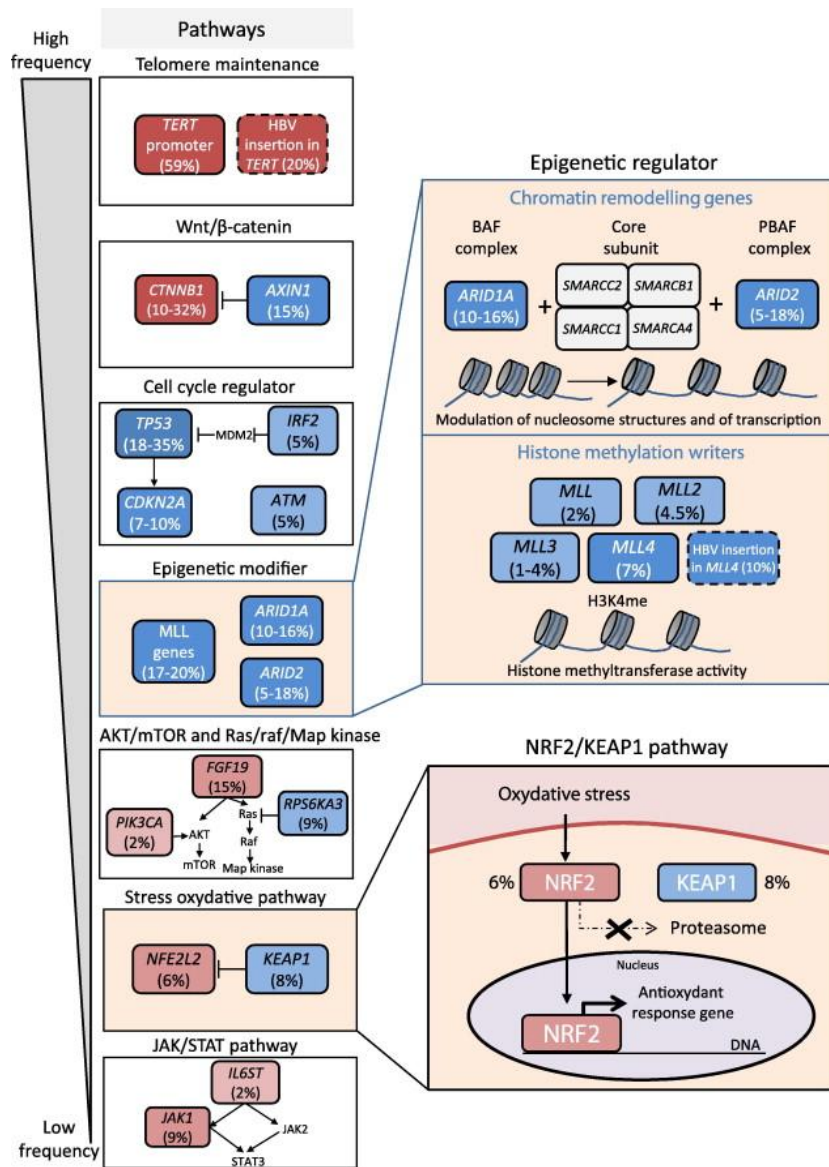


Figure 20. Major pathways involved in HCC.

Oncogenes are in red, tumour suppressor in blue. The incidence is indicated by percentages near genes (Nault and Zucman-Rossi, 2014b).

Peculiarity of CTNNB1-mutated HCCs

CTNNB1-mutated liver cancers have also a specific transcriptomic profile, with overexpression of target genes of β -catenin, which include the pericentrally expressed metabolic genes, like GS, and this transcriptional massive deregulation has metabolic consequences.

Indeed, the particular transcriptome of the CTNNB1 mutated liver tumours is associated with a very common intra-tumoural cholestasis. These tumours are never steatotic. Thirty per cent of CTNNB1-wt liver tumours, on the contrary, have lipids accumulation, the so-called steatotic phenotype (Figure 21) (Audard et al., 2007).

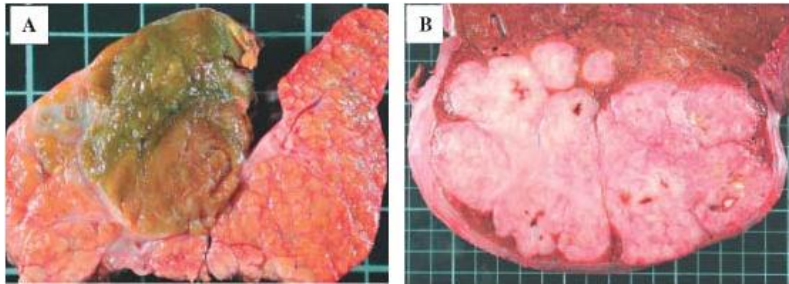


Figure 21. HCC different phenotypes.

In humans the HCC could have a cholestatic phenotype (A) or a steatotic one (B). They are respectively linked to a β -catenin activation or not (Audard et al., 2007).

Diagnosis

The early diagnosis of HCC is a current issue for the medical community. In general for internal tumours efforts have to be made to find non-invasive techniques able to detect and possibly stage a tumour.

1. **The imaging methods** are necessary for the surveillance of high risk patients, and is usually performed by ultrasonography at 6 to 12 months intervals; ultrasound has a sensitivity of 60-80% and specificity of over 90% (Jelic and Sotiropoulos, 2010; Llovet et al., 2003). In addition to complete the diagnosis the computed tomography (CT) and magnetic resonance imaging (MRI) are very useful, in particular to provide anatomical information. Moreover positron emission tomography (PET) is able to add also a functional characterisation (Park et al., 2015). Each of these modalities has advantages and disadvantages; hence different methods could be used together, in a multimodal analysis. For example MRI has a high spatial resolution meanwhile PET is highly sensitive (Kang

and Chung, 2008). The most common PET/CT analysis performed for cancer diagnosis is with fluorine-18 fluorodeoxyglucose (^{18}F FDG) as radiolabeled tracer. In this case the anatomical resolution of the CT is coupled with the functional information obtained from the PET. This examination is based on the increase of the glucose consumption in tumour metabolism, highlighting the Warburg effect: cancer cells will have an increased uptake of FDG than non-tumoral ones.

In the liver in particular it has been demonstrated that there are several ^{18}F FDG uptake patterns in HCC: higher, equal or lower than the non-tumoural adjacent liver, and FDG-PET is able to detect only 50-70% of the HCCs (Talbot et al., 2010). On the other hand the benign tumours (like adenomas, regenerative nodule, focal nodular hyperplasia) show in the major part of the cases a ^{18}F FDG uptake equal to the non-tumoral liver, with the exceptions of the null uptake in hepatic cysts and of a higher uptake in rare abscesses (Xia et al., 2015). Using this method, only rare false positive detections can be made, but not all the HCC are detectable by ^{18}F FDG PET/CT, which render actually this method not accurate.

Radiolabeled tracers are still improving and one recently promising tracer is the fluorine-18 fluorocholeline (Hara, 2001). In particular in prostate cancer it has been useful to detect the emergence of tumours and also to stage them, proving itself to be a better detector than the classical prostate-specific antigen (PSA) assay in blood. When PSA amount is less than 2.5 ng/mL it is considered as normal, when it is between 2.5 and 4 ng/mL the patient medical history has to be studied, and upper than 4 ng/mL the patient has to be screened by other more precise methods. ^{18}F Fluro-choleline PET has been a good imaging test even when PSA value is less than 5 ng/mL (Heinisch et al., 2006). ^{18}F Fluro-choleline PET is a good new tool also in other tissues; for instance, during a screening of a patient searching for prostate cancer recidivism, a squamous carcinoma in the left tonsil had been found (Treglia et al., 2015). Moreover it becomes a useful screening also for the lung cancer. In particular, by using the carbon 11 -choleline as a tracer, it has been possible to detect a large scale of pulmonary lesions. Furthermore this increased uptake is often associated

with an upper expression (both mRNA and protein level) of some enzymes involved in choline metabolism (Huang et al., 2015).

Very interestingly, recent studies have analysed the use of ^{18}F Fluro-choline PET for HCC diagnostic. Taking into account the tracer characteristics, ^{18}F Fluro-choline demonstrates, an increasing sensibility in detecting liver cancer, in comparison to ^{18}F FDG, and also an easier management of the molecule, in comparison to ^{11}C -Acetate PET, which is also better in sensibility than ^{18}F FDG, but difficult to use, because of molecule instability. A higher number of HCCs were diagnosed and in particular it was possible to detect in PET/CT images well differentiated HCCs, not visible with a classical ^{18}F FDG PET/CT (Figure 22) (Fartoux et al., 2012; Talbot et al., 2006, 2010).

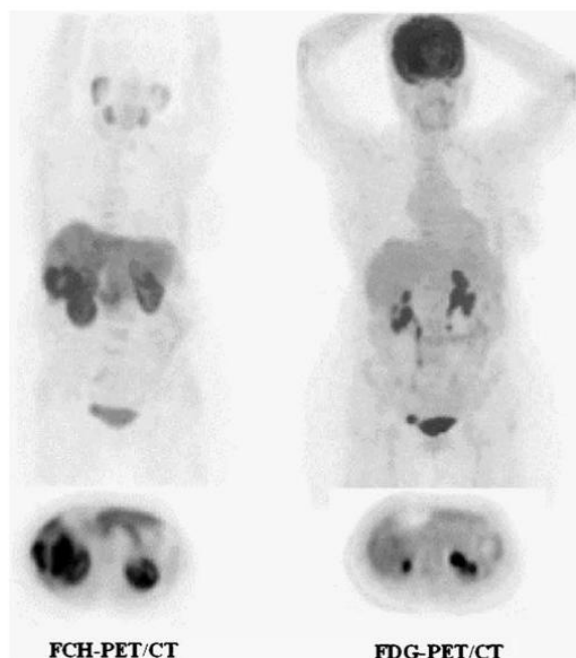


Figure 22. Fluorocholine and Fluorodeoxyglucose PET.

In the picture is shown the same patient; liver lesion was diagnosed only by F-choline PET and it is not visible with FDG (Talbot et al., 2010).

2. **Biomarkers in blood serum** could be analysed: AFP, des-carboxyprothrombin (DCP), lectin-bound AFP, tyrokinase with Ig and epidermal growth factor (EGF), interleukin-6 (IL-6) and squamous cell

carcinoma antigen (Voiculescu et al., 2014). The normal range for AFP is 10-20 ng/mL and its diagnostic sensitivity is around 60%, but up to 20% of HCC do not produce AFP and high levels of AFP could be expressed in regenerating nodules in viral cirrhosis (false positives) (Llovet et al., 2003).

3. **The histological examination** is the final step to confirm the diagnosis and is performed on tissue obtained by needle aspiration or biopsy. The histological examination is important at different levels: a simple eosin hematoxylin (HE) staining is able to show some morphological features that characterise an HCC, even if morphological similarities with other liver lesions exist. For this reason different markers are used to discriminate the HCC and to determine its differentiation degree (Figure 23).

Markers	Staining Pattern	Sensitivity, %	Specificity, %
ARG1	Cytoplasmic and nuclear	80-95	95-100
HepPar1	Cytoplasmic	70-80	~80
GPC3	Cytoplasmic	50-80	~95
pCEA	Canalicular	90	100
CD10	Canalicular	n/a	n/a
CD34	Sinusoidal	n/a	n/a

Abbreviations: ARG1, arginase 1; GPC3, glypican 3; HepPar1, hepatocyte paraffin 1; n/a, not available; pCEA, polyclonal carcinoembryonic antigen.

Figure 23. Principal histological markers in HCCs.

Proteins that help to characterise HCC differentiation (Chen and Lin, 2015).

Furthermore other specific markers, which allow a classification of the HCC in their different subgroups (explained in details later), exist. For example to identify the β -catenin hyperactivation in HCCs the immunostaining of the GS (or GLUL) is performed, and a GS strong immunostaining proves a β -catenin hyperactivation (Chen and Lin, 2015).

HCC management: treatments

Liver cancer can be treated by several different approaches, the choice of the best one for each patient is a very controversial and debated topic.

1. **Surgical**: these are high invasive surgeries, consisting in liver ablation or transplantation. In the first case the unhealthy part of the liver is removed, in the second case there is a complete substitution of the organ, with one from a healthy donor.
2. **Ablatives**: these are methods used for their function to downstage tumours, so as an intermediary palliative step to let patient be inserted in transplantation listing. It is possible to perform transarterial chemoembolization (TACE), which consists in a cytotoxic drug combination injection in the hepatic artery, followed by another injection to cause a vessel occlusion. Radiofrequency ablation (RAF) is performed in a minor range of situations (HCC has to be smaller than five centimetres, and the patient must not have a big amount of liver nodules) and it could be done under ultrasonography or CT guidance, or during a laparoscopic or open surgical procedure. Moreover other ablation procedures exist, like ethanol injection or cryotherapy, but they do not have such wide applications as the TACE and RFA.
3. **Radiotherapeutical**: The radioembolisation is based on Yttrium-90 microspheres injection in the hepatic artery to treat inoperable HCCs. The positive aspect is that it targets all the lesions present in the patient, even the undetectable ones. The microspheres not only block the blood flow to the cancer but also emit radiations that increase the damages induced in the tumour.
4. **Medical**: in the last few years classical medical approaches have started to be found for HCC. Sorafenib is a drug able to inhibit a big amount of kinases and for this reason to induce cell death. It has been approved by the food and drug administration (FDA) recently (in 2007) to treat HCC. Its

use is controversial because it is a very expensive drug and in the major part of the cases it is able to increase life expectancy only of few months (Jelic and Sotiropoulos, 2010).

The aim of the work

My thesis work has been divided in two main topics: the first consists in evaluating Hnf4 α impact *in vivo*, and its relationship with β -catenin signalling, in particular by analysing the consequences of its loss alone and together with an oncogenic β -catenin activation in hepatocytes.

The second topic concerned the deregulation in metabolism that can affect tumour onset and growth, focusing on choline pathway in a context of β -catenin activated liver tumorigenesis.

Mouse models

We used a strategy of hepatospecific and Tamoxifen inducible inactivation of APC or Hnf4 α genes. The transgenic mice overexpress the Cre recombinase: the TTR-CreTam transgene consists in a Cre recombinase transcribed under the control of the hepatospecific transthyretin promoter in a inactive conformation. After Tamoxifen administration, the conformation of the Cre changes into the active conformation. This activated Cre is able to “de-flox” the floxed exons.

Hnf4 α conditional knock-out liver

These transgenic mice harbor floxed Hnf4 α exons 4 and 5 (Hnf4 lox/lox) (Figure 24). They were interbred with TTR-Cretam mice. The resulting compound Hnf4 α lox/+/TTR-Cretam mice were then interbred with Hnf4lox/lox to obtain Hnf4 α lox/lox/TTR-Cretam mice. Hnf4 α lox/lox mice were used as controls. Mice are fed with Tamoxifen diet (400mg/Kg) for five days to reach a good invalidation.

After Cre recombination, the resulting DNA has exons 3 and 6 linked, and this produces a form of Hnf4 α with a frameshift mutation, able to originate a stop codon. The proteic product is only 128 aminoacids length.

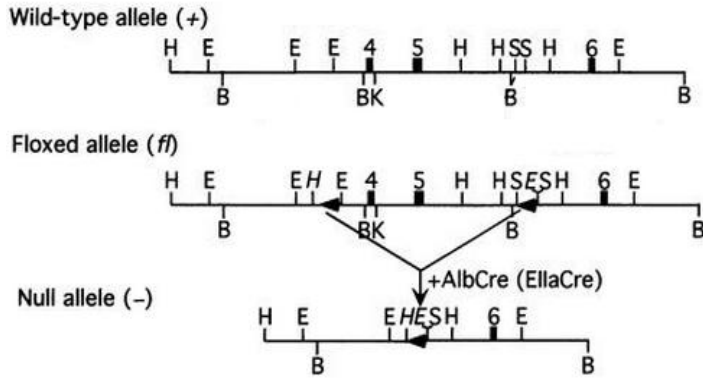


Figure 24. Hnf4 α knock-out strategy.
(Hayhurst et al., 2001)

APC conditional knock-out liver

Mice carrying the two floxed alleles on the 14th exon of the APC gene (*APC* lox/lox) (Figure 25) were interbred with TTR-Cretam mice. The resulting compound *APC* lox/+/TTR-Cretam mice were interbred with *Apc* lox/lox to obtain *APC* lox/lox/TTR-Cretam mice. *APC* lox/lox mice were used as controls.

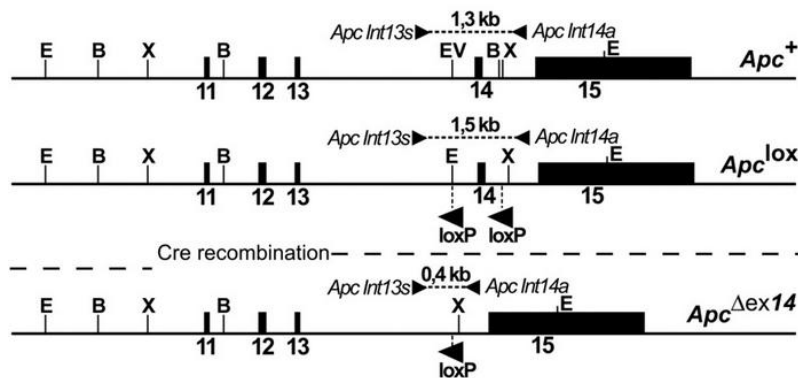


Figure 25. APC knock-out strategy.
(Colnot et al., 2004)

This model has been used in two different ways, in relation to the amount of Tamoxifen injected (Figure 26).

When a high dose (1.5mg) is injected, all hepatocytes lose APC, leading to a panlobular β -catenin hyperactivation, reached after 7 days. Mice do not survive for a long period to this protocol, developing a progressive

hepatomegaly and dying from hepatic encephalopathy, due to the deficiency in ammonia detoxification (Benhamouche et al., 2006).

These mice can be used as a pretumoral model, showing some tumoral characteristics, as the activation of metabolic targets and a proliferation phenotype.

On the contrary if a lower Tamoxifen dose is injected (0.75mg) β -catenin hyperactivation only occurs in few hepatocytes, which after months will develop a HCC that has the same characteristics of human HCC, mutated for β -catenin (Colnot et al., 2004; Gougelet et al., 2014, 2015).

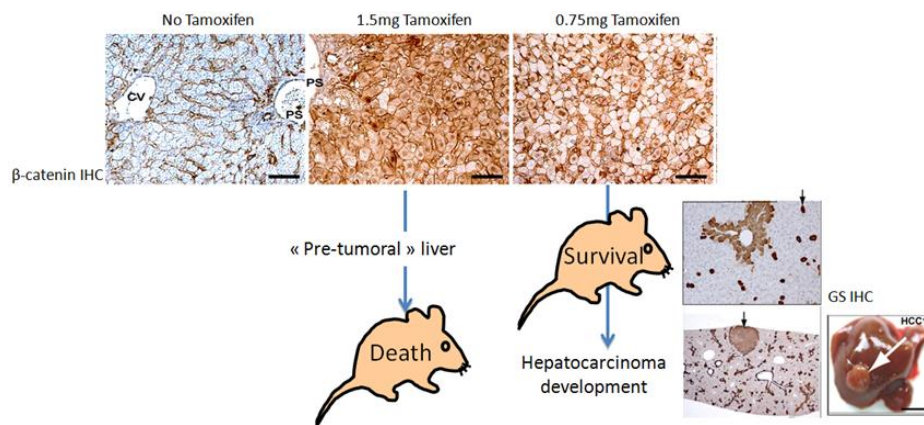


Figure 26. APC knock-out mouse model and the two different protocols.

Short term experiments mice are sacrificed 7 days after Tamoxifen injection (high dose: 1,5mg), instead long term experiment injected mice develop after months β -catenin mutated tumours (adapted from Colnot et al., 2004). β -catenin IHC reveals here the proportion of hepatocytes having an activation of β -catenin signalling (more than 90% with 1.5 mg dosage, and 10 to 30% with the 0.75 mg dosage)

An alternative protocol to the use of the transgenic TTR-CreTam mice is to inject them retro-orbital with Adenovirus expression the Cre recombinase (0.5×10^9 particules), to perform long term experiments perform long term experiments and follow HCC development (Colnot et al., 2004).

RESULTS

My thesis work has also correlated projects into which I have been involved, so my work results will be presented in four axes:

AXIS 1. The previous results of the team had focused on the ability of β -catenin to control pericentral gene transcription, and in particular genes able to control the metabolism. My project had therefore the aim to better understand the molecular mechanisms of this specialised liver targetome.

This topic has been addressed and published in *Hepatology*: “T-cell factor 4 and β -catenin chromatin occupancies pattern zonal liver metabolism” by Gougelet *et al.* in 2014. In this paper through a ChIP-Seq and RNA-Seq approach a cross-talk between β -catenin and Hnf4 α has been shown, *via* their common nuclear partner Tcf4. In particular Tcf4 factor is able to bind both Wnt Responsive Elements (WREs) and Hnf4 α Responsive Elements (HREs). The binding of Tcf4 together with β -catenin, to the WREs is related to gene activation; instead its binding on HREs correlates to negative β -catenin and positive Hnf4 α targets. Tcf4 action at WREs level leads to a transcriptional output that is linked to a metabolic program. In particular it positively controls bile acids, cholesterol and drugs metabolism. This feature is in accord with the particular “metabolic phenotype” of β -catenin activated tumours, which are mostly cholestatic and non steatotic. In this paper I validated the samples for metabolomic approach that was the key experiment to demonstrated fatty acids decrease in Apc knock-out livers, in comparison to controls. I confirmed this result also by performing Oil Red O staining and Thin Layer Chromatography. Moreover, the metabolomic approach demonstrated the bile acids accumulation, which fits with gene expression induction of Cyp7a1 and Cyp27a1, the master regulator of bile acids synthesis and also of Constitutive Androstane Receptor (CAR), involved as well in bile acids synthesis, and this gene expression increase is a characteristic also of human HCCs mutated for β -catenin.

Due to the modest participation to this work , this paper is inserted in the annexes of this manuscript.

Other results obtained thanks to metabolomic analysis were fundamental to initiate my the 3rd axis.

AXIS 2. The previous axis exploring β -catenin mechanism in controlling pericentral gene expression was able to identify a new actor: Hnf4 α .

To better understand the role of Hnf4 α in relation with β -catenin and knowing that Hnf4 α has been to be described as tumour suppressor gene, we decided to study Hnf4 α liver-specific conditional knock-out, both at short and long term. In the first case we got a total liver knock-out and we sacrificed mice after few days. In the second case, Hnf4 α was lost in all the hepatocytes, or in only few ones in combination with APC loss: we followed tumour onset and progression, in simple Hnf4 α , in simple APC knock-out and in double compound mice.

This study is presented in the following pages.

**HNF-4 α is not a tumor suppressor for hepatocarcinogenesis
driven by β -catenin activating mutations.**

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ABSTRACT

Hepatocyte nuclear factor-4 α (HNF-4 α) orchestrates the parenchyma formation in the adult liver, where it binds to the promoter of 12% of adult hepatic genes. A tumor suppressive role is currently conferred to HNF-4 α in liver cancer, since its amount is frequently impaired in hepatocellular carcinoma samples from patients with different molecular alterations. It has been recently shown that ectopic expression of HNF-4 α could limit tumor progression in a mouse model, which develops liver tumors induced by diethylnitrosamine, while no data has been published concerning the role of this factor in tumors with activating mutations of β -catenin. We report that reduced HNF-4 α was associated to higher levels of axin-2, cyclin D1 and Snail in a mouse model, which develops liver tumors following the hepatospecific deletion of *Apc*, the crucial inhibitor of the Wnt/ β -catenin pathway. However, we could not notice a significant impact of *hnf4 α* deletion in this mouse model on tumor development and progression, supporting that a HNF-4 α plays a non-classical tumor suppressive role. More interestingly, we could observe an unexpected phenotype in around 1/4 of *Hnf4^{ko}* mice, which presents a disorganization of the portal triad, characterized by a stenosis of the portal vein, accompanied with an increase in the number and size of hepatic arteries and bile ducts. This anormal portal structure recapitulates in fact the human idiopathic portal hypertension syndrome. We could correlate the presence of this altered portal triad with a higher expression of BMP-7, Snail and vimentin mRNAs, while there is no association with e-cadherin loss. This suggests that an original endothelial- and epithelial-to-mesenchymal transition occur following hepatic HNF-4 α loss.

INTRODUCTION

Hepatocyte nuclear factor-4 α (HNF-4 α) belongs to the nuclear receptor superfamily of ligand dependent transcription factors NR2A1 [1], which is most highly expressed in the liver [2-5], where it binds to the promoter of 12% of adult hepatic genes [6]. It plays a key role in liver development during gastrulation [7], and orchestrates the parenchyma formation in the adult

liver [8]. It also guides hepatoblasts to hepatocyte differentiation [9]. The conditional disruption of *hnf4α* in mouse liver showed that HNF-4α regulates a number of genes involved in lipid, glucose, amino acid and xenobiotic metabolisms [4]. In addition, numbers of studies showed a major role of HNF-4α in cell proliferation, particularly in liver [10-12]. Regarding to its anti-proliferative role, recent studies have reported that HNF-4α level was decreased in human hepatocellular carcinoma (HCC) [13-17], in contrast with a study by Xu *et al.* suggesting that HNF-4α expression was increased in HCC [18]. In mouse models, a deletion in *hnf4α* gene promotes hepatocarcinogenesis driven by diethylnitrosamine (DEN) [10], while HNF4α expression blocked liver tumor development in this model through NF-κB inhibition [16, 19]. More recently, HNF-4α was described as a major mediator of inflammatory programs in liver cancer [14, 20], and could regulate the epithelial-mesenchymal transition [16, 19, 21-23]. In conclusion, HNF-4α appears as a powerful therapeutic target in liver diseases [13, 14, 17].

Although the molecular pathogenesis of liver cancer is now well characterized, thanks to whole-exome sequencing [24], the mortality of HCC patients remains high due to the absence of efficient treatments. Recently, we proposed that a locked nucleic acid (LNA) approach against the microRNA miR-34a could be a potent strategy to treat HCC with activating mutations in the β -catenin gene, which constitute one-third of HCC patients [13]. In particular, the LNA-34a successfully slowed down the tumor progression in a mouse model developing HCC mutated for β -catenin, due to anti-proliferative activity and pro-apoptotic effects associated to caspase-2 and caspase-3 activation. This anti-proliferative impact of the LNA-34a was in part linked to an increase in HNF-4α level, which represses cyclin D1, supporting the protective role of HNF-4α in the tumors with activating mutations in the *β -catenin* gene. In this study, our goal was to assess the role of HNF-4α to prevent the development of tumors with an activation of the β -catenin pathway. This tumoral model is both relevant for modeling human HCC and distinct from that driven by DEN induction, in which tumors has been described as mostly mutated in the *Ha-Ras* gene. We thus create a tumoral mouse model with a concomitant deletion of *hnf4α* and of *Apc*. Using this

model, we showed that *Hnf4α* deletion has no impact on liver tumorigenesis driven by β -catenin activation. Interestingly, it appears that HNF-4 α loss could alter the portal triad architecture, a phenomenon reminiscent of a human disorder, the Idiopathic Portal Hypertension syndrome.

MATERIALS AND METHODS

Animals and reagents

The compound *Apc^{lox/lox}/TTR-Cre^{Tam}* mice, which received intra-peritoneally one injection of 1.5 mg tamoxifen (MP Biomedicals), constitute the so-called *Apc^{ko}* “pretumoral” and panlobular model, deleting *Apc* in more than 80% of the hepatocytes [13, 25]. *Apc^{lox/lox}/Hnf4 α ^{lox/lox}/TTR-Cre^{Tam}* mice were generated using *Hnf4α* floxed mice created by the Gonzalez laboratory [4]. The panlobular model of invalidation, deleting *Hnf4α* (thereafter called *Hnf4^{ko}*) with or without *Apc*, received a unique injection of 1,5mg tamoxifen, and Tamoxifen-enriched diet for five days (TD135859, 400 mg/kg, Harlan). Mice were either sacrificed 9 days after Tamoxifen or kept ten months for tumor follow-up. For the tumoral models, *Apc^{lox/lox}/Hnf4 α ^{lox/lox}* or *Apc^{lox/lox}* mice received a retroorbital injection of an adenovirus coding for the Cre-recombinase (0,5*10⁹ particules). Using this protocol, the *Apc^{lox/lox}* mice got the deletion of *Apc* in 10 to 50% hepatocytes, which was sufficient to obtain few isolated hepatocytes activated for β -catenin 90 days after tamoxifen injection. Tumors were obtained from these isolated hepatocytes after 6 to 8 months [26]. The tumor development was followed twice monthly by 2D-ultrasound (Vevo 770). Only male mice were analyzed throughout this study.

Immunostaining

Paraffin-embedded liver sections were treated as previously described [26]. The following antibodies were used: anti-HNF-4 α (1/250, Santa-Cruz, SC-8987), anti-cyclin D1 (1/100, thermo scientific MA5-14512), anti-glutamine synthetase (GS, 1/400, BD Transduction Laboratories, 61517), and Ki-67 (1/400, Leica ncl-Ki67p).

Immunofluorescence detection

Immunofluorescence study was performed in 5 μm sections of fixed paraffin-embedded liver, deparaffinized, hydrated and heated at 95°C for 35min. After the blocking step, the slides were incubated with primary and then with secondary antibody. The used primary antibodies were monoclonal anti Glutamine Synthase (GS, BD, diluted 1/300) and the polyclonal anti-HNF-4 α (SC, 1/1000). The secondary antibody was an Alexa 488 anti-rabbit for HNF-4 α (green) and an Alexa 514 anti-mouse for GS (red). Nuclei were evidenced by Hoechst (1/1000) and the slides were mounted with an anti-Fade mounting compound.

RNA extraction and RT-qPCR

Total RNA was extracted from liver fragments (\approx 100mg) or tumors with Trizol® reagent (Life Technologies). HNF-4 α (Taqman assay Mm01247713, Life technologies), GS (assay Mm00725701), axin 2 (assay Mm00432102), cyclin D1 (assay Mm00432359), PPAR α (Mm00440939), BMP-7 (Mm00432102), and E-cadherin (Mm01247357) mRNA levels were assessed with 1 μg total mRNA with normalization against ribosomal RNA. SYBR green experiments were performed for Snail (F: GGTCAGCAAAAGCACGGTT, R: CACACGCTGCCTTGTGTCT) and vimentin (F: CTGTTGCACCAAGTGTGC, R: TAGCAGGACACTATTGGCCG). Levels of miR-122 were determined on 2 ng total mRNA, with a specific Taqman miRNA assay (002245, Life Technologies), as compared to snoRNA135 (assay 001230).

Human samples

Forty-four patients treated for liver cancer in Cochin hospital were included in this study. All tumor samples were frozen after surgery in accordance with French law and ethical guidelines.

Lipid measurements

Hepatic triglycerides were separated by Thin Layer Chromatography (TLC). A 2:1 chloroform/methanol extraction was performed using Folch solution

[27]. Lipids were analyzed on a solid phase of silica and a liquid phase of petrol ether, acid acetic and diethyl ether. Silica slide of triglyceride stripe was cut and quantified with a kit based on absorbance measurement (Diasys). Oil Red O staining was performed on 10 μ m frozen liver sections fixed in 4% formaldehyde, stained with 0.3% Oil Red O and 36% Triethyl-phosphate for 30min and counterstained with Mayer Hemalun.

Statistical analysis

We assessed the significance of differences between two groups of samples in Wilcoxon tests if $n < 30$ and Student tests if $n > 30$. ANOVA was used to compare three groups of samples. $P < 0.05$ was considered statistically significant.

RESULTS

HNF-4 α is impaired during hepatocarcinogenesis driven by β -catenin-mutation

Our laboratory engineered few years ago a mouse model with β -catenin overactivation exclusively in the liver, by the tamoxifen-inducible and Cre-Lox-mediated inactivation of the Wnt/ β -catenin pathway inhibitor, Apc (Apc^{KO}) [26, 28]. The absence of Apc in single hepatocytes resulted in the clonal development of β -catenin-activated liver tumors with properties similar to those observed in human patients [25, 26]. In this mouse model, we observed that tumors with Apc deletion expressed a lower level of HNF-4 α mRNA level as compared to non-tumoral tissue (Figure 1A and [13]), supporting the potent tumor suppressive role of HNF-4 α in this subtype of tumors. HNF-4 α was also lost in human samples of HCC with activating mutations of β -catenin (Figure 1B and supplemental data from [13]). In addition, in Apc^{KO} mouse tumours, a higher level of HNF-4 α was correlated with a lower amount of Snail and cyclin D1 (Figure 1C and D-E, respectively). These two crucial actors of cancerous process are well-described negative targets of HNF-4 α [11, 21, 22]. Interestingly, in these tumors, a lower level of HNF-4 α was associated with a higher level of axin-2 mRNA, a positive target

of β -catenin (Figure 1F). This corroborates previous studies suggesting that HNF-4 α could impair β -catenin signaling ([16, 23, 25]). In conclusion, it appears that a loss of HNF-4 α in hepatocytes with β -catenin activation could participate to hepatocarcinogenesis through the modification of proliferation and differentiation program.

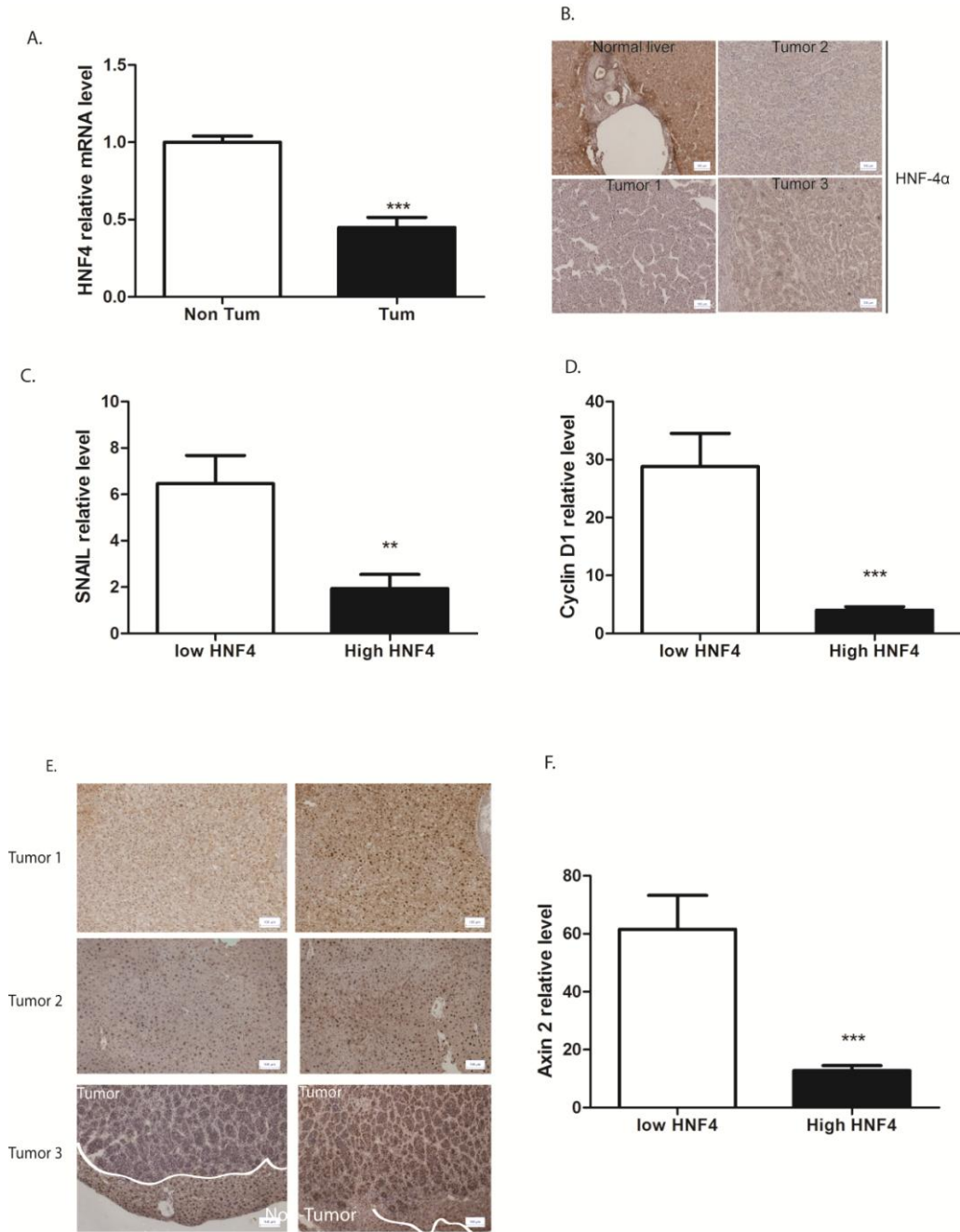


Figure 1: HNF4 α is reduced in tumors from Apc^{KO} mice.

A: HNF-4 α levels, relative to 18S, are determined by qPCR. Results are shown as the mean RQ (relative quantity) values for HNF-4 α for 35 tumors \pm SEM, as compared with non-tumor tissues. **B:** Loss of HNF-4 α expression in three human HCCs with β -catenin mutations, as compared to normal liver. **C-F:** SNAIL (**C**), Cyclin D1 (**D**), and Axin-2 (**F**) levels, relative to 18S, are determined by qPCR, and represented as previously, as compared with non-tumor tissues. **E:** Inverse correlation between Cyclin D1 and HNF-4 α by immunohistochemistry in Apc^{KO} tumors.

Liver-specific invalidation of *Hnf4α* promotes hepatocyte proliferation and lipid accumulation

To study the role of HNF-4 α in liver tumor development driven by β -catenin, we next decided to invalidate *hnf4α* gene specifically in hepatocytes through the use of TTR-Cre-ERT2 system (Figure 2A) in *hnf4α*^{flox/flox} mice [4]. Treatment of Cre+ mice with tamoxifen diet led to the deletion of *hnf4α* in around 80-98% hepatocytes, in comparison with Cre-negative mice, as demonstrated by qPCR and by immunohistochemical staining (Figure 2A). The deletion of *hnf4α* was also confirmed by a significant impairment of three of its targets: ApoCIII, PPAR α and miR-122 (Figure 2B). Deletion of *hnf4α* resulted in a net increase in liver to body weight ratio, which is associated with an induction in cyclin D1 mRNA level and a significant increase in Ki-67 immunostaining (Figure 2D). This confirmed accumulating evidence which indicates that downregulation of HNF-4 α results in increased cell proliferation ([10-12]). In addition, *hnf4α* deletion promotes cell architecture disorganization, suggested by the important cytoplasmic counterpart in liver section (Figure 2A and C). This was, in fact, due to lipids accumulation as revealed by triglycerides enrichment revealed by chromatography, and by oil red O staining (Figure 2D). This was in accordance with other previous studies, which suggest that HNF-4 α could orchestrate a transcriptional program regulating lipid metabolism and cell proliferation ([12, 25, 29, 30]).

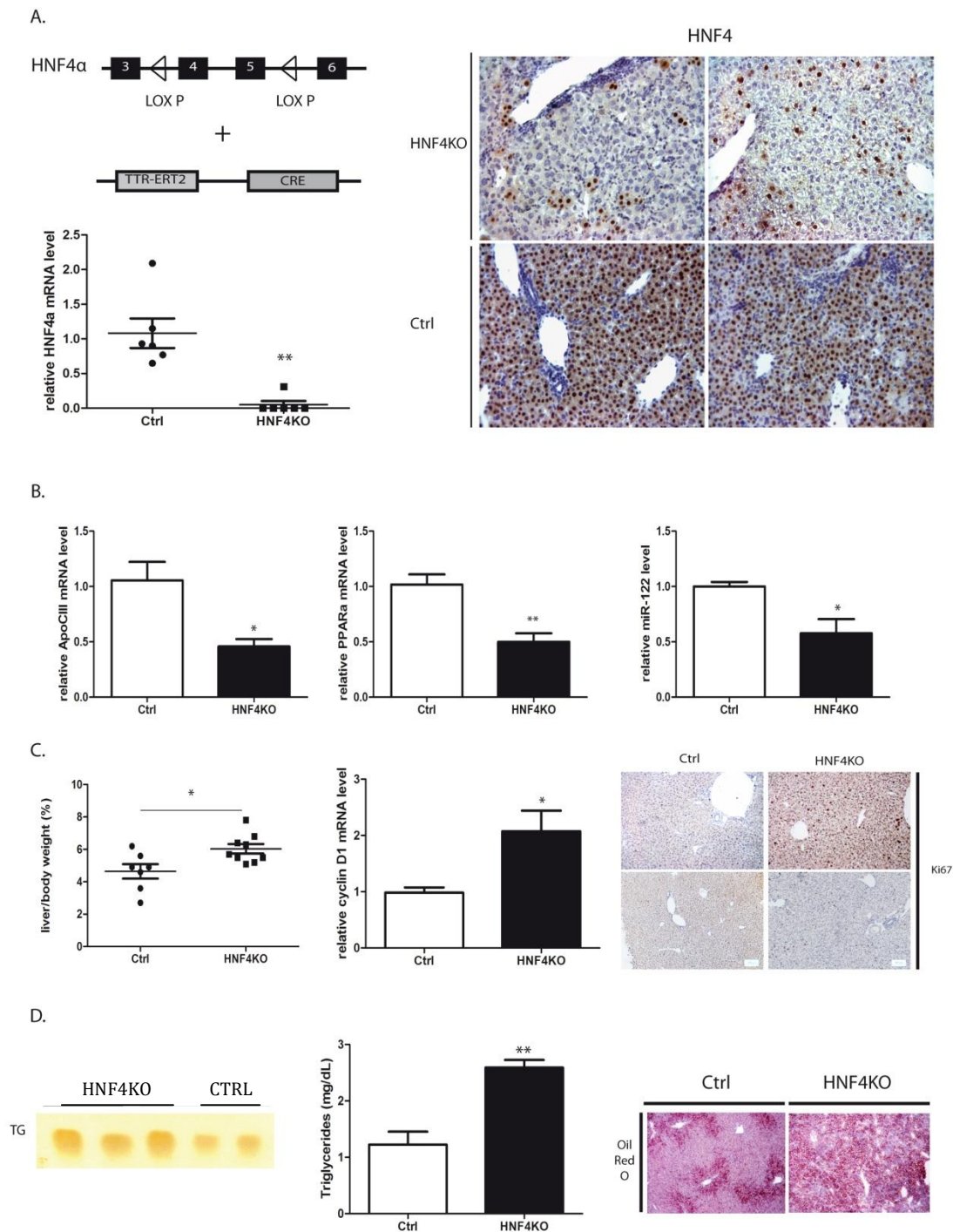


Figure 2: Characterization of *Hnf4*^{ko} mice

A: *hnf4α* is lost following tamoxifen diet, as revealed by qPCR and immunostaining, as compared to control mice without Cre recombinase. HNF-4 α levels, relative to 18S, are shown as the mean RQ (relative quantity) values for 6 mice \pm SEM, as compared to control mice. **B:** ApoCIII, PPAR α and miR-122 mRNA level, are determined by qPCR, and represented as previously, as compared to control mice (Ctrl). **C Left panel :** Liver to body weight ratio of *Hnf4*^{ko} mice (n=9) as compared to control (Ctrl) mice (n=7). **Middle panel:** Cyclin D1 mRNA level, relative to 18S, are determined by qPCR, and represented as previously, as compared to control mice

(Ctrl). **Right Panel:** Comparison of Ki-67 immunostaining between control (Ctrl) and Hnf4^{KO} mice. **D:** Comparison of triglyceride (TG) content in liver following *hnf4 α* deletion (Hnf4^{ko}) as compared to control mice (Ctrl) by Thin Layer Chromatography and oil red O staining. For TLC, results are represented as relative quantity normalized to loading.

***Hnf4 α* deletion alters liver zonal architecture**

A previous study reported that HNF4- α deficiency induced periportal expression of a pericentral marker, the glutamine synthetase, which is also a well-known target of β -catenin. We confirmed these data (Figure S1), further supporting the antagonistic link that we previously showed between HNF-4 α and β -catenin pathway in the liver [25]. Surprisingly, we observed another intriguing property occurring in 8 of the 28 Hnf4^{ko} mice studied (28%), consisting in a disorganization of the portal triad (figure 3A). This structural disorganization is characterized by a stenosis of the portal vein, accompanied with an increase in the number and size of hepatic arteries (ha) and bile ducts (bd) within the portal triad (Figure 3B). An increased number of small cells was also seen particularly around the portal vein, which were mostly proliferative (Figure 3B). As the loss of HNF-4 α has been involved in epithelial-mesenchymal transition *in vitro*, as reflected by a mislocalization of E-cadherin, we searched for E-cadherin immunolocalization *in vivo* [22]. However, E-cadherin was normally membrane-localized in the hepatocytes lining the disorganized portal triad (Figure 3B). The specific zonal pattern of E-cadherin, being periportally-restricted [31], was also preserved in Hnf4^{ko} livers, either being or not structurally disorganized (Figure 3C). Therefore, there is no impact of HNF-4 α on E-cadherin localization. It appears that the livers that develop this structural disorganization expressed a higher mRNA level of BMP-7 (Figure 3D), a direct target of HNF-4 α [12]. This protein has been involved in the preservation of the endothelial phenotype [32], and in the inhibition of endothelial to mesenchymal transition (EndMT) [33]. We found that the triad disorganization in Hnf4^{ko} livers was also correlated to Snail and Vimentin mRNA upregulation (Figure 3D), two markers of epithelial-to-mesenchymal transition (EMT) in liver [21]. Lastly, we searched for an evolution of this phenotype over one year, and for a potential tumor

development in mice with a panlobular *Hnf4^{ko}* liver. One mouse among the 7 studied developed a particular phenotype, with a worsened portal triad disorganization compared to short-term studies, together with the development of several hyperplastic nodules showing a strong accumulation of Glutamine Synthetase, revealing β -catenin activation but with no dysplasia (Figure 3E). Our data altogether link the loss of *hnf-4a* to portal vein stenosis together with a hypertrophy of bile ducts and hepatic arteries, through a mechanism that could imply EndMT actors such as BMP7, and EMT actors such as Snail and Vimentin. These features do not seem to be hallmarks events for carcinogenesis, as the β -catenin hyperplastic nodules observed were rare and non neoplastic. This phenotype rather presents interesting similarities with a human syndrome called idiopathic portal hypertension.

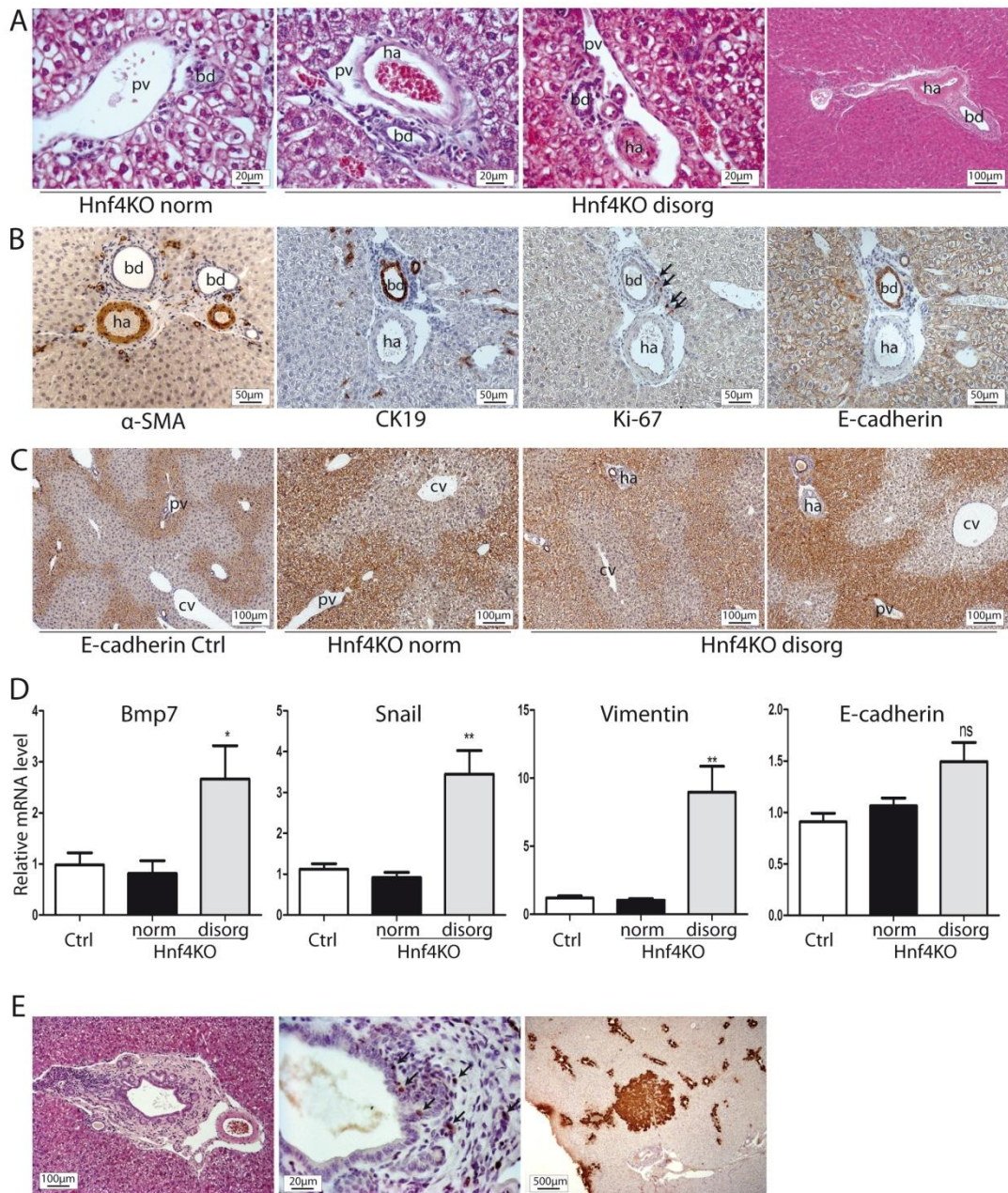


Figure 3: *hnf4α* deletion alters liver zonal structure.

A-C: Portal triad disorganization following *hnf4α* deletion is shown by haematoxylin/eosin (**A**), α-SMA, Cytokeratin-19 (CK19) and Ki-67 staining (**B**), and E-cadherin immunostaining (**B-C**). **D:** Comparison of Bmp-7, Vimentin, Snail and E-cadherin levels, relative to 18S, determined by qPCR and represented as previously, between control mice (Ctrl), *Hnf4^{KO}* mice with normal portal triad and those with disorganized triad (disorg). **E:** One *Hnf4^{KO}* mouse with disorganised portal triad develops a GS positive nodule 10 months after tamoxifen diet. cv : central vein, PV : perivenous, PP : periportal, HA : hepatic artery, bd : bile duct, ns : non-significant.

Successful *hnf4α* depletion in hepatocytes with activation in β -catenin signaling.

To elucidate the role of HNF-4 α in the subtype of HCC mutated for β -catenin, we fed compound *hnf4α*^{flox/flox}/*Apc*^{flox/flox}/*TTR-Cre-ERT*₂ mice with tamoxifen diet. This resulted in an efficient deletion of both *hnf4α* and *Apc* (*Hnf4*^{ko}/*Apc*^{ko}) in the double floxed mice, as revealed by HNF-4 α and GS level in qPCR (Figure 4A). However, no changes in liver to body weight ratio (Figure 4B), in cyclin D1 mRNA level (Figure 4C), and in Ki-67 staining (Figure 4D), was observed in *Hnf4*^{ko}/*Apc*^{ko} mice as compared to *Apc*^{ko} mice. Regarding of these data, *hnf4α* deletion does not seem to alter the proliferation rate and the metabolic properties of hepatocytes with an activation of the WNT/ β -catenin pathway in the pretumoral state.

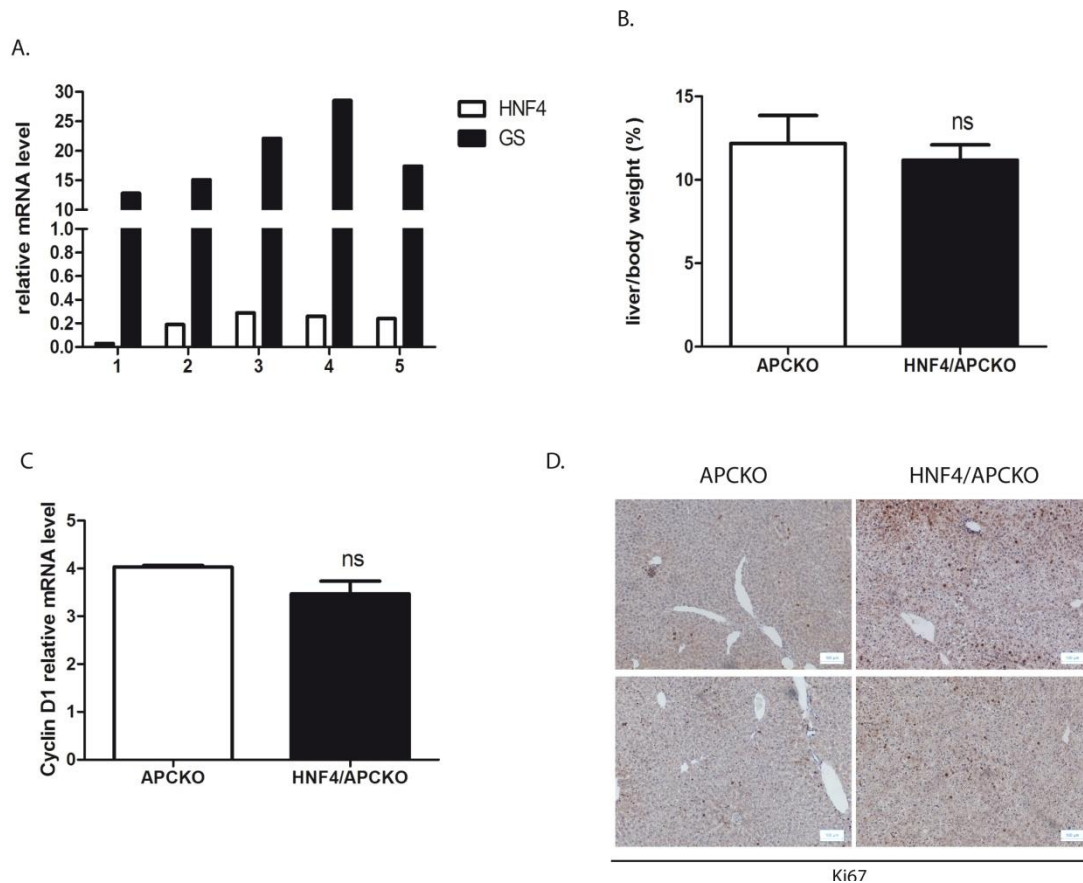


Figure 4: *hnf4α* deletion in hepatocytes with β -catenin activation did not exacerbate hepatocyte proliferation.

A: *hnf4α* and *Apc* are efficiently lost following tamoxifen diet, as revealed by HNF-4 α and GS mRNA level measurement in qPCR in 5 different samples. **B:** Liver to body weight ratio of *Hnf4*^{ko}/*Apc*^{ko} mice (n=9) as compared to control (Ctrl) mice (n=7). **C-D:** Comparison of cyclin D1 mRNA level (C), and Ki-67 immunostaining (D) between *Apc*^{ko} and *Hnf4*^{ko}/*Apc*^{ko} mice. ns: non-significant.

***Hnf4α* depletion has no impact on liver tumors with activation in β -catenin signaling.**

We next assessed the role of HNF-4 α in the development of tumors with activating β -catenin signaling. Injection of $0,5 \times 10^9$ pfu of an adenovirus encoding the Cre recombinase, succeeds to invalidate both *Apc* and *hnf4α* in single hepatocytes, as revealed by the presence of an immunostaining for GS in *Hnf4^{KO}* (Figure 5A). No mice developed tumors after a focal unique *hnf4α* invalidation. However, in absence of both *hnf4α* and *Apc*, ultrasonography revealed that tumors appeared after 5,5 months, as soon as tumors only invalidated for *Apc* (Figure 5C). Immunostainings revealed that tumors in the *Hnf4^{ko}/Apc^{ko}* mice were GS positive and HNF-4 α negative (Figure 5B). Conversely, single *Apc^{ko}* tumors were GS positive and had a variable decreased *Hnf-4α* staining compared to the non-tumoral counterpart (Figure 1A). The number of tumors and their growth were not distinguishable between *Hnf4^{ko}/Apc^{ko}* mice and *Apc^{ko}* mice (Figure 5C). Their differentiation grade was also similar: in both cases, tumors were either well differentiated with frequent glandular structures, or moderately differentiated (Figure S2). No increase in progenitor-like cells or mesenchymal cells could be detected, and accordingly Snail mRNA amount was not modified (Figure 5D). Their similar proliferation rate was associated to identical cyclin D1 mRNA level (Figure 5D). In conclusion, a loss of HNF-4 α concomitantly with β -catenin activation did not exacerbate HCC development as compared to β -catenin activation only.

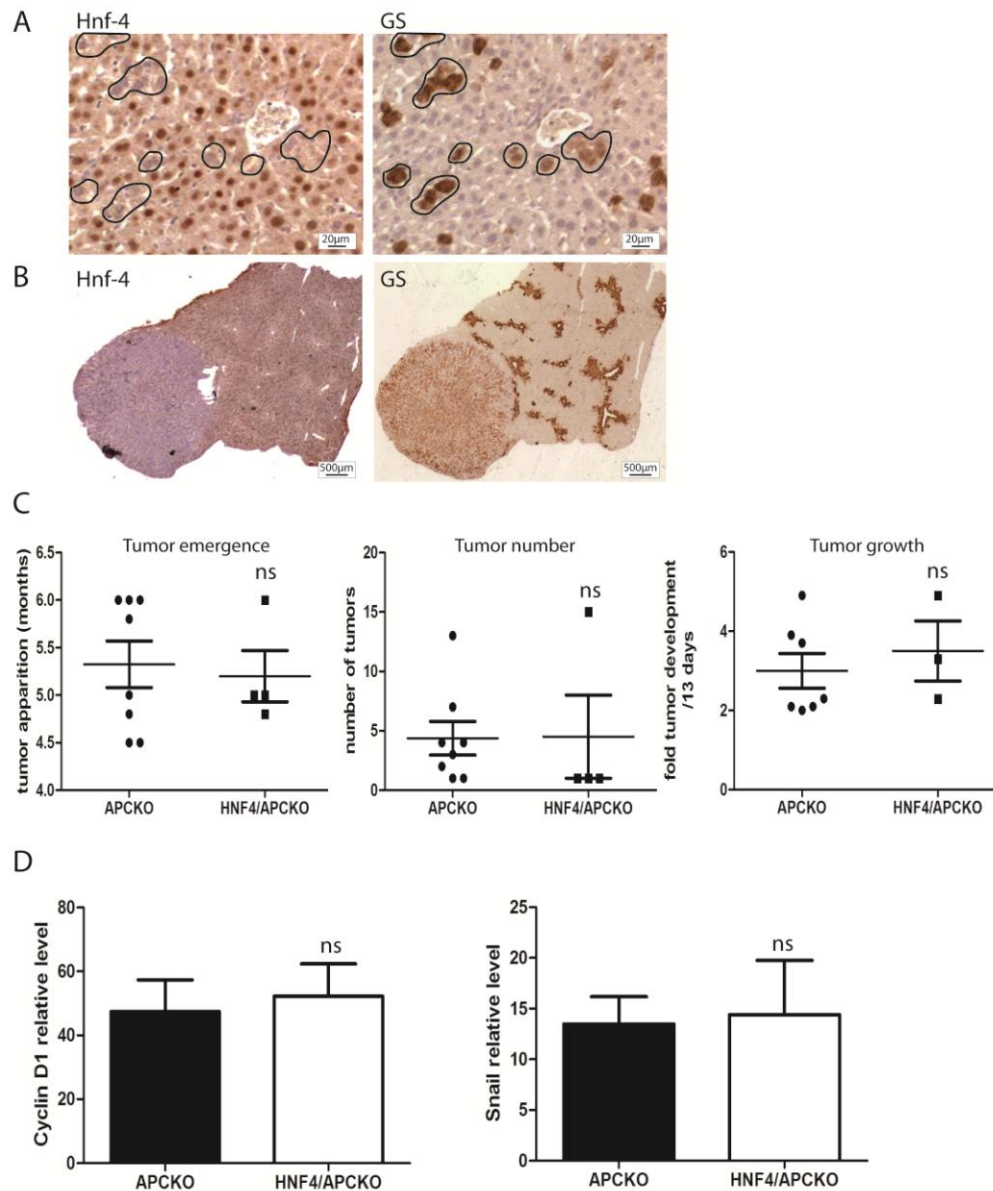


Figure 5: *hnf4α* deletion did not accelerate the development of tumors from *Apck^{ko}* mice. **A-B:** Characterization of *Hnf4^{ko}/Apc^{ko}* mice by HNF-4α (left panel) and GS immunostainings (right). **C: left panel:** Number of months for tumor apparition detected by ultrasounds following adenovirus injection. The results are expressed as the mean of months ± SEM normalized with respect to *t0*, for mice with *Apc* deletion and with (n=8) or without *hnf4α* (n=4). **Middle panel:** Numbers of tumors detected during ultrasound following. **Right panel:** Tumor development was followed by 2D ultrasound bimonthly. The results are expressed as the fold-change in volume over 13 days ± SEM normalized with respect to *t0*, for mice with *Apc* deletion and with (n=8) or without *hnf4α* (n=4). **D-E:** cyclin D1 (**D**) and Snail (**E**) mRNA levels determined by qPCR and represented as previously, as compared with non-tumor tissues. All the results were non-significant (ns).

DISCUSSION

Although HNF-4 α is described in numerous studies as a tumor suppressor in liver cancer, it appears in ours that a loss of Hnf4 α is not systematically a pro-oncogenic signal. Firstly, only one mouse with a panlobular loss of HNF-4 α developed a nodule 10 months after adenovirus injection. Using our model of tumor development with focal activation of the Wnt/ β -catenin pathway, a concomitant deletion of *hnf4 α* did not accelerate either tumor apparition or progression (Figure 5C). Although we have previously shown that HCC with β -catenin activation expressed low level of HNF4 α [13], it seems that this decreased expression is even sufficient for β -catenin-dependent liver tumorigenesis, and does not require its total removal. This has to be linked to the study of Ning *et al.*, showing that HNF-4 α -mediated tumor suppression in rat treated with DEN was less pronounced when β -catenin is constitutively activated [16]. They showed that HNF-4 α -mediated liver tumor suppression is partially due to β -catenin downregulation, and this correlates well with our data showing that the expression of axin-2, a target of β -catenin, decreases when HNF-4 α amount is high in tumours (Figure 1F). We also found in *Apc*^{ko} tumors that a high expression of Snail is correlated with a low expression of HNF-4 α (Figure 1C). This is consistent with the work of Yang *et al.*, who showed that the Wnt/ β -catenin pathway negatively regulates HNF-4 α *via* Snail induction in Hep3B cells [23]. We showed that Snail is not controlled by HNF-4 α in normal livers, excepted when triad disorganization occurs. Tumors with no *hnf4 α* and *Apc* also present a similar Snail mRNA level as compared to *Apc*^{ko} tumors, and they are not associated with an EMT phenotype. Altogether, these data suggest that β -catenin overactivation is the major signal inducing a maximal Snail expression to impair HNF-4 α amount, and that *hnf4 α* genetic deletion per itself is not a major inducer of epithelial-mesenchymal transition *in vivo* in adult hepatocytes. This contrasts with a previous report of Tripodi's group showing such a role for HNF-4 α [22]. This was done *in vitro* in hepatoma cells (not β -catenin activated) and *in vivo* in a model of *Hnf4 α* invalidation obtained using a constitutive Albumin-Cre transgenic mouse. In this latter model, Cre recombination occurs in perinatal hepatocytes. We can hypothesize that the discrepancies between

their study and ours can be due to the distinct maturity of the hepatocyte targeted, and that our study reflects what happens when *Hnf4α* is lost in an adult hepatocyte.

However, HNF-4α seems to play a crucial role in the structural morphology of the portal triad, since a loss of *hnf4α* promotes a disorganization of the portal vein with a hypertrophy of the hepatic arteries in around 28% of mice invalidated for *hnf4α* (Figure 3A). We could correlate the presence of this remodeling with a significant induction of BMP-7, Snail and vimentin mRNA in the entire liver of mice *Hnf4^{ko}* (Figure 3C). This suggests that probably some epithelial-mesenchymal and endothelial-to-mesenchymal transitions could occur in the portal regions following *hnf4α* deletion, which could also be related to miR-122 reduction (Figure 2C), as described in cardiac endMT [34]. Surprisingly, we could not notice an increase rate of portal disorganization in the double mutant *Hnf4^{ko}/Apc^{ko}* (data not shown), although it has been suggested that β-catenin and TGF-β/BMP/SMAD could synergize in endMT process in heart [35]. In the next steps, it would be interesting to isolate these structures by laser micro-dissection to better understand the molecular mechanisms involved in this process. In particular, a study of the TGFβ pathway appears crucial. Indeed, TGFβ1 could activate hepatic stellate cells to produce extracellular matrix proteins. It thus appears important to determine the level of TGFβ secretion and SMAD2 phosphorylation. Indeed, our model is reminiscent of the syndrome of idiopathic portal hypertension, associating portal vein stenosis, and misregulation of TGFβ/BMP/SMAD signalings [32]. Since portal hypertension has been demonstrated these recent years as a significant risk factor for HCC [36], it appears important to understand how HNF-4α could promotes portal remodeling and perhaps favor HCC development.

Lastly, our study showed that HNF-4α genetic loss is not required to exacerbate tumor development mediated by β-catenin activation. This is surprising considering our previous study showing that β-catenin-activated tumors can be treated with an inhibitor against miR-34a [13], and that this LNA acts partially through a restoration of high HNF-4α levels in these tumors, corroborating previous studies that showed tumor suppression

effect of HNF-4 α when it is expressed after DEN injection [16, 19]. This underlines the fact that HNF-4 α is not a classical tumor suppressor, and that this suppressive role could be finely tuned through: (i) a modulation of the just-right dose of HNF-4 α required for hepatocyte homeostasy ; (ii) a context dependency, occurring at either the genetic level, i.e. the presence of β -catenin mutations, or at the cellular level, i.e. the degree of maturity of the hepatocyte targeted.

ACKNOWLEDGMENTS

We thank Pierre-Alexandre Just for his help for TLC experiments. We warmly thank Dr A. Ribeiro for the gift of hnf4 α ^{flox/flox} mice and for helpful discussion.

GRANT SUPPORT

This work was supported by the *Ligue Nationale Contre le Cancer (Labellisation Program 2011-2013 and 2014-2016)* and the *Agence Nationale de la Recherche (WNT-METABOLIV. 2010-2014)*.

ABBREVIATIONS:

Apc : Adenomatous polyposis coli ; Apc^{ko} : specific inactivation of *Apc* in the liver with a liver-specific and tamoxifen-inducible; bd: bile duct; Cre-loxP; Ctrl: control; cv: central vein, DEN: diethylnitrosamine; disorg: disorganized; EMT: epithelial-mesenchymal transition; EndMT: endothelial-to-mesenchymal transition; GS : glutamine synthetase ; HA: hepatic artery; HCC : hepatocellular carcinoma ; HNF-4 α : hepatocyte nuclear factor 4 α ; ns : non-significant ; PV : perivenous ; PP : periportal ; TG: triglyceride; TLC: Thin Layer Chromatography; Tum: tumor

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AXIS 3. As previously anticipated the metabolomic analysis initiated in the team had underlined a general deregulation of lipids in APC knock-out livers (See the Hepatology in the annexes). More specifically triglycerides are decreased and on the contrary bile acids, sphingolipids and phospholipids were increased.

Due to this latter observation, I focused on choline metabolism, due to its dual role, first in the synthesis of phospholipids and their derivatives (sphingolipids and lysophospholipids), and second, as a methyl-group donor, two fundamental elements in cancer onset and progression.

The other reason to focus on this metabolism was the recent emergence of the ¹⁸Fcholine PET to detect liver tumours.

Thus we wondered whether ¹⁸Fcholine PET positivity could be a signature of β -catenin mutated HCCs. Molecular dissection of choline metabolism was analysed in mice both at day 7 (APC knock-out pretumoral model) and in APC knock-out tumours, thanks to radiolabeled fluxes experiments and to alterations of dietary choline intake.

The results are presented in the following pages.

Genotyping β -catenin-mutated Hepatocellular Carcinoma through F-Choline Positron Emission Tomography imaging.

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ABSTRACT

Hepatocellular Carcinoma (HCC) is the third cause of cancer death worldwide, mostly due to its resistance to treatments. It therefore needs an early and non invasive diagnosis. Imaging methods such as Fluoro-deoxyglucose Positron Emission Tomography (FDG-PET) detects only sixty percent of HCCs and until now, it has not been shown to be able to genotype tumors, a prerequisite to treat patients with more efficient and pathway-specific targeted therapies.

One third of the HCCs is characterized by activating mutations in *CTNNB1* encoding β -catenin. These tumors show unique transcriptomic, histological and metabolic specificities and previous results showed that they are FDG-PET negative.

In this study, we demonstrated that the liver metabolic signature of β -catenin is affecting choline metabolism. Choline is an essential nutrient contributing to phospholipid synthesis and methylation processes. As Fluoro-choline PET (FCH-PET) imaging in liver tumors is under development, we searched for a correlation between *CTNNB1*-mutations and FCH-PET positivity in tumors. This correlation was found in 100% of human HCCs tested with a specificity of 100%. The same correlation was found in mouse HCCs, by the use of two models, one dependent on β -catenin activation, the so-called *Apc*-knock-out model, and the other independent on β -catenin status, i.e. the diethylnitrosamine-induced model of HCC.

By suppressing choline and methionine intakes from the diet, we showed that the hepatomegalic phenotype developed following β -catenin activation was reversed in the absence of choline. Such a reversal was also shown for β -catenin-dependent DNA methylation. In fact, we showed that ^{14}C -choline was incorporated in intracellular phospholipids in primary hepatocytes and tumors in proliferation. But interestingly, ^{14}C -choline was also incorporated in secreted phospholipids only in hepatocytes and tumors with a β -catenin activation, and we linked this phenomenon with an increase in bile synthesis in these livers. We finally showed that ^{14}C -choline was a potent methyl group donor for DNA methylation, and this occurred only in a context of β -catenin-activated tumors.

In conclusion, this study demonstrates that a simple non-invasive and routinely FCH-PET analysis can be used to genotype β -catenin-mutated HCCs. This increased metabolism of choline has unique consequences in β -catenin-activated hepatocytes and could be a therapeutic target for this category of HCCs.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common liver primitive cancer and the third leading cause of cancer-related death worldwide. HCC development is a multistep process, driven by genetic, viral and environmental factors. In eastern countries, its onset has increased, as a consequence of hepatitis and cirrhosis due to the infection with the hepatitis B and C viruses, to alcohol consumption and to metabolic dysfunction (Knudsen et al., 2014).

The prognosis of HCC remains unfavorable in humans, and its non-invasive early diagnosis a thorny issue. Moreover, the genetics of these tumors is complex, and there is a need for an early diagnosis of the altered signaling pathways, that could allow targeted therapies to be successfully administered. Biomarkers in blood serum are used, the most common being the α -fetoprotein (AFP) assay, which however has a quite low sensitivity (60%), and has no specificity in terms of altered signaling pathway. Imaging methods have emerged to follow high-risk patients without needing the use of invasive methods. The ^{18}F -Fluoro Deoxyglucose (^{18}F -FDG) Positron Emission Tomography (PET) and its hybrid version including computed tomography (PET/CT) has been useful to detect many cancers, but its use is limited in the liver because of a low sensitivity (60% overall) (Talbot et al., 2010). FDG-PET positivity is based on the addiction of tumor cells to glucose, a well-documented property known as the Warburg effect, that consists in a high rate of glycolysis directed to lactate production, conferring to the tumor a metabolic and growth advantage (Bensinger and Christofk, 2012).

The inability of FDG-PET to detect all HCCs could be linked to heterogenous tumoral phenotypic traits. Indeed, one major genetic mutation characterize

one third of the HCCs, and consists in activating mutations in *CTNNB1*, encoding β -catenin, leading to an aberrant and constitutive activation of β -catenin signaling (de La Coste et al., 1998; Miyoshi et al., 1998; Schulze et al., 2015). The Wnt/ β -catenin pathway plays a dual role in the adult liver: through its limited activation to the pericentral area of the liver, it patterns the so-called hepatic metabolic zonation (Benhamouche et al., 2006; Torre et al., 2010). When activated by oncogenic mutations, its aberrant accumulation leads to liver tumorigenesis (Cavard et al., 2008). Interestingly, β -catenin-mutated HCCs have peculiar characteristics: a low proliferation rate, a high differentiation grade and a better prognosis than non-mutated ones (Boyault et al., 2007; Hoshida et al., 2009). They also exhibit specific metabolic properties: they are mostly cholestatic (Audard et al., 2007), they overexpress a panel of genes defining metabolic zonation (Gougelet et al., 2014), among which is the glutamine synthetase, a target of β -catenin and a histological marker of these tumors (Audard et al., 2007).

In previous studies, we used genetically-engineered mice developing β -catenin-activated HCCs, through the hepatic loss of the main break of β -catenin signaling, the Adenomatous Polyposis Coli (*Apc*) tumor suppressor gene. These HCCs are quite similar to their human counterpart with *CTNNB1* activating mutations, sharing their transcriptome and metabolic characteristics (Colnot et al., 2004; Gougelet et al., 2014). Using this model in a FDG-PET approach, we demonstrated that β -catenin-activated livers had no addiction to glucose (Chafey et al., 2009). An elegant study showed thereafter that genetic mutations in liver tumors pattern tumor metabolism; they either elicit a Warburg-type response, or a more complex metabolic response characterized by glutamine synthetase overexpression and glutamine production with only a mild FDG addiction (Yuneva et al., 2012). Altogether, these data suggest that an accurate PET determination could discriminate and genotype HCCs, and this could be a significant breakthrough to treat patients with targeted therapies without a need for invasive genotyping methods.

We focused on a ^{18}F -fluorocholine (FCH) PET approach, because we previously showed its higher sensitivity compared to FDG-PET, in order to

detect well-differentiated HCCs (Fartoux et al., 2012; Talbot et al., 2010). Choline is an essential nutrient that allows the synthesis of phosphatidylcholine, a major lipid of the membranes. In the liver, it is also used for VLDL and bile formation. Choline is also used as the major methyl-group donor of the cell. For these reasons, cancer cells may be characterized by their ability to incorporate choline actively, and as an example, FCH-PET is now routinely used for the detection of prostate cancer (Glunde et al., 2011). An increased choline metabolism is mostly characterized by an increase in Choline kinase α , whose expression is controlled by a number of oncogenic pathways, such as Ras, Mapk, PI3K-AKT, AP1 and HIF1 (Glunde et al., 2011). We postulated that β -catenin pathway could also be such a signal increasing choline metabolism.

In the present study, we revisited metabolomics of the β -catenin-activated mouse liver, and found a deregulation in choline products. We then assessed that CTNNB1-mutated hHCCs were revealed by their FCH-PET positivity, contrary to non-mutated HCCs. These features were also found in genetically- or chemically-engineered tumoral mice. By using *in vitro* and *in vivo* methods, we found a role of choline in regulating liver size and DNA methylation, two aspects that can be crucial for liver tumorigenesis.

MATERIALS AND METHODS

Animals, tumor follow-up and MCD diet

Mice carrying the two floxed alleles on the 14th exon of the *Apc* gene (here referred to as *Apc*^{lox/lox}) were interbred with TTR-CreTam mice to generate *Apc*^{lox/lox}/TTR-CreTam mice (Colnot et al., 2004; Tannour-Louet et al., 2002). Two-month old male *Apc*^{lox/lox}/TTR-CreTam mice were *Apc*-invalidated with an intraperitoneal injection of either a low (0.75mg) or a high (2mg) dose of Tamoxifen, generating so-called *Apc*^{ko} animals. In our experimental settings, Tamoxifen-injected *Apc*^{lox/lox}/Cre-negative mice were used as controls (Ctrl). Animals injected with 2mg developed a panlobular activation of β -catenin; they were sacrificed 6 or 7 days after Tamoxifen injection and used as a pretumoral model for either short-term molecular studies (Day 7) or primary

hepatocyte culture (Day 6). The mice injected with 0.75mg Tamoxifen activated β -catenin in single hepatocytes (Gougelet et al., 2015): they were kept for long-term tumorigenesis studies.

C57Bl/6 male mice (Janvier, France) were injected with Diethylnitrosamine (DEN) at 14 days after birth. Tumor development of DEN-injected and *Apc^{ko}* mice was followed up by ultrasonography from 5 months after Tamoxifen or DEN injection (Small animal imagery facility, Cochin Institute). When tumors reached 2mm diameter, they were analyzed by micro-PET (Tenon Hospital, Paris), and thereafter mice were sacrificed for tumor sampling and analysis.

For the studies of mice fed a Methionine and Choline Deficient (MCD) diet, two-month male mice were given a MCD diet (SNIFF, Germany) during 7 days. Then the mice were injected with Tamoxifen (2mg/mouse) and another 7-day MCD diet period was done before sacrifice. The efficiency of the diet was assessed by the appearance of small lipid droplets in the MCD control mice, due to the defect in VLDL formation in these mice.

All animal procedures were carried out according to French governmental regulations, and were approved by the ethical committee of Paris Descartes University.

Micro-PET analysis in mice

PET imaging was performed on *Apc^{ko}* and DEN-injected with a Mosaic animal PET system (Philips Medical systems, Cleveland, OH, USA). Mice were imaged with ¹⁸F-Fluorocholine (Ison or Advanced Accelerator Applications, France). Mice were anesthetized with a mixture of 1.5% isoflurane and oxygen. Animals were injected intravenously in the retro-orbital sinus with 5 ± 2 MBq. For ¹⁸F-Fluorocholine, static PET acquisitions were performed during an exposure time of 10 minutes, 15 min after injection. Images were reconstructed and data were analyzed using PETView and Syntegra–Philips software (PETView; Philips Medical Systems). Tracer uptake was quantified using maximum standardized uptake value (SUVmax, g/mL). SUVmax = measured maximal volumic activity in the tumor (Bq/mL) x body weight (g) / injected activity (Bq).

Bile flow rate

Seven days after 2mg Tamoxifen injection, Apc-floxed fasted mice were anesthetized under isoflurane and underwent midline laparotomy. Thereafter, their common bile duct was ligated and the gallbladder cannulated. Bile was collected up to 90 minutes for both knock-outs and controls.

Patients, PET/CT analysis and histomorphological characterization

Data of 18 patients with one or two HCC nodules on MRI or contrast-enhanced CT were analyzed. They all gave their written informed consent to be included in a prospective phase III study comparing FCH and FDG PET/CT for the detection of HCC (Eudract 2006-0000538-11). Each patient underwent both FDG and FCH PET/CT examinations on two different days in a random order within less than 2 months. Subsequently, 15 patients underwent surgical resection of HCC in less than 2 months after the last PET/CT. Three patients underwent a tumor biopsy after PET/CT.

PET/CT examinations and interpretations were performed as previously described (Fartoux et al., 2012). Briefly, for both FDG and FCH PET/CTs, lesions were considered positive if they corresponded to non physiological foci with high radiotracer uptake.

The resected specimens were treated after surgery in accordance with French law and ethical guidelines. A part was suddenly frozen in liquid nitrogen, the other was fixed in 4% formol to be included in paraffin. The number of lesions and the maximal diameter of the main HCC nodule were determined on the resected specimen. Histological differentiation grade was assessed.

Other cohorts of human HCCs with genetically well-characterized HCCs were used: one from Cochin Institute (Table S1), and one constituted of 155 normal and tumoral samples used for an Affymetrix microarray analysis (Boyault et al., 2007).

Mutation screening

Genomic DNA samples were extracted thanks to the NucleoSpin Triprep, Macherey-Nagel kit. Mutations analysis in the exon3 of β -catenin gene was

done on amplified DNA (25ng) by PCR (PF : AAC CCT GGC TAT CAT TCT GCT; PR : AGG ACT TGG GAG GTA TCC ACA), followed by a Sanger sequencing.

RNA extraction and qPCR

RNA was extracted with Trizol® (Life technologies™) or thanks to the NucleoSpin Triprep, Macherey-Nagel, depending on the available tissue. RNAs were dosed and a Reverse Transcription PCR was performed (Thermo scientific). The primers and the reagents for qPCRs were either SybRgreen or Taqman (see table S2; Thermo scientific). Normalizations were done against 18s ribosomal RNA. The PCRs were done on a LightCycler II apparatus (Roche).

Immunohistochemistry

Immunohistochemistry (IHC) was undertaken using 5 µm sections of fixed paraffin-embedded liver (human and mouse). The sections were deparaffinized, hydrated and antigen retrieval was performed in a hot-water bath at 95°C for 20min with citrate buffer pH6 (Vector H-3300). After the blocking step, the slides were incubated with primary antibody for 1H at RT (see table S3) and after with biotinylated secondary antibody (1/750 for mouse, 1/200 for rabbit antibodies). IHC were revealed with an ABC (Avidin Biotin Complex) – DAB (3, 3'-diaminobenzidine) system. Nuclei were then evidenced by Mayer's hemalun solution and the slides were mounted after dehydration.

Metabolomic Profiling

Metabolomic profiling of 16 frozen samples (8 Apc knock-out livers, 8 control livers; 100 mg/sample) was performed with Metabolon, Inc (Durham, USA). Part of these results have been described elsewhere (Gougelet et al., 2014). Liquid chromatography/mass spectrometry in positive and negative modes and gas chromatography/mass spectrometry were used. A total of 469 metabolites were measured. No metabolites were filtered out based on a 25% missing data criteria.

[Methyl ¹⁴C] Choline metabolism

Isolation of control or transgenic hepatocytes by collagenase perfusion has been described elsewhere (Gougelet et al., 2015). Primary hepatocytes were cultivated first in Williams E medium for 24 h, then during the last 2 h in Williams E containing 0.01 mM [Methyl-¹⁴C] choline (0.1 Ci/mol).

The tumors or the non-tumoral adjacent tissues were carefully dissected out and then chopped down into 1 to 2 mm³ pieces. One hundred to 150 mg of these explants were incubated for 2 hours at 37 °C into Williams E media containing 0.01 mM [Methyl-¹⁴C] choline (0.1 Ci/mol). The medium was then collected for lipid secretion assay : 3 ml were layered under 5 ml of 150 mM NaCl and centrifuged for 18 h at 10°C at 110 000 g. [Methyl-¹⁴C]-Choline incorporation into secreted lipids was directly assayed by scintillation counting of 500 µl from the top fraction.

The primary hepatocytes or the explants were washed 3 X in PBS (phosphate buffered saline) then mechanically homogenized in PBS. One fifth of the homogenate was used for protein measurement using the Bradford assay (Bio-Rad laboratories, USA). The lipid soluble fraction was then extracted from the remaining homogenate with chloroform/methanol (2/1, v/v), and separated by thin layer chromatography on silica gel plates. After iodine staining, the different lipid species were collected and ¹⁴C incorporation assayed by scintillation counting.

¹⁴C-methylated DNA was assayed on a water-soluble fraction after a phenol/chloroform (1/1, v/v), pH 8.0 extraction. Two more chloroform extractions of the aqueous phase were done. After RNase A treatment, genomic DNA was pelleted by 300 mM sodium acetate pH 4.8 and ethanol (1/3, v/v). DNA was quantified by spectrophotometry at 260 nm, and [Methyl-¹⁴C] incorporation into DNA was measured by scintillation counting of the whole genomic DNA fraction.

DNA methylation measurement

Input DNA was extracted from frozen livers by a classic phenol-chloroform-isoamyl-alcohol/chloroform or obtained using NucleoSpin Triprep, Macherey-Nagel. DNA methylation was quantified using Epigentek kit,

measuring 5-methylcytosine levels by the use of capture and detection antibodies and then quantification through an ELISA-like colorimetric reaction.

Statistical analysis

We assessed the statistical significances of the data using GraphPad Prism 6 software. The tests used were parametric and assumed a Gaussian distribution, as tested by R software.

RESULTS

β -catenin has an impact on choline metabolism in mouse livers

In an attempt to identify the metabolic landscape generated by β -catenin in the liver, we previously adopted a metabolomics strategy, enlightening biliary acids and glutamine products as being accumulated in *Apc^{ko}* livers, whereas triglycerides and fatty acids were dramatically decreased (Gougelet et al., 2014). We revisited these data and found among the accumulated metabolites, phospholipids from the lysolipid subfamily, affecting only products of the choline but not those of the ethanolamine pathway (Figure 1A, B). We also found an increase in intracellular choline after feeding, and in its intermediate product CDP-choline, generated by the Kennedy pathway. Sphingolipids accumulated much more, and the pathway of choline oxidation generating Betaine and Methionine was altered, with a lesser amount of Betaine, and a higher amount of Methionine and Homocysteine. Then, we used a previous screening of mRNA-Seq done on the same samples as the metabolites, and found an association of these deregulations with an increased gene expression of the choline transporters *Oct*, of the *Cept1* enzyme of the Kennedy pathway and of the *Sgms* enzymes of the sphingolipid synthesis (Figure 1B, Table S4 for a full overview). Altogether, these data suggest that choline uptake and metabolism is a target of β -catenin overactivation.

A

SUB PATHWAY	CHOLINE PATHWAY	PLATFORM	FASTED APC KO/CTRL	FED APC KO/CTRL	ETHANOLAMINE PATHWAY	PLATFORM	FASTED APC KO/CTRL	FED APC KO/CTRL	
Glycerolipid metabolism	choline	LC/MS pos	0.88	1.31	ethanolamine	GC/MS	0.91	1.24	
	choline phosphate	LC/MS pos	0.85	0.85	phosphoethanolamine	LC/MS neg	1.46	1.08	
	cytidine 5'-diphosphocholine	LC/MS pos	1.83	3.23	cytidine-5'-diphosphoethanolamine	LC/MS pos	1.18	1.37	
Lysolipid	1-myristoyl-GPC	LC/MS pos	1.76	1.47	1-palmitoyl-GPE	LC/MS neg	1.34	0.99	
	1-palmitoyl-GPC	LC/MS pos	3.59	1.29	2-palmitoyl-GPE*	LC/MS pos	2.50	1.08	
	2-palmitoyl-GPC*	LC/MS pos	2.33	1.50	1-stearoyl-GPE	LC/MS neg	1.19	0.90	
	1-palmitoleyl-GPC*	LC/MS pos	3.01	1.52	1-oleoyl-GPE	LC/MS neg	1.36	1.30	
	2-palmitoleyl-GPC*	LC/MS pos	1.35	1.33	2-oleoyl-GPE*	LC/MS neg	1.05	1.11	
	1-heptadecanoyl-GPC	LC/MS pos	2.39	1.06	1-linoleyl-GPE*	LC/MS neg	0.53	0.62	
	1-stearoyl-GPC	LC/MS pos	2.96	1.50	2-linoleyl-GPE*	LC/MS neg	1.08	1.30	
	2-stearoyl-GPC*	LC/MS pos	1.51	1.03	1-arachidonoyl-GPE*	LC/MS neg	0.79	1.04	
	1-oleoyl-GPC	LC/MS pos	4.00	2.37	2-arachidonoyl-GPE*	LC/MS pos	2.73	1.36	
	2-oleoyl-GPC*	LC/MS pos	3.49	1.57	2-docosapentaenoyl-GPE*	LC/MS pos	2.34	1.28	
	1-linoleyl-GPC	LC/MS pos	1.57	1.14	2-docosahexaenoyl-GPE*	LC/MS pos	4.17	1.62	
	2-linoleyl-GPC*	LC/MS pos	2.77	1.09	INOSITOL PATHWAY		PLATFORM		
	2-eicosatrienoyl-GPC*	LC/MS pos	1.50	1.43	1-palmitoyl-GPI*	LC/MS neg	1.26	0.80	
	2-eicosatrienoyl-GPC*	LC/MS pos	1.48	1.26	1-stearoyl-GPI	LC/MS neg	0.85	0.87	
	1-arachidonoyl-GPC*	LC/MS pos	1.94	1.73	1-stearoyl-GPI*	LC/MS neg	0.72	0.76	
	2-arachidonoyl-GPC*	LC/MS pos	4.00	2.58	2-stearoyl-GPI*	LC/MS neg	0.82	0.96	
	2-docosapentaenoyl-GPC*	LC/MS pos	2.60	1.95	1-arachidonoyl-GPI*	LC/MS neg	1.12	1.84	
	1-docosahexaenoyl-GPC*	LC/MS pos	3.17	1.64	2-arachidonoyl-GPI*	LC/MS neg			
	2-docosahexaenoyl-GPC*	LC/MS pos	6.42	2.32	BETAINE/METHIONINE/FOLATE PATHWAY		PLATFORM		
	Sphingolipid	sphinganine	LC/MS pos	2.65	3.28	betaine	LC/MS pos	1.00	0.52
sphingosine		LC/MS pos	2.85	2.74	dimethylglycine	GC/MS	1.11	0.73	
palmitoyl sphingomyelin		GC/MS	0.96	1.33	S-adenosylhomocysteine (SAH)	LC/MS neg	0.79	0.75	
stearoyl sphingomyelin		GC/MS	2.51	2.26	methionine	LC/MS pos	0.93	1.30	
					homocysteine	GC/MS	1.50	4.03	
				5-methyltetrahydrofolate (5MeTHF)	LC/MS neg	0.64	0.26		

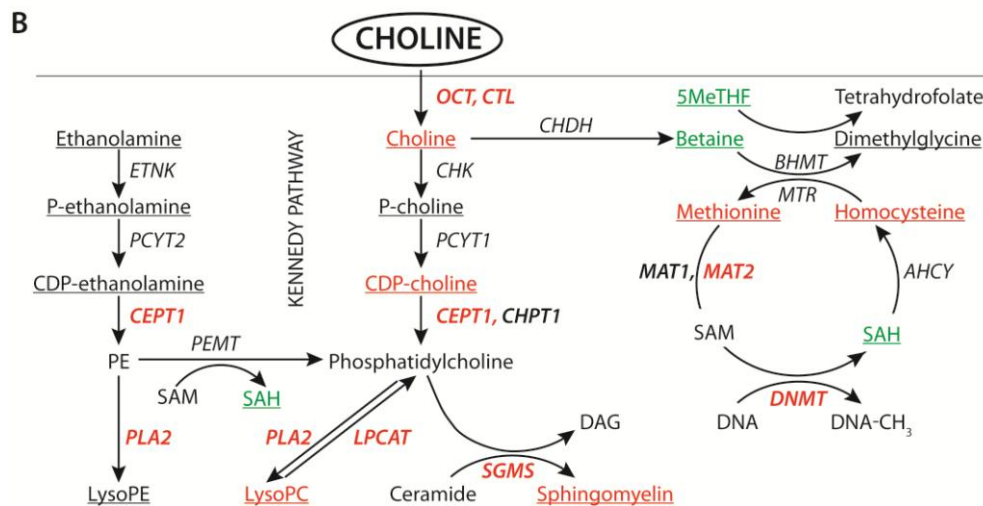


Figure 1 : Metabolites resulting from choline metabolism accumulate after β -catenin overactivation in mouse liver. (A) Metabolomic analysis assessing the metabolites differentially accumulated in Apc^{ko} versus Apc^{Ctrl} livers at Day 7 after Tamoxifen-mediated inactivation of Apc . In red and yellow, significant fold accumulations ($p < 0.01$) ; in blue, less significant ones ($p < 0.05$) ; in green and yellow, significant decreased amounts ($p < 0.01$). (B) Choline metabolism and its deregulations in Apc^{ko} livers. The metabolites measured in the metabolomic study are underlined. In red appear those which are accumulated, and in green those with a decreased amount. In italics are depicted the enzymes and transporters involved in choline metabolism, in red when their mRNA expression is deregulated (Table S4, for a complete overview of the mRNA-Seq data focused on choline metabolism).

Genotyping human β -catenin-mutated HCCs with FCH-PET

A systematic double PET/CT screening, i.e. FDG-PET and FCH-PET, was performed on a cohort of patients with HCCs, before resection or biopsy of the tumor. Eighteen patients were selected with distinct etiologies, distinct tumor sizes and differentiation gradings (Table 1). Blood AFP was also fluctuating. We assessed the status of β -catenin activation on paraffin-embedded tissue by a GS immunostaining (a score of 3 corresponded to *CTNNB1*-mutated status (Audard et al., 2007)), and on frozen tumors by *CTNNB1* exon-3 sequencing on one part, and on the other part by qPCR assessment of β -catenin target genes, GS and Axin2, on tumors (T) compared to their non tumoral adjacent liver tissue (NT) (the threshold chosen was a T versus NT ratio upper than 5). The positivity for PET was either only visually estimated, or confirmed by SUVmax ratios between T and NT, using a threshold of 1.5.

We had to exclude 6 patients from this series: two because the biopsy performed was exhausted and we could not assess GS immunostaining, 2 others because their PET showed a complex pattern, as FDG and FCH-PET analyses showed both positive and negative areas. The last 2 tumors excluded had discordant data between GS immunostainings that were positive, and *CTNNB1* sequencing or/and qPCR assessments, which were negative: this suggests an intra-tumoral heterogeneity.

For the 12 informative patients, 6 were FCH positive, among which only one was FDG positive, and all of these were *CTNNB1*-mutated (mut) (Table 1); the six other patients were FHC negative, among which three were FDG positive and all of them were *CTNNB1*-wild-type (wt). An illustration of these data is shown in Figure 2 for one patient of each genotype. But the complementary assessment by immunostainings of the enzymes of the Kennedy pathway (CEPT1, CHKA, PCYT1A) was not informative to discriminate between both genotypes (Figure 2 and data not shown). In each case, the tumors expressed these enzymes but the stainings were not different between T and NT. However, the intratumoral staining for CEPT1 looked higher in *CTNNB1*-mut than in *CTNNB1*-wt tumors (Figure 2C). To further assess this observation, we looked at gene expression on three cohorts of HCCs: the one used in this

study with PET characterization (Figure 3A), and two cohorts of genetically-characterized HCCs (Figure 3B,C). The third cohort had been characterized by a genome-wide microarray analysis (Boyault et al., 2007), and we searched for a differential gene expression (mut *versus* wt) in the orthologous genes described in Table S4 for mice, relevant for acting in choline metabolism. We found that *CHKA* and *PCYT1A* expression were upregulated in HCCs regardless of their mutation status, but they were found increased in *CTNNB1*-mut tumors compared to *CTNNB1*-wt tumors (Figure 3C). *CEPT1* tended to be overexpressed in this series in *CTNNB1*-mut tumors. These deregulations were specific for HCC, as their expression in adenomas were not distinguishable from normal livers, regardless of their genetic alteration. In the three cohorts, there was either a trend or a significant increased expression of *CHKA*, *CEPT1* and *PCYT1A* mRNAs, the three main enzymes of the choline Kennedy pathway, in *CTNNB1*-mut/FCH-PET positive HCCs compared to *CTNNB1*-wt ones.

Altogether, these data showed that in the case of homogenous tumors, PET-FCH positivity is powerful to discriminate *CTNNB1*-mutated HCCs, whereas it is not the case for FDG-PET. This is accompanied with a mild increase of expression of the enzymes of the choline Kennedy pathway, not seen in adenomas, suggesting that this is specific for HCCs.

Table 1: Cohort of 18 patients with ¹⁸F-Choline and ¹⁸FDG PET/CT characterization.

The differentiation grade of the tumors is depicted as follows: W = well differentiated, MW = Moderately well, M = Moderately, P = Poorly. The histological characterization of GS immunostaining is as follows: 3 = diffuse and intense staining, 2 = heterogenous and/or moderate staining, 1 = low intensity of staining and/or few tumoral cells with positive staining.

Id	Patient characteristics					Tumor characteristics & Histology				β-catenin status					PET-Scan			
										DNA	IHC	mRNA T/NT		p-catenin status	F-Choline		FDG	
Patient Id Sex	Age	Cirrhosis	Etiology	AFP	Nodule size (mm)	Multi-focal	Differentiation grade	CTNNB1 mutations	GS	GS	Axin 2	p-catenin status	SUV Ratio T/N T		PET FCh	SUV Ratio T/N T	PET FDG	
CTNNB1-mutated, FCh-POS																		
7749	M	69	0	HCV	4	120	0	W	S33P	3	45	24	+	2.24	+	1.24	-	
9543	M	61	1	HBV+O H	7	20	0	W	S37F	3	66	37	+	1.85	+	1.24	-	
4341	F	52	1	HCV+O H	96	50	0	MW	G34V	3	97	24	+	2.43	+	0.35	-	
5493	M	62	0	HFE	6.7	80	0	MW	H36P	3	406	101	+	2.6	+	nd	-	
6742	M	55	0	OH	35	25	1	M	S45P	3	31	8.8	+	nd	+	nd	+	
961	M	66	1	OH	7	50	1	M	nd	3	nd	nd	+	nd	+	nd	-	
CTNNB1-wild type, FCh-NEG																		
1519	M	57	1	VHC	448	14	0	MW	wt	2	5.7	2.8	-	1.37	-	1	-	
2635	F	66	0	VHC	87	51	0	MW	wt	2	0.5	7.1	-	0.97	-	1.77	+	
612	M	49	0	VHB	12	57	0	MW	wt	1	1.3	1.9	-	0.9	-	1.74	+	
5205	M	54	1	VHC	12	20	0	MW	wt	1	4.9	4.3	-	nd	-	nd	-	
4340	M	52	1	VHB	700	90	0	M	wt	1	1.1	3.4	-	0.94	-	3.25	+	
5895	M	68	1	OH	8	47	0	W	wt	1	5.2	1.3	-	1	-	1	-	
Tumor heterogeneity																		
909	M	70	0	NASH	59	70	1	W/ M	S33P	3	8.5	3.5	?	nd	+ / -	nd	+ / -	
9125	M	70	1	VHC	50	80	0	MW	wt	3	1.3	1.8	?	2.1	+	1	-	
4379	M	37	1	VHB	194	20	0	M	wt	2	6.7	1.2	?	nd	+ / -	nd	+ / -	
5162	M	60	1	VHC	4620	50	1	P	wt	3	0.8	0.5	?	nd	+	nd	+	
Non informative (biopsy with no frozen tissue)																		
8215	M	49	1	VHC	3123 0	100	0	P	nd	nd	nd	nd	?	1.5	+	2.1	+	
6340	M	70	1	NASH	1125	40	1	W	nd	nd	nd	nd	?	2.5	+	1	-	

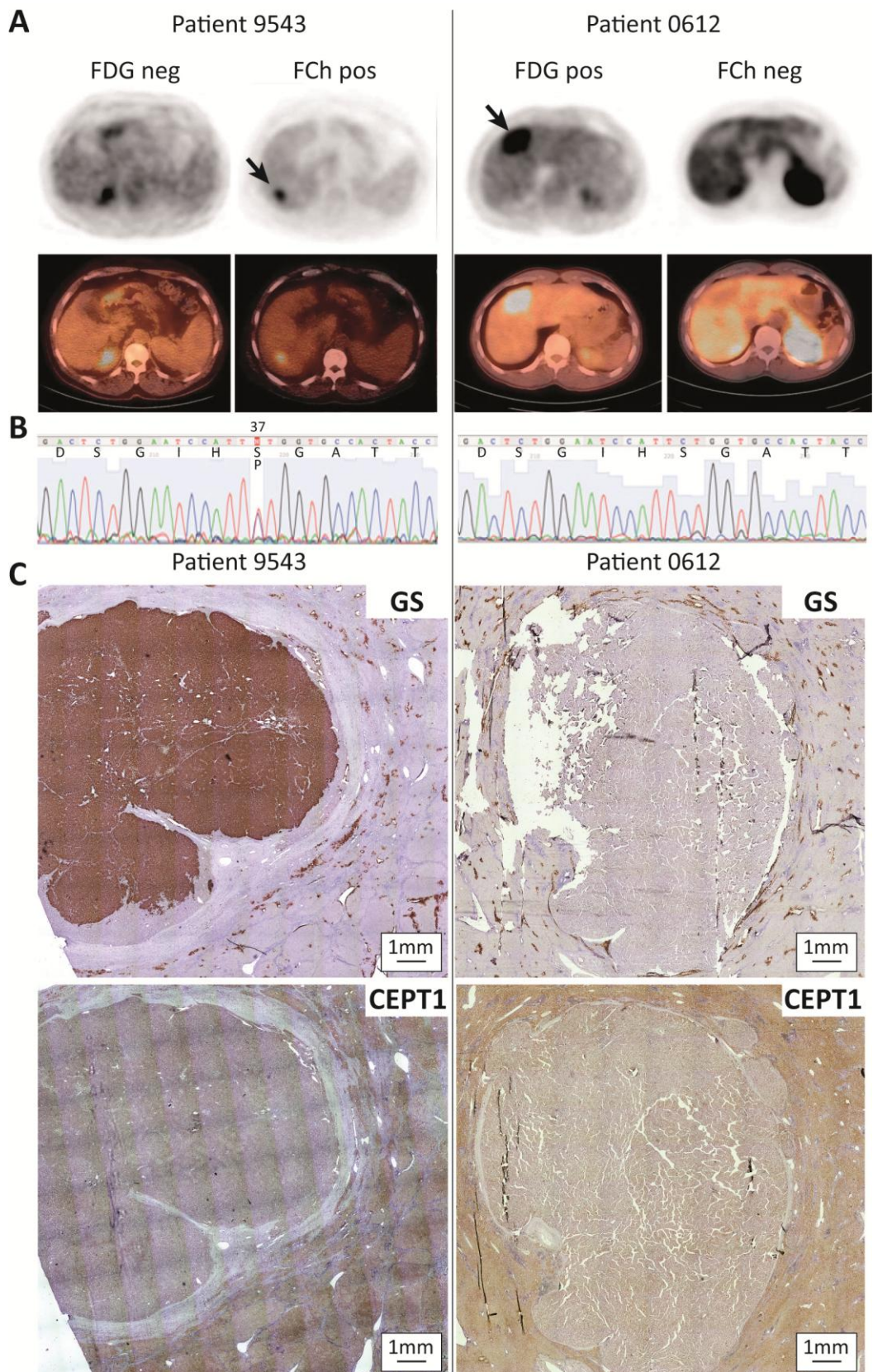


Figure 2 : Correlation between PET/CT analyses and β -catenin activation in patients with HCCs. On the left, one example of patient with F-Choline positive and β -catenin-activated HCC is shown, and on the right, one example of patient with F-Choline negative PET and non β -catenin-activated HCC. These data derive from

Table 1. (A) PET/CT data : FDG and FCH, up PET and down PET/CT fusion. (B) Sanger sequencing of *CTNNB1* gene. The heterozygous S37P mutation of patient 9543 is shown by a double band for nucleotides T and C. (C) Up, GS immunostainings of the tumors, illustrating β -catenin activation, is diffuse and positive for patient 9543, and low for patient 612. Down, immunostainings for CEPT1 showing immunoreactivity in the 9543 tumor, which is lower in 612 tumor : in both cases the staining in the tumor is lower than the non-tumoral counterpart.

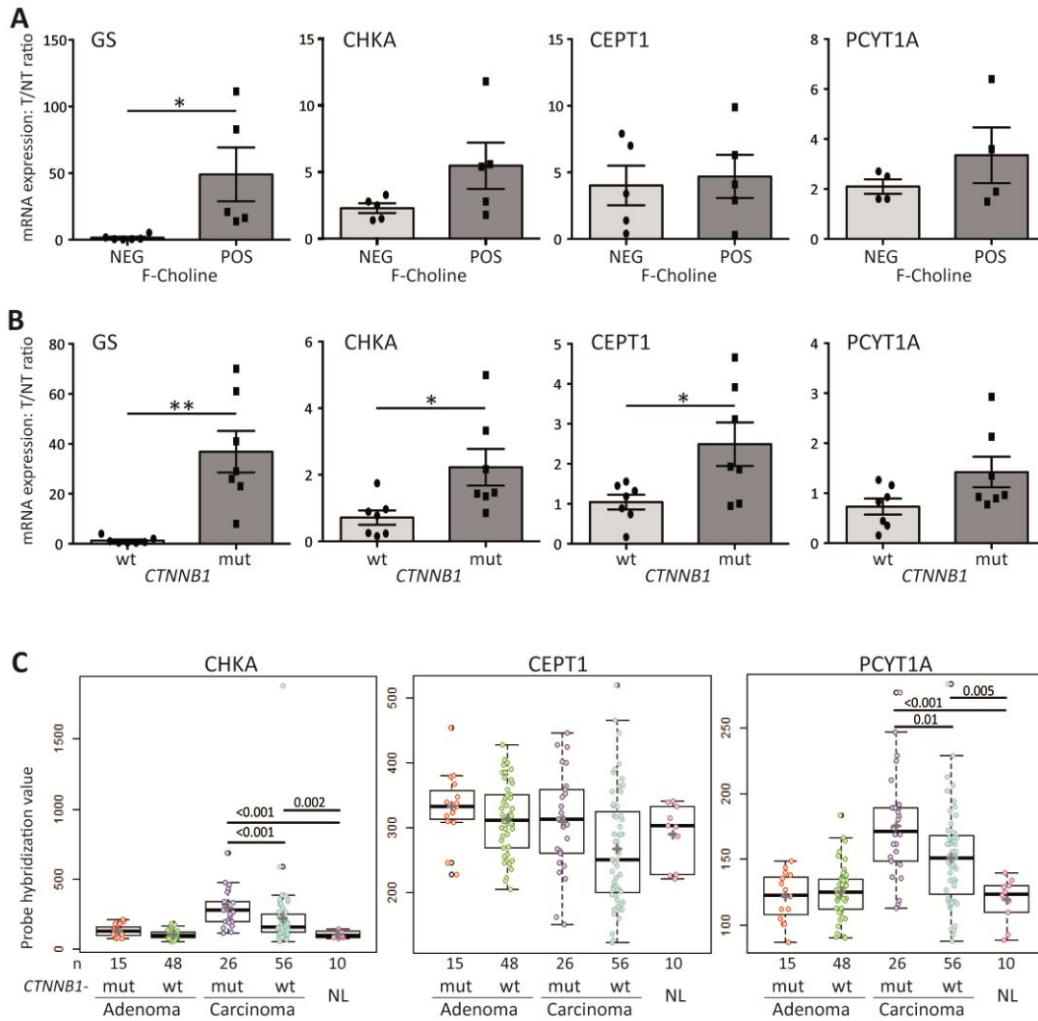


Figure 3 : In human HCCs, gene expression of choline metabolism enzymes correlates with FCh uptake and *CTNNB1* mutations. In (A,B) the PET and the Cochlin cohorts are studied by qPCR; in (C) the cohort described in (Boyault, Hepatology 2007) was analyzed by microarray. The tumors samples are divided in FCh-POS (n=5 or n=4), FCh-NEG (n=5 or n=4) in (A) ; and *CTNNB1*-mut (n=7) and *CTNNB1*-wt (n=7) in (B). Student t-tests with Welch corrections have been done in (A,B) and two-way ANOVA in (C). *p<0.05, **p<0.01.

FCH-PET is also efficient to genotype mouse β -catenin-activated tumors

To assess whether the Apc^{ko} model of mouse liver tumors can be used to unravel the mechanism and consequences of choline deregulation by β -catenin, we first measured the uptake of choline in Apc^{ko} livers (Figure 4A). This was done *in vivo* by FCH-microPET and revealed an increased uptake of choline in these livers compared to controls (Figure 4B). A similar result was obtained *in vitro* on primary cultures of hepatocytes: the uptake of Methyl- ^{14}C -Choline by Apc^{ko} hepatocytes was increased compared to controls (Figure 4C).

Next, we developed two mouse models of HCCs: one is linked to Apc inactivation and β -catenin gain-of-function (Colnot et al., 2004), whereas the second is induced by diethylnitrosamine (DEN) and is independent from β -catenin status (Aydinlik et al., 2001). After the emergence of tumors within 5 to 8 months (screened by ultrasonography), FCH-PET was done (Figure 4D,E). Tumor size and numbers do not differ between both models, as assessed by the liver to body weight ratio (Figure 4D). In the 7 Apc^{ko} tumoral livers studied, FCH-positive spots were systematically detected, corresponding to the location of tumors after sacrifice (Figure 4D, E). In mouse tumors, *Cept1* mRNA expression was specifically induced in Apc^{ko} tumors and not in DEN-induced ones, both at the mRNA and at the protein level (Figure S1B). *Chk α* but also *Chk β* were as well induced but were not specific for β -catenin status. In the 10 DEN-induced tumoral livers, 3 cases of FCH positivity were detected, rather occurring in larger tumors (Figure 4D, E). In conclusion, β -catenin favors choline uptake by the hepatocyte, and β -catenin-activated tumors in mice can be characterized, as in humans, by their FCH positivity, which is higher than in the non-activated DEN-induced tumors.

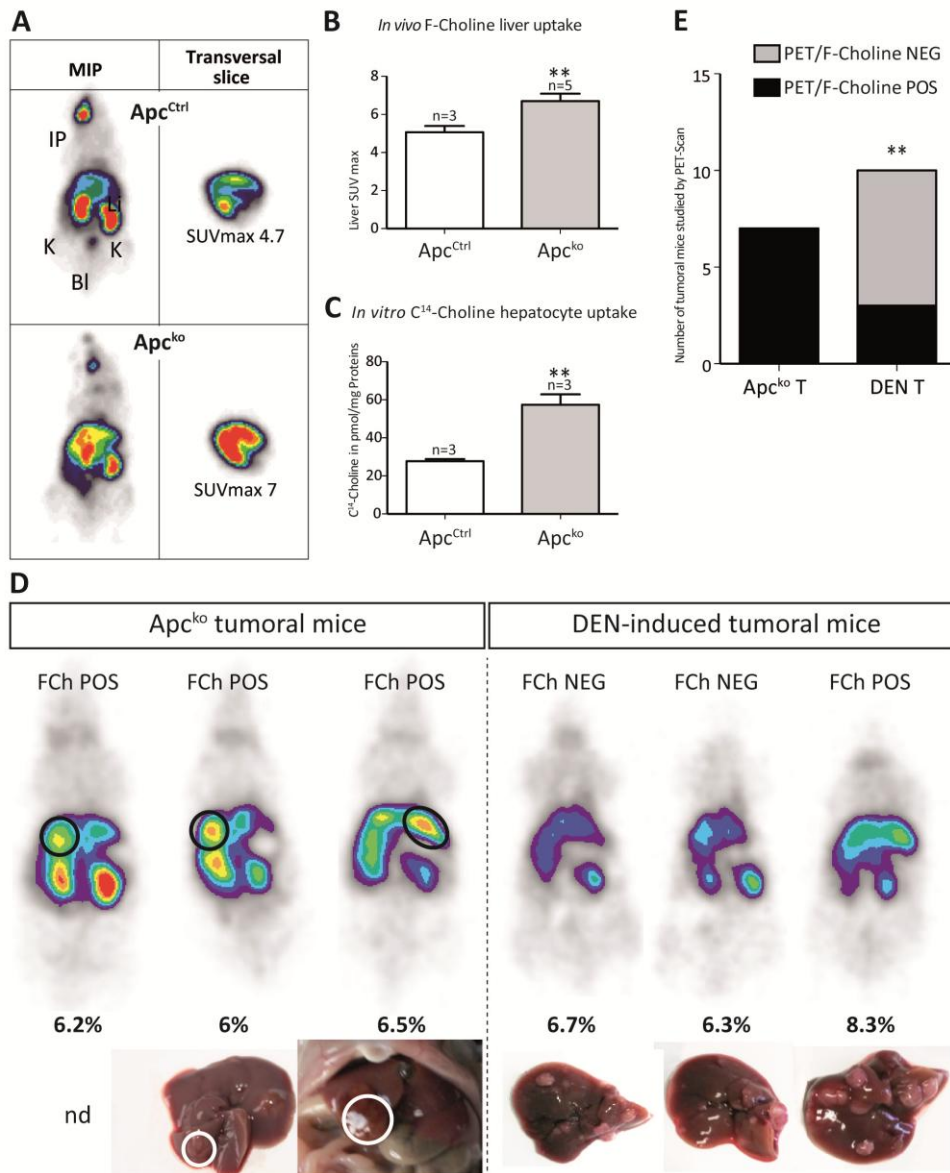


Figure 4 : Increased uptake of F-choline in mice with β -catenin overactivated livers and tumors. (A) one representative example of microPET performed on mice with Apc^{KO} panlobular inactivation (Day 7 after tamoxifen). MIP = Maximum Intensity Projection, SUVmax = Maximum Standardized Uptake Value. (B) Graphic representation of the liver SUVmax in Apc^{KO} versus Ctrl mice. (C) Uptake of ¹⁴C-choline in primary hepatocyte cultures. In (A,C), **p<0.01 (Student t-test with Welch correction). (D) Longitudinal slices of F-Choline PET for tumoral mice. The mice were sacrificed one week after PET : tumors are shown and the liver to body weight ratio mentioned ; (E) Summary of the F-Choline PET experiments in mice, **p<0.01 (Fisher exact t-test).

Choline dietary intake controls Apc^{ko} liver size and β -catenin-dependent DNA methylation

In order to define whether the choline preferential uptake of β -catenin-activated hepatocytes has functional consequences, we deprived mice from choline and methionine dietary intake. We submitted Apc^{ko} mice to a Methionine-Choline Deficient diet (MCD). The MCD diet is able to suppress choline substrate for phospholipid synthesis, while the coupling with Methionine deficiency also suppresses choline as a methyl group donor (Figure 1B). This diet was administered during 15 days (Figure 5A), because longer administrations generate long term inflammatory and cancer consequences. These carcinogenic effects are due to a dramatic steatosis originating mainly from the absence of VLDL formation and lipid export (Mato and Lu, 2011). As our purpose was to identify immediate consequences of choline depletion, we checked by Oil Red O stainings that small lipid droplets began to accumulate by 15 days (D15) (data not shown), demonstrating the efficiency of the depletion at D15 and justifying this time point proposed for our experiment (Figure 5A).

When mice were fed a conventional diet, 7 days after Tamoxifen injection the panlobular Apc inactivation led to a severe hepatomegaly linked with hepatocyte hyperproliferation ((Benhamouche et al., 2006; Colnot et al., 2004), and Figure 5B, C). Interestingly, under a MCD diet, this hepatomegaly was completely abolished (Figure 5B). We expected that this hepatomegaly could be linked to a default in hepatocyte proliferation, which requires membrane formation and thus phospholipid production. However, the proliferation of hepatocytes at day 7 remained constant (Figure 5C). No difference in the size of the hepatocytes could be detected by comparing Apc^{ko} livers in either diet (data not shown).

Nevertheless, we tested another parameter potentially controlled by choline intake, which is DNA methylation. Under a conventional diet, Apc^{ko} hepatocytes tended to be overmethylated on cytosines, and this DNA methylation was dramatically down regulated when mice were fed a MCD diet (Figure 5D). Moreover, the expression of the DNA methyltransferases

Dnmt1 and Dnmt3a was induced in *Apc^{ko}* livers in conventional diet. This increase was completely abolished when *Apc^{ko}* mice are fed a MCD diet. In summary, we have shown that depleting both choline and methionine intakes, leads to a reversal of the hepatomegaly of *Apc^{ko}* mice, whose cause is still to be deciphered. It also leads to a reversal of DNA methylation, suggesting that choline has a major role as a methyl group donor in β -catenin-activated hepatocytes.

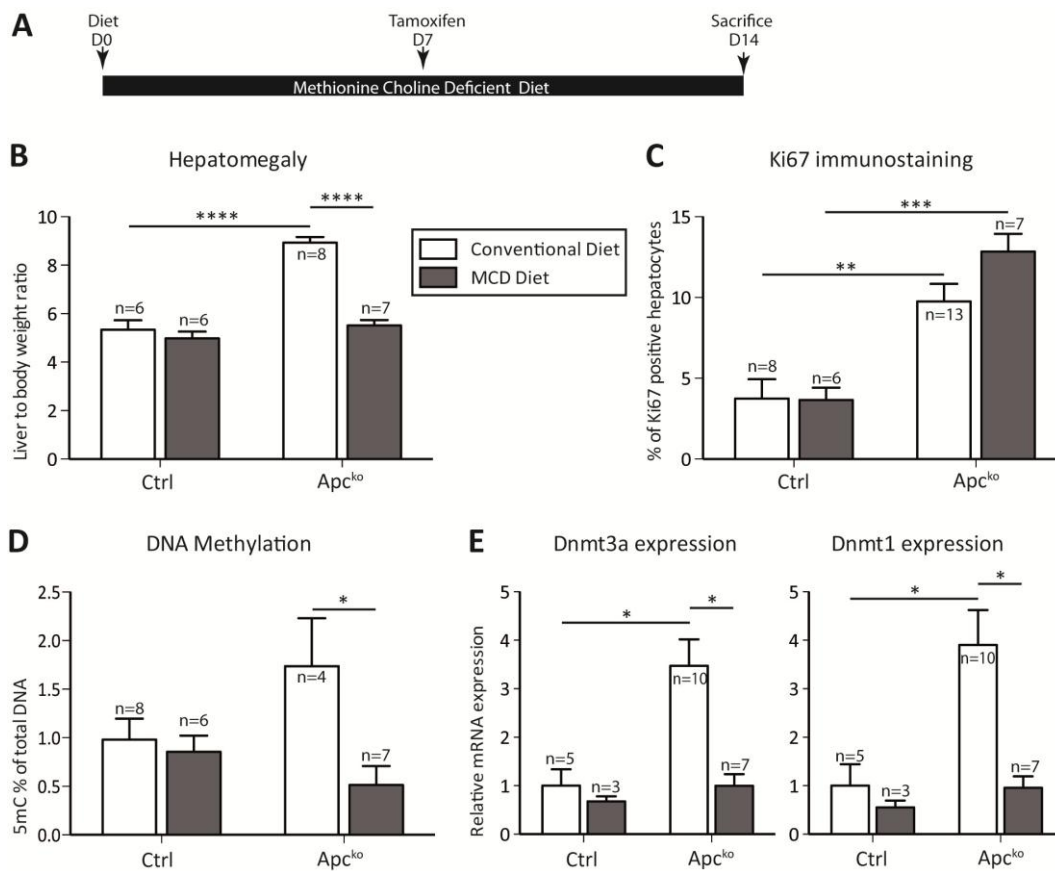


Figure 5: Functional consequences of abolishing choline dietary intake through a Methionine-Choline Deficient (MCD) diet. (A) Experimental design ; (B) Assessment of hepatomegaly through liver to body weight ratio measurement at sacrifice; (C) Proliferation as a percentage of Ki67-positive hepatocytes ; (D) DNA methylation on cytosines ; (E) Gene expression. Two-way ANOVA with Tukey multiple comparisons tests were done.

β -catenin specifically orientates choline fate to secreted phospholipids and to methylated DNA

To better understand the fate of choline and its specificity regarding β -catenin status, we analyzed its incorporation in phospholipids and its contribution to DNA methylation. Two models of cultures incubated with Methyl- ^{14}C -Choline were chosen: primary hepatocytes and tumor explants (either of DEN-induced or Apc^{ko} origin) (Figure 6). Firstly, we detected a significant accumulation of ^{14}C in intracellular phospholipids, in both primary hepatocytes and tumors of either model (Figure 6A). We expect that this incorporation was linked to hepatocyte proliferation, as Apc^{ko} primary hepatocytes, as well as DEN-induced and Apc^{ko} tumors are strongly proliferating (Figure 6B).

Secondly, we measured the accumulation of ^{14}C in secreted phospholipids (Figure 6C): in the liver, they should be represented by the VLDL production, but also by the bile secreted in the medium which is rich in phospholipids. Interestingly, both Apc^{ko} primary hepatocytes and tumor explants strongly incorporated choline in their secreted phospholipids. This was not the case for DEN-induced tumors, meaning that this effect should be specific for β -catenin activation. We previously showed that Apc^{ko} livers produced less blood VLDL than normal livers (Gougelet et al., 2014), suggesting that the incorporation of choline into VLDL is not a major fate in Apc^{ko} livers. Interestingly, we also showed that β -catenin activation was an inducer of bile acid synthesis through a transcriptional increase in *Cyp7a1* and *Cyp27a1* gene expressions (Gougelet et al., 2014). Moreover, *CTNNB1*-mut HCCs are often cholestatic (Audard et al., 2007). These data suggested that choline could be preferentially incorporated in secreted bile phospholipids. To test this hypothesis, we measured the bile flow of Apc^{ko} livers, and found that there is a trend in a faster bile flow, and consequently to an increased bile synthesis, potentially requiring more synthesis of both phospholipids and bile acids.

Third, we searched for ^{14}C incorporation in tumor DNA that would endorse the role of Choline as a methyl group donor for DNA methylation. We found

that DNA from Apc^{ko} tumors was enriched in ^{14}C , contrary to DEN-induced tumors.

Altogether we have shown here that ^{14}C -Choline is used to synthesize phospholipid membranes, and this is associated with hepatocyte proliferation. Moreover and specifically in a context of β -catenin activation, ^{14}C -Choline contributes to the synthesis of secreted phospholipids, which are probably bile phospholipids, and to DNA methylation.

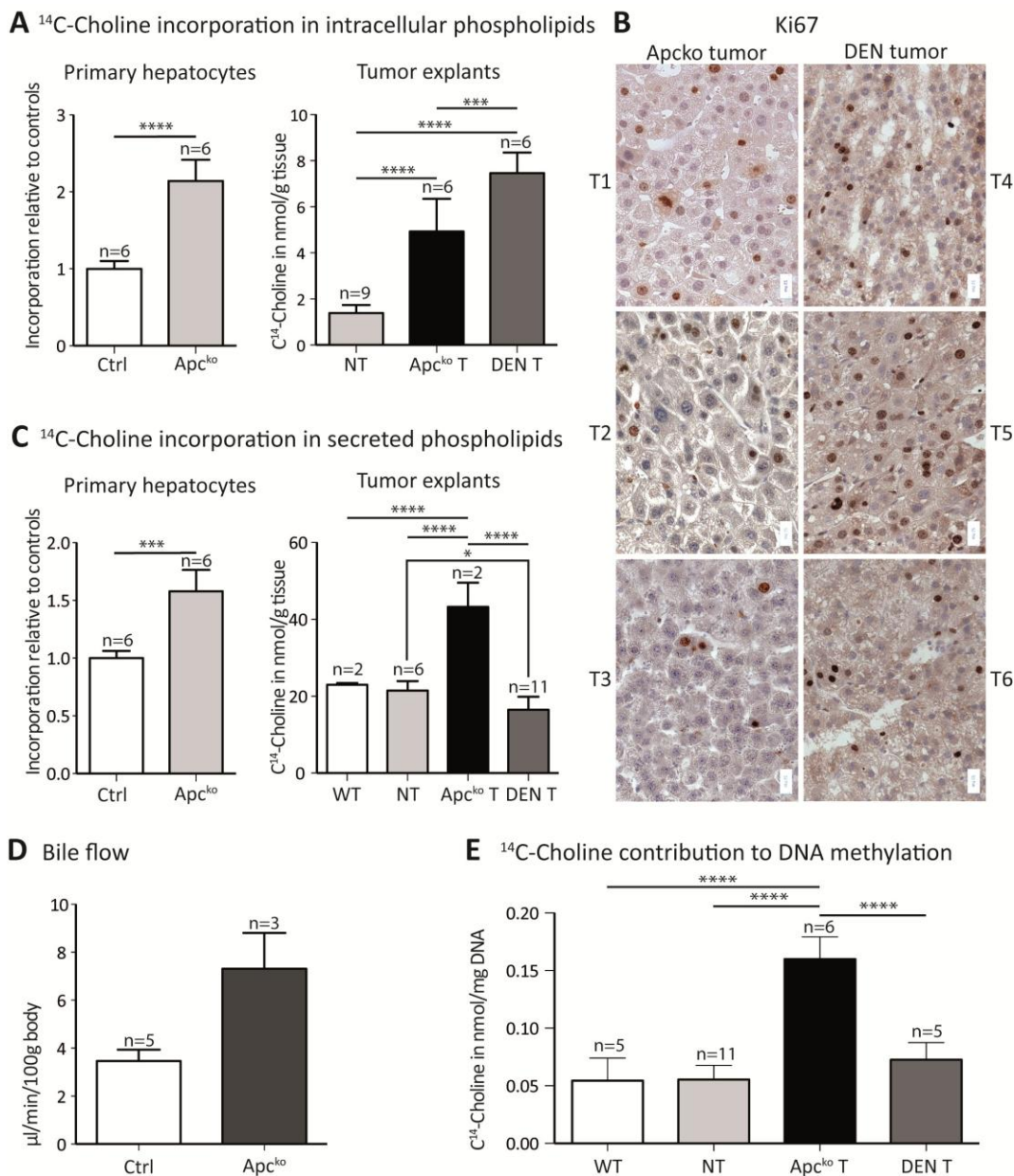


Figure 6 : Fates of ¹⁴C-choline in β -catenin-activated mouse hepatocytes and tumors. Choline incorporation in (A) intracellular phospholipids, (C) secreted phospholipids, was assessed in primary hepatocytes and (*Apc*^{ko} and DEN) tumor explants ; (B) Equivalent proliferation in *Apc*^{ko} and DEN tumors was assessed by Ki67 immunostaining ; (D) Bile flow tended to be faster in *Apc*^{ko} mice ; (E) Choline contributes preferentially to DNA methylation in *Apc*^{ko} tumors. Student t-tests or two-way ANOVA with Tukey multiple comparisons tests were done.

DISCUSSION

The major breakthrough of this study was to uncover that an imaging non-invasive method would be able to genotype tumors. To our knowledge, this has never been described before in the cancer field. We are aware that the cohort that we used is a limited series and requires an extension to validate this potentiality for FCH-PET to genotype *CTNNB1*-mutated tumors. However, the same correlation was found in mice, reinforcing the strength of our data. In fact, in a previous study, we showed that FCH-PET was more sensitive than FDG-PET to detect well-differentiated HCCs (Talbot et al., 2010), and this fits well with the fact that *CTNNB1*-mut HCCs are globally better differentiated than *CTNNB1*-wt ones (Cavard et al., 2008; Hoshida et al., 2009).

But our data also show that an increased choline uptake would not only be a diagnostic tool, but could be a therapeutic target. In fact, the depletion of choline and methionine in diet leads to immediate functional consequences, occurring in *Apc*^{ko} hepatocytes and not in control ones. Particularly, the effect of choline deprivation to suppress hepatomegaly was dramatic. We have not brought here the evidence that this is due to an impaired hepatocyte proliferation linked to a default in phospholipid amount needed for membrane synthesis. We showed in a previous work that the proliferation of *Apc*^{ko} hepatocytes occurred from day 3 after Tamoxifen and was maximal at Day 4 (D4), rather than at day 7 (Benhamouche et al., 2006). We can thus hypothesize that MCD diet could alter hepatocyte proliferation at D4, time point that has to be tested for proliferation in new experimental settings.

Choline plays another key role in β -catenin dependent liver tumorigenesis process. It takes part in DNA methylation. We showed here that *Apc*^{ko}

hepatocytes tended to have an increased DNA methylation on cytosines compared to controls. We also showed that this methylation was choline-dependent and β -catenin specific for several reasons. First, it is reversed when choline is depleted, second ^{14}C is found in DNA as soon as 2 hours after Apc^{ko} hepatocytes are cultivated in the presence of ^{14}C -Choline, third this flow of ^{14}C in DNA is not found in DEN-induced tumors, despite the fact that they overexpress the DNA methyltransferases Dnmt1 and Dnmt3 at the same level as Apc^{ko} tumors (Figure S1C). HCC is considered as a CpG Island Methylator Phenotype (CIMP+), with many promoters being hypermethylated (Li et al., 2010). Correlations between methylation patterns and different etiologies have been made (Neumann et al., 2012), and the methylation of specific loci has been associated with *CTNNB1*-mut HCCs (Nishida et al., 2007). DNA hypermethylation is thought to be tumor promoting and choline addiction by tumor cells could play a role in β -catenin dependent tumorigenesis through this means and not only through the synthesis of phospholipids.

A last role that appears to be specific for β -catenin-activated liver tumors is the incorporation of choline in secreted phospholipids. Our hypothesis that these secreted phospholipids could come from an increased bile synthesis relies upon different points. The first one is that the bile flow is faster in Apc^{ko} livers, and this goes well with our previous study, showing a direct impact of β -catenin on bile acid synthesis (Gougelet et al., 2014). This is also supported by the inverse phenotype found in mice with a hepatospecific β -catenin knock-out that have a decreased bile flow (Yeh et al., 2010). Lastly, this fate of choline is particularly relevant in tumorigenesis, due to the cholestatic phenotype found in *CTNNB1*-mut HCCs that could be related to an increased bile production (Audard et al., 2007). As the bile can be toxic due to hydrophobic bile acids, it can be postulated that this choline-derived bile accumulation should have pro-carcinogenic effects.

Thus, the involvement of choline in the Kennedy pathway to produce phospholipids has both β -catenin independent consequences on hepatocyte proliferation, and β -catenin-dependent consequences on secreted phospholipids. The global link of choline-derived with cell proliferation

constitutes probably the reason for which the enzymes of the Kennedy pathway, i.e. CHKA, CEPT1, PCYT1A are induced in HCCs regardless of their genotype, with only a trend to further increase in *CTNNB1*-mut tumors compared to wt ones. Moreover, we were not able to identify in tumors a choline transporter that would be a target of β -catenin, and explaining the increased uptake of choline. But there are many choline transporters (the specific ones belong to the OCT or the CTL family), and moreover choline can also be transported into the cell by facilitated diffusion (Bieze et al., 2014).

Due to this plethora of potential oncogenic roles for choline in β -catenin-activated HCCs, it would be tempting to test in our mouse cancer models, anticancer drugs targeting the choline pathway, such as MN58b and TCD-717, two specific inhibitors of the choline kinase α , able to reduce cell proliferation, the latter being approved by the FDA for a phase I clinical trial (Lacal and Campos, 2015).

Lastly, we propose here a tool to diagnose β -catenin-activated HCCs. The question is what is the interest to specifically diagnose this subcategory of liver cancers? As already specified in the introduction, the therapeutic tool currently used to treat patients with HCCs is mainly the resection. There are some indications to treat these patients with Sorafenib, a tyrosine kinase inhibitor, but with no major therapeutic effects (Peck-Radosavljevic et al., 2010). We expect that the information of the genetic mutation involved in HCCs would allow the patients to access to targeted therapies. We actually have shown in several studies that β -catenin-activated liver tumors have particular characteristics, in terms of transcriptome, histomorphotype, and metabolism. They have also a better prognosis and should be treated distinctly than *CTNNB1*-wt tumors. In a recent study, we have shown that their miRNome is distinct from *CTNNB1*-wt tumors (Gougelet et al., 2015). We have applied a therapeutic strategy based on the use of a Locked Nucleic Acid blocking the effect of miR-34a, a target of β -catenin, and we have shown that this LNA-34a was efficient *in vivo* for reducing the progression of β -catenin-activated mouse tumors. This effect is supposed to be specific for β -catenin activation. But we can expect that in the near future, direct and druggable inhibitors of β -catenin signaling will be characterized as

antitumoral agents. A recent screen performed *in vivo* using a zebrafish model of liver cancer has identified such molecules. Among them, one antidepressant, amitriptyline, was successful to decrease tumor burden in a mouse HCC model (Evason et al., 2015). Nevertheless, a prerequisite to use these targeted therapies will consist in an efficient genotyping of HCCs, done as early as possible and in a non-invasive way. Genotyping HCCs with FCH-PET could pave the way for a powerful use of these targeted therapies.

ACKNOWLEDGMENTS

We thank the staff of the Animal Facilities of Cochin Institute, and of the Small Animal Imagery Facility of Cochin Institute for his efficiency in ultrasonography screening. We also thank Jean-Noël Talbot and Claire Provost (LIMP, Tenon Hospital, Paris) for helpful discussions, and Benoit Terris for the access to HCC samples of Cochin's patients.

FUNDINGS

Chiara Sartor and Nadia Senni got fellowships from the French Ministry of Research, and the Ligue Nationale Contre le Cancer (LNCC). This work was supported by the Ligue Nationale Contre le Cancer (Labellisation Program 2011-2013 and 2014-2016), and the Agence Nationale de la Recherche (WNT-METABOLIV, 2010-2014).

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SUPPLEMENTAL MATERIALS

Table S1 : Cohort of patients from Cochin Hospital

Samples	Etiology	qPCR ^{GS} T/NT	IHC ^{GS}	CTNNB1 [?] mutation	IHC/mut [?] p53	Sex	Age
F65186	HCV	nd	0	wt	nd	M	44
F95045	HCV	0.4	0	wt	no [?] mut	M	64
F69924	HBV	4	1	wt	no [?] mut	M	54
F72514	HCV	2	0	wt	no [?] mut	M	46
F85548	HCV	0.7	0	wt	1	M	55
F65483	OH	nd	0	wt	nd	M	67
F77178	HBV ^{OH}	0.08	1	wt	nd	M	53
F50472	HVC	29	2	T41A	0	F	78
F59541	OH	61	2	I35S	0	M	61
F49311	HBV	26	2	D32G	0	M	79
F59093	OH	8	2	S45P	no [?] mut	M	74
F62751	?	23	2	S45P	no [?] mut	M	77
F64236	HBV	41	2	T41A	0	M	53
F50582	OH	70	2	D32G	0	M	53

Table S2 : Oligonucleotides used for qPCR analyses

GENE	Primers [?]		Technology
	MOUSE	HUMAN	
CHKalpha	LP:TAGCTCAGAACCCGCCTAAA [?] RP:ACTCAGGCTCCTCCTGTTGA	LP:TTTGGCCCTTGCATCTCATT [?] RP:AGTCCTCCCCACAGTCACAC	SybRgreen
CEPT1	LP:TGGGAATGGCTAGTTGGAAG [?] RP:AATGAAAAGGCCACAAGCAC	LP:CTTGAGCCCTTAATGCAAG [?] RP:TTGCCTGTTTCCCATCAAT	
CHKbeta	LP:TGGAACAGTACCTCCCAAGC [?] RP:CAAGTCCTGGATCTGCTTC	LP:TCTTCTGGGGTCTGTGGTC [?] RP:CTTCTGCTCGTTGTTCTCTC	
DNMT1	LP: [?] ACCGTTCTACTTCTCGAGGCCT A RP: [?] GTTGCAGTCTCTGTGAACACTGT GG	-	
DNMT3	LP:CTGCAATGACCTCTCCATT [?] RP:CGGCCAGTACCCTCATAAAG	-	
PCYT1A	Mm00447774_m1	Hs00192339_m1	Taqman
GS	Mm00725701_s1	Hs00365928_g1	

Table S3 : Antibodies used in immunohistochemistry studies

Protein	dilution	Reference Antibody	secondary Biotinylated Ab
Glutamine Synthetase	1/400	BD Transduction LaboratoriesTM. Ref: 610518	mouse 1/750
CTP Phosphocholine Cytidyltransferase alpha (CCT1 or Pcyt1α)	1/100	US Biological. Ref: 7948-36	rabbit 1/200
Choline/ethanolamine phosphotransferase 1 (CEPT1)	1/200	ProteinTechTM. Ref: 20496-1-AP	rabbit 1/200

Table S4 : mRNA expressions of choline-related genes extracted from mRNA-Seq data (analyzed in Gougelet *et al.* Hepatology, 2014). In yellow and red, the most significant gene expression differences (fold ratio >2 AND p<0.05), in red, less significant ones (2> fold ratio >1.5 AND p<0.05).

Symbol	Aliases	Description	Keyword	Expression (WT/mice) (arbitrary value)	Apcko/Ctrl fold	Apcko/Ctrl p-value
Ahcy	CuBP, SAHH	adenosylhomocysteinase	Homocysteine	84	0.76	0.23
Ahcy1	Ahcy-rs3, CAL, Rbit	putative_adenosylhomocysteinase_2	Homocysteine	30	1.59	0.03
Ahcy2	Adohcyase3	putative_adenosylhomocysteinase_3_isoform_1	Homocysteine	5	1.20	0.49
Bhmt		betaine-homocysteine_S-methyltransferase_1	Betaine	560	0.81	0.94
Bhmt2		betaine-homocysteine_S-methyltransferase_2	Betaine	64	1.00	0.99
Cept1		choline/ethanolaminephosphotransferase_1	Choline	8	1.89	5.1E-03
Chdh		choline_dehydrogenase_mitochondrial_precursor	Choline	78	1.32	0.20
Chka	CK, EK/alpha, Chetk-alpha, Chk, Chok, Etk, Etnk-alpha	choline_kinase_alpha_isoform_1	Choline	13	1.31	0.21
Chkb	Chetk, Chkl, Ek, Ek/Ek, Ek/Ek-beta, Ekb, Ekekb, Ek	choline/ethanolamine_kinase	Choline	12	1.30	0.25
Chpt1		cholinephosphotransferase_1_isoform_2	Choline	26	1.27	0.26
Dnmt1	Cxxc9, Dnmt, Dnmt1o, MGMT, MTase, Met-1, Met1, MomeD2, D	DNA_(cytosine-5)-methyltransferase_1_isoform_1	Methionine	4	2.41	5.9E-04
Dnmt3a		DNA_(cytosine-5)-methyltransferase_3A_isoform_2	Methionine	7	2.23	1.1E-03
Dnmt3b	MmullIB	DNA_(cytosine-5)-methyltransferase_3B_isoform_3	Methionine	1	1.18	0.72
Etnk1	EK1	ethanolamine_kinase_1	Ethanolamine	24	1.02	0.88
Etnk2	EK12, Eki2, Tuc1	ethanolamine_kinase_2	Ethanolamine	140	1.59	0.07
Lpcat1	Aytl2, LPCAT, Id11	Lysophosphatidylcholine_acyltransferase	lysophospholipides	0.01	3.94	0.02
Lpcat2	Aytl1, Aytl1a, Pafat1	lysophosphatidylcholine_acyltransferase_2	lysophospholipides	0.02	8.63	3.9E-04
Lpcat2b	Aytl1b	lysophosphatidylcholine_acyltransferase_2B	lysophospholipides	0	not detected	
Lpcat3	C3f, Rcc3f, Lpcat, Lpeat, Lplat5, Lpsat, Mboat5, Moact5, Oact5, P TG	lysophospholipid_acyltransferase_5	lysophospholipides	33	1.00	0.40
Lpcat4	Agpat7, Aytl3, LPEAT2	lysophospholipid_acyltransferase_LPCAT4	lysophospholipides	0	5.06	0.08
Mat1a		S-adenosylmethionine_synthase_isoform_type-1	Methionine	1030	0.60	0.12
Mat2a		S-adenosylmethionine_synthase_isoform_type-2	Methionine	11	1.93	0.00308
Mat2b		methionine_adenosyltransferase_2_subunit_beta	Methionine	6	1.78	0.02
Mtr	MS	methionine_synthase	Methionine	2	1.68	0.13
Mtrr	MSR	methionine_synthase_reductase	Methionine	2	1.64	0.14
Pcyt1a	CTalpha, Ctalpha, Ctpct, Ctalpha	choline-phosphate_cytidyltransferase_A	Choline	29	1.34	0.18
Pcyt1b	CTTbeta	choline-phosphate_cytidyltransferase_B_isoform	Choline	0.03	0.76	0.59
Pcyt2	ET	ethanolamine-phosphate_cytidyltransferase	Ethanolamine	224	1.09	0.74
Pemt	PEAMT, PEMT2, Pempt, Pempt2	phosphatidylethanolamine_N-methyltransferase	Choline	87	0.98	0.92
Pla2g10	PLA2GX, GXsPLA2, PLA2-X	group_10_secretory_phospholipase_A2_precursor	lysophospholipides	0	not detected	
Pla2g15	ACS, LLPL, Lpla2, Lypla3	group_XV_phospholipase_A2_precursor	Phospholipase	5	1.87	0.01
Pla2g1b	Pla2a, S PLA21B	phospholipase_A2_precursor	lysophospholipides	0	not detected	
Pla2g6	PNPLA9, PLA(2)beta, PLA2, iPLA2beta	85_kDa_calcium-independent_phospholipase_A2	lysophospholipides	16	2.69	8.9E-06
Sgms1	Mob, Sms1, Sor1, Mem23	phosphatidylcholine:ceramide	Phospholipase	2	5.43	1.5E-10
Sgms2		phosphatidylcholine:ceramide	Phospholipase	18	1.74	0.01
Slc22a1	Lx1, Oct1, Orct, Orct1	solute_carrier_family_22_member_1	Choline	131	3.84	4.3E-07
Slc22a2	Oct2, Orct2	solute_carrier_family_22_member_2	Choline	0.04	2.90	0.22
Slc22a3	EMT, Oct3, Orct3, Sca22a3	solute_carrier_family_22_member_3	Choline	1	16.00	4.9E-23
Slc44a1	CTL1	solute_carrier_family_44_member_1	Choline	11	1.25	0.31
Slc44a2	CTL2	choline_transporter-like_protein_2_isoform_1	Choline	0.05	5.17	1.3E-04
Slc44a3	CTL3	choline_transporter-like_protein_3	Choline	2	1.14	0.66
Slc44a4	CTL4, NG22	choline_transporter-like_protein_4	Choline	0	2.11	0.74
Slc44a5	CTL5	choline_transporter-like_protein_5	Choline	0	not detected	
Slc46a1	HCP1, Pcf	proton-coupled_folate_transporter	folate	22	0.88	0.58
Slc5a7	CHT1	high_affinity_choline_transporter_1	Choline	0	not detected	

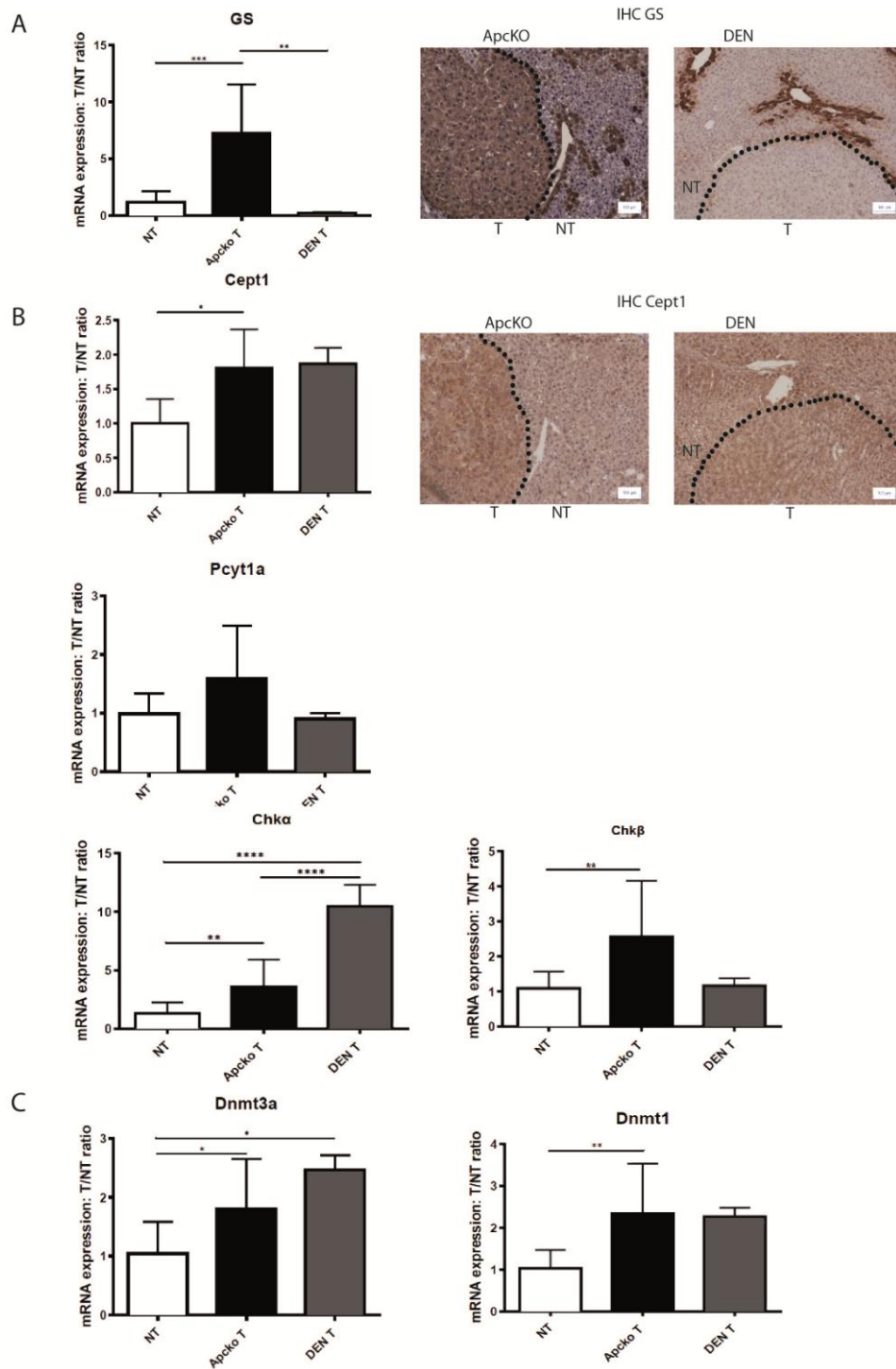


FIGURE S1 : (A) *Apc*^{ko} and DEN-induced tumors show (A) respectively a β -catenin activation or not, according to the expression of GS, and to its protein accumulation revealed by immunostaining. (B) Expression of enzymes from the Kennedy pathway : *Cept1*, *Chk α* and β are significantly induced in *Apc*^{ko} tumors compared controls. *Cept1* protein accumulates only in *Apc*^{ko} and not in DEN tumors. *Pcyt1 α* shows a trend to increase but is not significant. A global trend of increased expression also occurs in DEN tumors. (C) Expression of the DNA methyltransferases : In both DEN-induced and *Apc*^{ko} tumors, they have an increased expression, in comparison to controls.

AXIS 4. In order to better identify molecular mechanisms of β -catenin-dependent liver carcinogenesis, the laboratory studied the role of the micro-RNAs in cancer onset and progression, with the aim to also identify new therapeutic targets. The sequencing of miRNAs was performed in control and APC knock-out mouse hepatocytes to detect differentially-regulated miRNAs. One of these, miR-34a, was specifically increased in APC knock-outs. This increase correlated with a decrease in Hnf4 α expression, both at mRNA and protein level. Moreover the level of caspase-2 was also decreased in these mice. Strong of these results and knowing from already described axis 1 and 2 that Hnf4 α could influence liver tumorigenesis, a therapeutic approach was tested. A locked nucleic acid directed against miR-34a, LNA-34a, was used to specifically block miR-34a expression in mice bearing APC knock-out tumours. Results are very encouraging as LNA-34a is able to block miR-34a, consequently re-establishing Hnf4 α level and so reducing proliferation (through cyclin D1 decreased expression) and increasing apoptosis, through an increase in caspase-2 level. All these effects are able to reduce not only tumour progression, but also its onset.

This is a very useful result that could be used for the treatment of β -catenin mutated liver tumours in humans.

However, this treatment should be specific for β -catenin mutated HCCs, so their detection by FCh/PET would be a pre-requisite for this approach.

My contribution to this work was done during my M2 and the beginning of my thesis. I generated the compound mice with a hepatospecific and tamoxifen-inducible loss of both APC and Hnf4 α , and characterised them at the molecular and phenotypical levels. This contribution was important for the article, but only marginal for my thesis and that is way the article is in the annexes in this manuscript.

Discussion

My PhD was focused on two main topics, both strongly related to β -catenin functions in this organ.

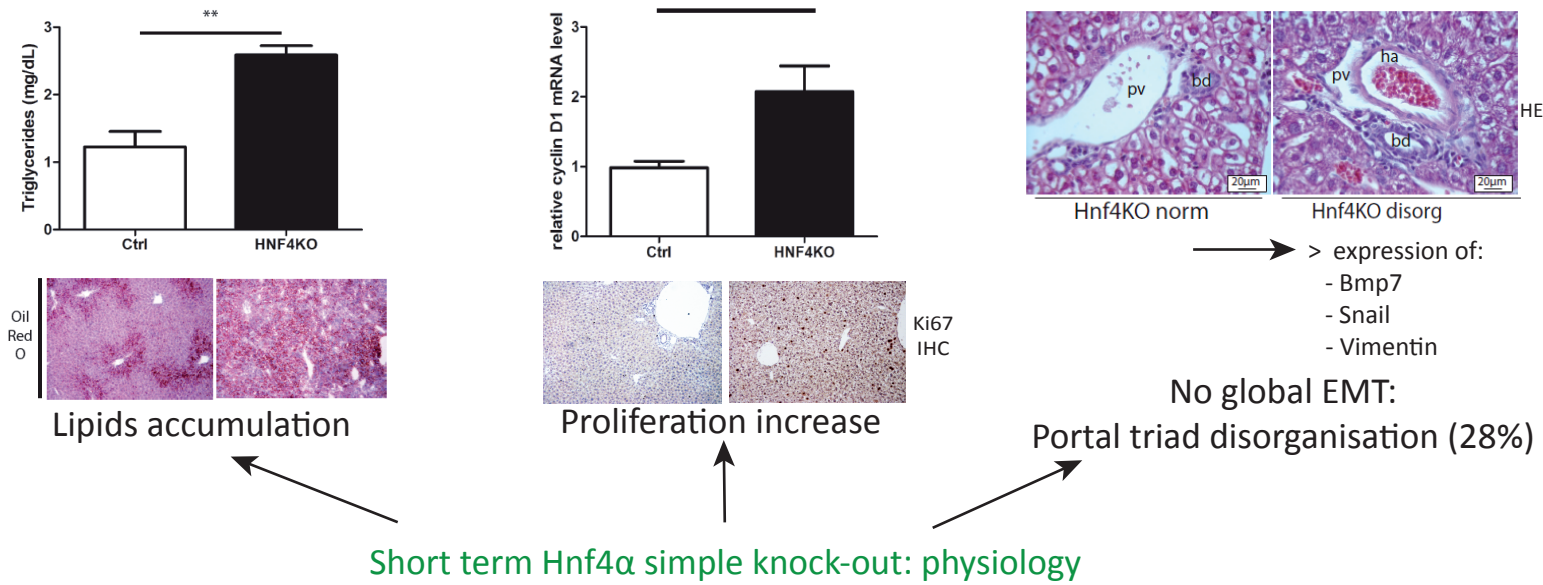
(1) The first main line considered Hnf4 α role in the liver, *per se* and in relation to WNT signalling pathway, both in healthy liver and during a carcinogenesis process.

The results are summarised in the following Graphical Abstract #1.

(2) The second one was focused on β -catenin-activated hepatocellular carcinoma. In particular I studied a peculiar metabolic change in these tumours, the choline pathway addiction, and its exploitation for genotyping tumours.

The results are summarised in the following Graphical Abstract #2.

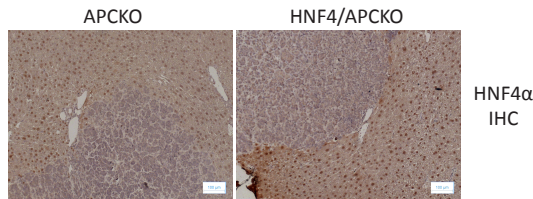
GRAPHICAL ABSTRACT #1



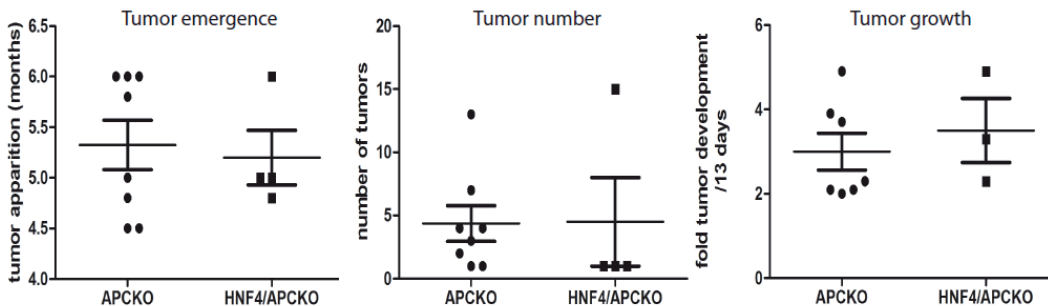
Long term knock-out: Hnf4α role in β-catenin activated tumors

β-catenin activated tumors ↔ β-catenin activated tumors in Hnf4α absence

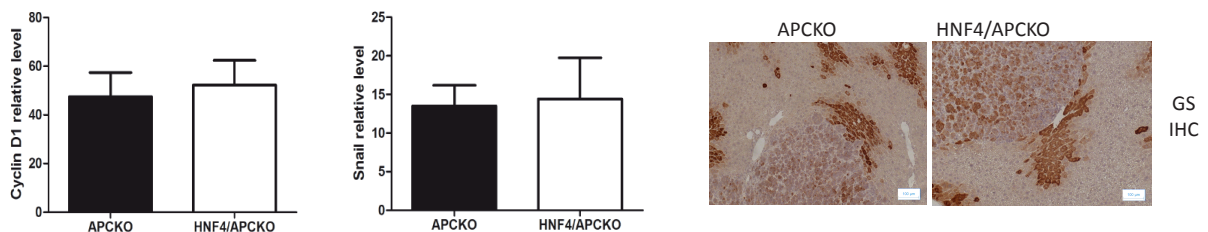
1. Hnf4α expression in tumors: down in Apc KO, absent in HNF4/APC KO



2. Tumor onset and growth do not change



3. Tumors phenotype is identical



1.Hnf4 α in the liver: from liver homeostasis maintenance to liver carcinogenesis

1.1 Hnf4 α and β -catenin negative crosstalk

Hnf4 α is a fundamental transcription factor in the liver, able to control the expression of many liver-specific genes (Odom et al., 2004). Our team previous results, published in 2014 in *Hepatology* (article inserted in annexes section), underlined an antagonistic crosstalk between Hnf4 α and β -catenin in their transcriptional activity (Figure 27) (Gougelet et al., 2014).

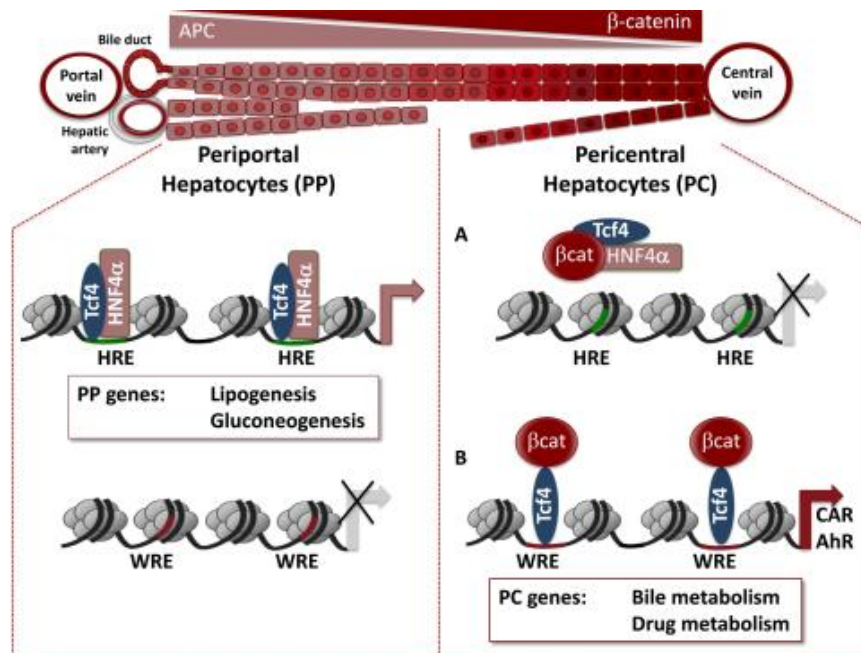


Figure 27. β -catenin in regulating Tcf4 chromatin occupancy determines metabolic zonation.

β -catenin and APC expression are at the top of the picture. In periportal hepatocytes, where β -catenin signalling is absent, Tcf4 can associate with Hnf4 α and bind to the Hnf4 α Responsive Elements (HREs), inducing periportal genes expression. In pericentral hepatocytes, where β -catenin is activated, (1) it allows the Tcf4 binding on WNT Responsive Elements (WRE), inducing pericentral genes expression and (2)titrates Hnf4 α to avoid its binding to HRE and thus inhibits the expression of PP program. (A) β -catenin repressed-genes, (B) β -catenin induced-genes (Berasain and Avila, 2014).

These results are in line with those of Cattin *et al.*, in 2009 that showed a negative relationship between the two signalling pathways. In the case of Hnf4 α loss, WNT/ β -catenin signalling was more active in intestinal epithelial cells (Cattin *et al.*, 2009). Furthermore the laboratory results demonstrating a direct binding of Tcf4 on the HREs, as shown in the figure, have been confirmed elsewhere (Ning *et al.*, 2014; Yang *et al.*, 2013).

We decided to take advantage of the liver-specific conditional knock-out model (Hayhurst *et al.*, 2001) to investigate *in vivo* the physiological role of this presume negative crosstalk with β -catenin. I used the simple knock-out of APC as a comparison, assuming that in both models a negative regulator of β -catenin was removed. The more obvious common phenotype is that the GS expression expanded in the lobule (instead of being only in pericentral areas), but the effect was not as strong as in APC knock-outs. This result is in accord with Stanulović *et al.* study (Stanulović *et al.*, 2007).

From our study, Hnf4 α appears as a minor actor in the metabolic zonation, in comparison to the effect of β -catenin in controlling this phenomenon. Moreover *in vivo* the Hnf4 α and β -catenin antagonism has a more complex dynamics than that hypothesised.

1.2 Hnf4 α in the Epithelial Mesenchymal Transition (EMT)

During liver development, E-cadherin expression is controlled by Hnf4 α , as Hnf4 α null mice do not pass gastrulation phase, as a consequence of a lack of E-cadherin (Parviz *et al.*, 2003). E-cadherin is necessary to cell adhesion maintenance and a switch from E-cadherin to N-cadherin plays a crucial role in the EMT, a process involved in cancer aggressiveness. This is for example the case for the extrahepatic cholangiocarcinoma, as shown by Araki *et al.* (Araki *et al.*, 2011). A role of Hnf4 α in controlling the EMT involved genes has been shown by Santangelo *et al.* in cell culture model and also in liver cancer in the articles of Ning *et al.* and Yang *et al.* (Ning *et al.*, 2014; Yang *et al.*, 2013).

However we showed that mice with Hnf4 α loss did not have a massive parenchyma disorganization and EMT. In particular, they keep an intact E-cadherin immunostaining, like controls.

Remarkably a percentage of animals (28% of Hnf4 α knock-outs) presented a characteristic phenotype: a portal triad disorganisation (see in the graphical abstract at the top), with an incomplete penetrance onset. The portal triads present a strong stenosis of the portal vein, and an increase in the hepatic artery branches and bile ducts.

Furthermore in the Hnf4 α disorganised knock-outs the expression of vimentin, snail and bmp7 is increased, both in comparison to control mice and to Hnf4 α not disorganised knock-outs.

An increased in the expression in Snail family genes (Snail and slug) is known to be associated with the EMT, as these factors are able to inhibit the E-cadherin and so to let the EMT process happen (Cano et al., 2000).

These findings of the literature suggest that Hnf4 α loss leads to EMT in embryonic stage or in *in vitro* experiments on hepatoma cells. But our results show that *in vivo* the situation is more complex, as Hnf4 α loss has an action limited to the portal area. Moreover the incomplete penetrance of this phenomenon suggests that this action is dependent on other factors that have not yet been depicted that do not act through an E-cadherin change.

The portal triad disorganisation could be related to different molecular mechanisms: we can hypothesize (1) an EMT; on the other hand (2) it is possible also that the epithelial cell deficient in Hnf4 α , is producing some different signals, able to induce an Endothelial to Mesenchymal Transition (EndMT) in surrounding cells. (3) The observation that in periportal disorganised areas there is an increased in biliary ducts suggests another hypothesis, which is the possibility of a transdifferentiation of the hepatocytes that have lost Hnf4 α , into a biliary fate. This would be in accord with the fact that during development Hnf4 α signalling is the determinant of the hepatocyte phenotype, whereas its absence triggers a cholangiocyte fate (Figure 6).

A possible element to investigate the second hypothesis is the eventual involvement of miR-122, which plays an active role in the heart EndMT (Ghosh et al., 2012) and is decreased in Hnf4 α knock-outs livers.

Previously we discussed about the fact that this local phenotype is influenced not only by the loss of Hnf4 α , but also by others factors, not already discovered. One possible hypothesis is the involvement of the Tgf β pathway that plays a crucial role in EMT process. Indeed it is known that, during development, Tgf β directs EMT process, and, during cancer-associated EMT, it plays a role in inducing EMT process. Its action is mediated by smads proteins (the phosphorylated form of Smad2/3) that are able to activate transcriptionally the effectors of EMT process (Derynck et al., 2014, for review).

Snail family of transcription repressors are such effectors stimulating the EMT process, and Cano *et al.* demonstrated that the overexpression of Snail is able to induce a strong transition in phenotype, in concomitance with mesenchymal genes expression and loss of E-cadherin expression (Cano et al., 2000).

Moreover in the review of van Meeteren *et al.* the molecular mechanism underlying EndMT induced by Tgf β is described: there is first a Tgf β -dependent activation of Smads proteins and other involved pathways, which leads to the upregulation of Snail family of transcription repressors that are able to finally change gene expression, inducing the EndMT. It is also important that the upregulation of Snail factors has to be concomitant to the inhibition of their inhibitor, GSK-3 β (van Meeteren and ten Dijke, 2012).

Interestingly in literature a human pathology, the idiopathic portal hypertension has the same phenotype found in a subset of Hnf4 α knock-outs: Kitao *et al.* demonstrating that in the cells around the portal space there is an increased staining of P-smad2, the smad that, with the smad3, when phosphorylated, mediates Tgf β signalling (instead of smad1/5/8 that mediate Bmp7 signalling). Moreover an increased of Tgf β in patients serum, which inversely correlates with Bmp7 in serum was found (Kitao et al., 2009).

Also in Araki *et al.* article, the mediated E/N-cadherin switch that stimulates the EMT is related to the Tgf β signalling activation (Araki et al., 2011).

As in our experimental setting, I found Bmp7 to be overexpressed, but not Tgf β , I tested by immunohistochemistry the antibody against Smad4, the common mediator between Tgf β and Bmp7 signalling, and against Smad 1/5/8, specific for Bmp7 signalling. With this approach I was not able to demonstrate neither Tgf β nor Bmp7 involvement. It would be very interesting to further investigate this aspect, for example by measuring in mice serum the levels of Tgf β and Bmp7, and correlate them to portal space alterations. Another approach would be the laser microdissection that allows focusing the analysis on this specific area of the portal trias. After RNA extraction from microdissected portal triads (using paraffin embedded tissue), it would be possible to test the expression of direct Tgf β targets in a local way.

Also the WNT/ β -catenin signalling plays a role in the EMT, during embryonic development and also in some pathologies (like diabetic nephropathy and carcinomas) (Derynck et al., 2014). Moreover Tgf β /Bmp and WNTs signals have been shown to cooperate in some EMT process, like the cardiac development (Song et al., 2013) or in mammary epithelial cells and carcinomas, WNTs signals are cooperating with Tgf β in inducing EMT (Scheel et al., 2011).

In our case Hnf4 α loss is inducing the GS expression, suggesting a mild activation of β -catenin signalling in periportal area. An opened question is if this effect on β -catenin signalling can play a role in the EMT process, both in a Tgf β -dependent or -independent way.

1.3 Is Hnf4 α alone a tumour-suppressor gene?

The functions regulated by Hnf4 α (EMT, cell proliferation and differentiation) let us suppose that Hnf4 α could be a tumour suppressor gene in the liver.

Moreover, the Hnf4 α knock-outs have lipid accumulation in the liver parenchyma. In humans, lipid accumulation induces steatohepatitis that, after a while, provokes a fibrotic state of liver, which often degenerates into cirrhosis (Browning et al., 2004; Ekstedt et al., 2006; Farazi and DePinho, 2006; French et al., 1993; Kleiner et al., Nonalcoholic Steatohepatitis Clinical Research Network, 2005; Paradis et al., 2009; Zakhari and Li, 2007).

Furthermore, in literature many studies demonstrated that Hnf4 α , in some way, acts as a tumour suppressor, both in human HCC and in rodent models. Indeed, its absence correlates to cancer aggressiveness, and re-establishing its level delays tumour onset and growth (Flodby et al., 1995; Gougelet et al., 2014; Kalkuhl et al., 1996; Ning et al., 2014; Walesky et al., 2013; Yin et al., 2008).

In my PhD I explored the role of Hnf4 α loss in inducing cancer onset. In my cohort of animals, only one Hnf4 α panlobular knock-out mouse showed some nodules, but no HCC was found. This result lets us conclude that the absence of this factor cannot play a role *per se* in causing tumour onset, but rather needs additional genetic events to become oncogenic.

In their last study, Schulze *et al.* performed whole-exome sequencing in a cohort of 243 HCCs and did not find any Hnf4 α mutation, supporting our conclusions (Schulze et al., 2015).

1.4 Hnf4 α tumour-suppressor role in β -catenin carcinogenesis

Hnf4 α tumour-suppressor role had previously been tested in a context of liver tumourigenesis induced by DEN, which is a model of cancer onset not dependent of β -catenin (Ning et al., 2010, 2014; Walesky et al., 2013). That is why we focused our study on the Hnf4 α tumour-suppressor role in a context of β -catenin activated HCCs.

When APC and APC/Hnf4 α knock-outs were done in single hepatocytes, I did not found any differences in tumour emergence, in the total number of tumours per mouse and in their growth (first graphical abstract, in the bottom part). All the tumours showed a strong decrease in Hnf4 α expression, and that confirmed previous experiments performed in our laboratory, where simple APC knock-out tumours have a low Hnf4 α expression (Gougelet et al., 2015). GS, Snail and Cyclin D1 expression were not significantly different in both mouse model tumours, but in β -catenin simple tumours, Snail and Cyclin D1 levels were inversely-correlated with Hnf4 α expression.

From our findings and those of the literature, the following scenario can be hypothesized: (1) firstly oncogenic β -catenin is able to regulate Snail expression at a level that is sufficient to decrease Hnf4 α expression (our results, and Yang et al., 2013). (2) This decreased in Hnf4 α expression is sufficient to elicit an oncogenic effect. This is supported by the fact that the re-expression of Hnf4 α has been found to be anti-tumoral (Ning et al., 2014); (3) This latter conclusion is based on our results showing that a concomitant loss of APC and Hnf4 α is not influencing the tumour onset, in comparison to simple APC loss.

1.5 Restoring Hnf4 α levels for cancer therapies

Hnf4 α is not a tumour suppressor gene alone. But our results and those of the literature suggest that a just-right dose of Hnf4 α is required to render it anti-tumoral when oncogenic mutations occur.

Indeed, in DEN-dependent cancer onset, Hnf4 α expression is decreased during tumour growth, and its re-expression is preventing nodules formation (Ning et al., 2014).

In β -catenin-dependent cancer, our results show that it is not playing a role of classical tumour suppressor gene, but it is also decreased during the tumoral process. Similarly to DEN model, the laboratory shows that a re-expression of Hnf4 α through LNA-34a treatment during this kind of tumourigenesis is very useful to decrease tumour proliferation and the onset of new tumours in the same liver (Gougelet et al., 2015).

Different features could explain how Hnf4 α could be a tumour suppressor. Hnf4 α decreased expression in HCCs is associated with EMT and high proliferation (Ning et al., 2014). Moreover Hnf4 α is able to participate to the control of inflammation, in particular Hnf4 α blocks interleukin 6 Receptor (IL6R), through transcriptional activation of miR-124. In HCC progression Katziapostoulou *et al.* demonstrated that a feedback loop is established, allowing the IL6R expression and the Hnf4 α repression (Hatziapostolou et al., 2011).

Lastly, the results of the team have provided an emerging targeted therapy for β -catenin-dependent HCCs, by the use of LNA-34a, which is partially dependent on Hnf4 α restored expression (Gougelet et al., 2015). This proof-of-concept study showed that LNA-34a (miR-34a inhibitor) is able to block β -catenin-dependent HCCs in two ways: (1) a Hnf4 α -independent way, by inducing a proapoptotic program through the activation of caspase2/caspase3 cascade; (2) a Hnf4 α dependent one, blocking cell proliferation, through the mediation of Hnf4 α in blocking Cyclin D1 (Gougelet et al., 2015).

It will be very interesting to test in clinical trials on patients affected by β -catenin-dependent HCCs, the LNA-34a strategy, due to the promising results in the mice.

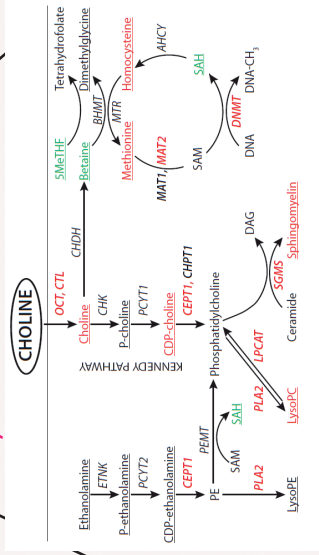
GRAPHICAL
ABSTRACT #2

β -catenin-activated
hepatocyte

CHOLINE

CHOLINE

2) Both USEs increase:



Phosphatidylcholine
(PC) production

Bile

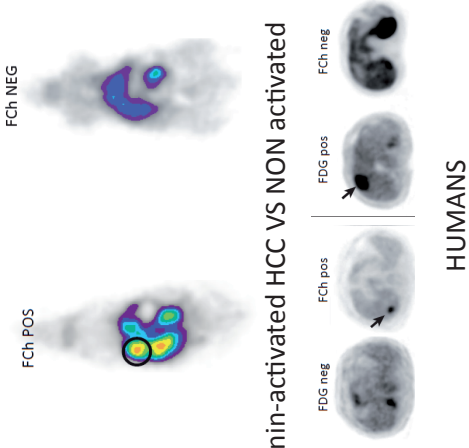
Phospholipids

Plasma Membrane
Proliferation

Secreted phospholipids
Tumor explants

MICE

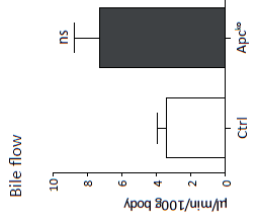
1) UPTAKE increase:
Fch PET



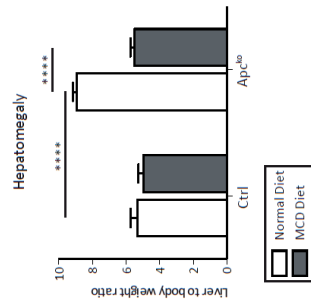
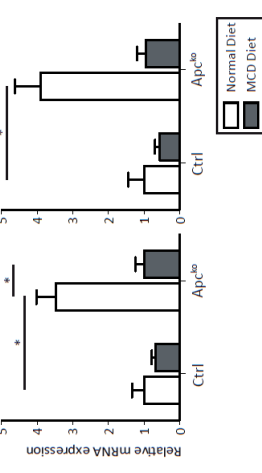
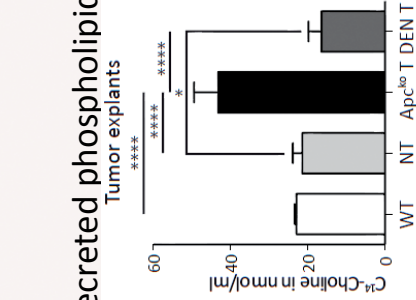
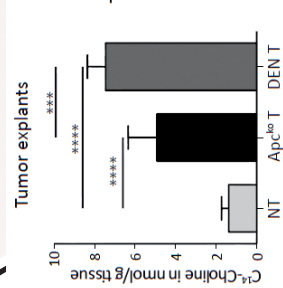
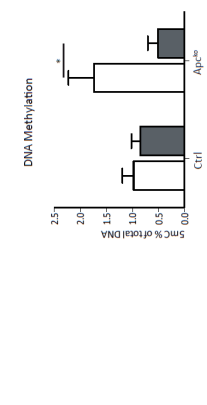
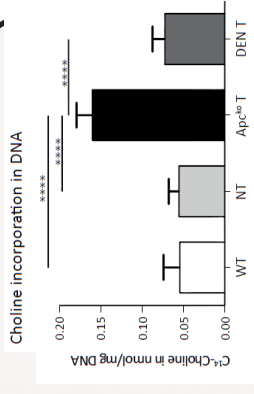
β -catenin-activated HCC VS NON activated

HUMANS

3) Phenotype rescue
by MCD diet:



Methyl group
donor



3) Phenotype rescue
by MCD diet:

Secreted phospholipids
Tumor explants

Plasma Membrane
Proliferation

3) Phenotype rescue
by MCD diet:

2. Choline metabolism in β -catenin-dependent tumour

2.1 β -catenin HCCs have a peculiar metabolic phenotype

There are three main arguments showing that *CTNNB1*-mut hepatocellular carcinoma, constituting approximately one third of the HCCs, is a homogenous group of tumours displaying a particular metabolism.

(1) *CTNNB1*-mut human HCCs are distinguished from *CTNNB1*-wt ones by their GS immunostaining, but also by their cholestasis, by their better differentiation associated with a conserved functional metabolism of adult hepatocytes. They are never steatotic contrary to 30% of *CTNNB1*-wt HCCs (Audard et al., 2007).

(2) These tumours have a specific transcriptome, because β -catenin plays a role of transcription factor. This feature is found both in human β -catenin-mutated HCCs and in *Apc^{ko}* model of β -catenin activation. This distinct transcriptome is reminiscent of that expressed by pericentral hepatocytes (overexpressing GS and other metabolic enzymes). At least for this reason β -catenin dictates to the hepatocyte a metabolic signature that we have been able to reveal, thanks to our metabolic approaches (Benhamouche et al., 2006; Gougelet et al., 2014). This metabolic signature concerns four metabolic pathways: the anabolism of glutamine, the bile acids synthesis, the lipogenic pathway decrease, the choline pathway increase (our current study).

(3) β -catenin-activated livers have no clear Warburg effect. In previous study, we had shown that β -catenin was repressing gluconeogenesis, while enhancing glycolysis. But this glycolysis is not accompanied by an increase in fluorodeoxyglucose uptake (as assessed by FDG-PET analyses) (Chafey et al., 2009). This was at the basis of our current work, and it has been elegantly assessed by Yuneva *et al.*, showing that *MYC* -induced liver tumours were typical Warburg tumours and they seem to be β -catenin-independent (Yuneva et al., 2012). On the other hand, *MET*-induced tumours overexpress

GS, and for this reason they are expected to be β -catenin-activated ones. *MET*-tumours present a low Warburg effect and are characterised by an accumulation of glutamine. These tumours are able to fuel their tumoral energetic requirements in a different way than *MYC* ones, and we can hypothesise, from our results, that the addiction to choline of β -catenin-activated tumours could be a way to ensure a proper proliferation.

2.2 Is β -catenin directly controlling choline metabolism?

The role of β -catenin as a transcription cofactor may suggest that the enzymes and transporters of choline metabolism could be direct targets of β -catenin. In this case the gene regulation pathways should be conserved between human and mice liver tumours.

(1) Which is the choline transporter used for choline uptake?

The mRNA-Seq showed an increased expression of two classes of transporters: OCT and CTL. In particular I have confirmed the increase of expression of OCT1 (*Slc22A1*) by qPCR in mice, but not in humans. This results is not so surprising because choline is transported by the OCT group of transporters through a passive transport. The speed and direction of this transport are dependent only on choline concentration in the cell and in extracellular space. Therefore OCT expression is not a limiting step for choline uptake. Moreover, it has also been described that choline could be transported by facilitated diffusion across the membrane. For this reason, the need for a specific transporter is not established (Bieze et al., 2014).

(2) Are the enzymes of the Kennedy pathway β -catenin targets?

In the APC “pre-tumoral” model we found that the expression of CEPT1 was induced. qPCR approach demonstrated that the situation is complicated in tumours: in β -catenin-activated ones there is a significant increase of expression of *Cept1*, *Chk β* and *Chk α* . But also in DEN tumours there is an

increase in these genes expression, and this is not so different than in β -catenin-activated ones.

In the case of CEPT1 and Pcyt1 α , a IHC approach was tested, and even if there is a trend for β -catenin-activated tumours to have more staining of these two enzymes in the tumours, the situation is not clear. In DEN tumours the staining is not different between the tumour and the non-tumoral adjacent tissue. Moreover, in human tumours, the results are different than in mice. The expression of Chk α and CEPT1 are significantly induced in β -catenin-activated tumours. Pcyt1 α expression shows a trend to increase in our samples, even if in microarray study in a cohort of patients affected by both adenoma and carcinoma it is found induced specifically in β -catenin-activated carcinoma. IHC approaches for CEPT1 and Pcyt1 α were tested in human tumours but the results did not shown a clear association between a positive IHC and β -catenin-activated tumours.

(3) Are the enzymes of the Methionine and the DNA methylation pathway targets of β -catenin?

In the transcriptome of APC knock-outs pre tumoral livers, Dnmt1 and Dnmt3a DNA methyltransferase expressions were increased. Dnmt1 has already been described as a direct target of WNT/ β -catenin (Campbell and Szyf, 2003), but the expression of both DnmTs is induced also in DEN-derived tumours. This is consistent with the frequent induction of DnmTs during carcinogenesis, whatever their origin. MAT2a and MAT2b are also increased. They are methionine adenosyltransferases enzymes, allowing the synthesis of the SAM metabolite, the critical methyl group donor. MAT2a and MAT2b, contrary to MAT1 are frequently overexpressed in liver cancers, as they allow a permanent recycling of homocysteine to methionine in order to ensure an efficient protein synthesis (Mato and Lu, 2011).

To summarise, both results in Kennedy pathway and methyl-group donor one depict the complex situation *in vivo*. Furthermore, for all the genes concerned, we controlled our ChIP-Seq data, and we did not find a direct binding of β -catenin and/or its DNA-binding partner Tcf4 in their promoter.

Thus, the molecular way to reach the increase in choline metabolism seems complex, different in mice and humans, with no evidence for an involvement of β -catenin as a direct transcriptional regulator for the genes concerned.

2.3 Functional consequences of choline pathway deregulation

The hyperactivation of β -catenin have functional consequences in hepatocytes, due to this choline pathway addiction. In particular thanks to the Methionine Choline Deficient (MCD) diet we were able to analyse different aspects.

(1) Membrane phospholipids production to sustain cell proliferation.

As previously anticipated choline can give rise to phosphatidylcholine that is the main component of the plasma membrane phospholipids. The need for membrane formation is essential during proliferation processes. APC knock-outs pre-tumoral livers have an increased proliferation. Thanks to radiolabelled ^{14}C -choline we demonstrated that in β -catenin-activated hepatocytes there are more phospholipids derived from choline, than in normal hepatocytes. This increased proliferation of APC knock-outs hepatocytes is reflected by a dramatic hepatomegaly 7 days (D7) after Tamoxifen-induced Apc inactivation that has already been described before (Benhamouche et al., 2006; Colnot et al., 2004). The hepatomegaly is not present if animals are fed with MCD diet, suggesting that choline is necessary to support the increase in proliferation of β -catenin-activated hepatocytes. However, the staining of Ki67 protein, a proliferative marker, was high in APC knock-out hepatocytes at D7, whatever the diet. This failure to link the absence of hepatomegaly in choline-deficient diet with a decreased proliferation can be technical. Indeed the limit of this experiment was the timing, because the Ki67 staining was tested at the end of the protocol, whereas the peak of APC knock-out hepatocytes proliferation is maximal at day 4, as previously demonstrated by the team (Benhamouche et al., 2006).

Further investigation has to be done at this peculiar time, to see if the MCD diet is really able to decrease the proliferation rate in APC knock-out livers at this time point.

Furthermore another hypothesis could be that the absence of the hepatomegaly phenotype does not occur by decreasing the proliferation, but by the production of smaller hepatocytes, in the APC knock-outs mice under MCD diet, in comparison to APC knock-outs mice under normal diet. This hypothesis is of interest, because betaine, a metabolite generated by choline oxidation and which is less abundant in *Apc^{ko}* livers, is an osmolyte regulator controlling hepatocellular hydration (Hoffmann et al., 2013). Therefore, we measured the mean size of the hepatocytes in each condition, but there were no significant difference linked to mice diets.

By the use of radiolabelled ¹⁴C-choline the increased use of choline to produce intracellular phospholipids was underlined both in β -catenin activated tumours and DEN-derived ones. We hypothesize that it is due to the high rate of proliferation of DEN-derived tumours that need a big amount of phospholipids to produce new plasma membranes. Therefore the use of choline for producing membrane phospholipids seems to be a phenotype linked more to the tumours proliferation, than to the β -catenin status.

(2) Phosphatidylcholine in secreted lipids: bile or VLDL production?

Phosphatidylcholine can also be secreted. In this case, there are two possibilities. It can participate to the formation of the VLDL membrane, and be secreted with the VLDL. As they are also a major constituent of the bile, the other option is their secretion into the bile, in order to increase bile acids solubility (Figure 28).

By performing fluxes experiments with ¹⁴C-choline we demonstrated that in β -catenin activated hepatocytes there is an increased use of choline to produce secreted phospholipids.

In comparison to normal liver or adjacent healthy tissue both β -catenin activated hepatocytes and tumours have an increased amount of secreted

phospholipids derived from ^{14}C -choline. This effect is specific for β -catenin activated tumours, because it is not the case for DEN-induced ones.

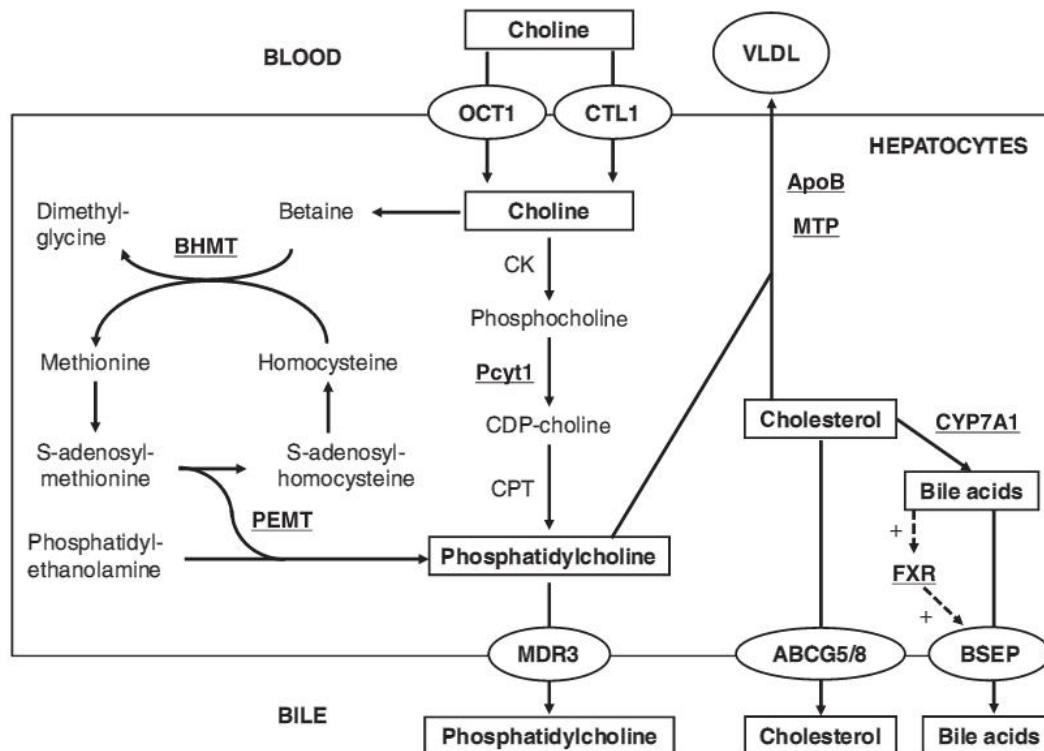


Figure 28. Choline/phosphatidylcholine metabolism.
(Kohjima et al., 2015)

In the laboratory we demonstrated that in the APC knock-outs there are less VLDLs released in the blood circulation (Gougelet et al., 2014). The hypothesis that phospholipids neosynthesised from choline would be used to form VLDLs is therefore infirmed by this results. On the contrary in APC knock-outs there is an increase in bile acids production, and β -catenin is indeed able to directly control the expression of Cyp27a1 and CAR and indirectly the expression of Cyp7a1 (Gougelet et al., 2014).

In the literature the work of Yeh *et al.* demonstrated that β -catenin knock-outs have a reduced bile flow, and the bile composition is different from the controls. They pointed towards the link between active β -catenin and a metabolic phenotype, proving β -catenin capacity, in the liver, to stimulate

cholesterol and bile metabolisms and to slow down lipids one (Yeh et al., 2010).

The transcriptome of β -catenin activated hepatocytes shows an increased expression of the PC-specific flippase (MDR2 in mice, and MDR3 in humans) able to secrete the PC in the bile. However, I was not able to confirm this increased expression in our murine model of HCC, and MDR3 does not change in human HCCs, whatever their mutational status. Nevertheless, the bile flow in these mice has a trend to increase, even if the cohort of mice is too small to be significant. It will be very important to enlarge the cohort of animals and moreover to analyse the bile composition in bile acids and in phospholipids, in APC knock-out mouse liver.

These additional analysis will be able to support, or not, our hypothesis that in β -catenin activated hepatocytes choline is re-directed to produce a sufficient amount of phosphatidylcholine, necessary to sustain the increased bile production.

This would be consistent with the cholestasis present in β -catenin activated HCC subgroup.

(3) Methyl-group donor for DNA methylation.

Choline can be oxidised into betaine to give rise to methionine and then to S-adenosylmethionine, which is the main methyl group donor in the cells. We found in APC pre-tumoral knock-out livers an increase in DNA methylation on cytosines. By performing fluxes with ^{14}C -choline we demonstrated that, in β -catenin-activated tumours, DNA methylation derived from the radiolabelled methyl group of ^{14}C -choline was three-fold higher than in healthy tissue or in wild-type animals. In DEN-derived tumours there was no increase, demonstrating that this is an effect specific for β -catenin-activated tumours. Moreover if APC pre tumoral animals are fed with a MCD diet this hypermethylation phenotype is not present. These data prove that the choline from diet is the fundamental methyl-group donor to sustain this hypermethylation in β -catenin-activated hepatocytes.

In the last years many studies focused on the “epigenomics of cancer”, considering that epigenetic changes can modulate gene expression, helping

cancer onset and growth. Indeed a deregulation of epigenetic mechanisms is playing an active role together with the genetic alterations in tumour pathogenesis (Jones, 2013).

Moreover HCC is a cancer that shows an accumulation of genetic mutations and epigenetic alterations. A crescent number of studies evaluate the levels of the two proteins responsible for, respectively, maintenance and *de novo* DNA methylation, DNMT1 and DNMT3a. They were found increased in HCC, in comparison to healthy tissue, and their increase correlated with HCC progression (Choi et al., 2003; Park et al., 2006; Saito et al., 2003). Calvisi *et al.* in their study showed a crescent genome hypomethylation in the HCCs, but an increase in local CpG hypermethylation (Calvisi et al., 2007). More recently studies correlate this methylation pattern with the different aetiologies (Neumann et al., 2012), and furthermore Nishida *et al.* found a specific association between in β -catenin mutated HCCs and a hypermethylation in precises loci (Nishida et al., 2007).

During HCC progression, it is important to study DNA methylation both as a tool to determine the outcome of the patient, but also to better understand the molecular mechanism underlying cancer onset. DNA hypermethylation is associated with either gene repression or transcription: if it is present in the gene promoter, it is associated with gene inactivation, instead a hypermethylation in the gene body is positively-correlated with gene expression (Yang et al., 2014). In our current state of knowledge we do not know if the choline is used by β -catenin-activated hepatocytes to methylate genes as a marker of repression or activation. It will be really instructive to analyse how this change in DNA methylation is able to participate to the oncogenic process in β -catenin-activated HCCs. The goal is to identify, by differential methylome analysis, which are the hypermethylated targets of β -catenin, and if they can play a role in liver tumourigenesis.

It is a very promising field of study also because some drugs, as 5-azacytidine and 5-aza-2'-deoxycytidine, are available for chemotherapy, even if these specific DNMTs inhibitors are basically used for leukemia treatment and not for solid tumours (Ghoshal and Bai, 2007).

Methylation changes could also occur on histones, the DNA-packaging proteins, to influence gene expression. It will be interesting to analyse if also the histone methylation is changed in APC knock-out mice and if this is correlated with the choline supply, both in APC pan-lobular knock-outs and APC knock-out derived tumours.

2.4 Differential diagnosis of β -catenin activated HCCs and perspectives

Our study has revealed that choline addiction in HCC is associated with β -catenin activated tumours, both in humans and mice.

β -catenin-activated tumours (in both organisms) in PET analysis fix specifically F-choline in the tumour, and they do not fix FDG. β -catenin-activated tumours are more avid in choline uptake, than the surrounding healthy tissue. On the contrary the other subgroup of HCCs does not fix the F-choline, demonstrating that F-choline PET could be use as a tool to perform a differential diagnosis between HCC subgroups.

β -catenin-activated tumours are very different from the other HCCs, like anticipated, and for this reason, we actually think that they should be treated with different and specific tools. For instance, as described in the section of the discussion about Hnf4 α , our team developed a very promising therapy, based on the administration of LNA-34a, able to block the tumour growth, in the case of HCC with β -catenin activation (Gougelet et al., 2015). Moreover Evason *et al.* recently discover the effect of some antidepressants (involved in the serotonin signalling) able to decrease the hepatomegaly consequent to β -catenin activation in the liver, and also in decreasing the tumour growth in *MET*-derived tumours (with β -catenin activation) in Zebrafish model (Evason et al., 2015).

We are therefore optimistic that in the near future β -catenin targeted therapies could be efficient to treat HCCs. Our present study will be essential, as FCH-PET screening will be able to diagnose early and in a non-invasive way β -catenin-activated HCCs that will be then treated with these targeted therapies.

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Annexes

1) Résumé:

La voie de signalisation WNT/ β -caténine est impliquée dans de nombreuses processus cellulaires, du développement à la physiologie. Dans le foie adulte, elle est nécessaire pour établir et maintenir la zonation métabolique, condition préalable pour la fonctionnalité de l'organe, mais elle est aussi cause d'un pourcentage non négligeable (11-32%) de carcinomes hépatocellulaires (CHC), qui surviennent après mutation activatrice du gène codant la β -caténine.

Mes travaux se sont inscrits dans la continuité de travaux de l'équipe auxquels j'ai participé, et ont eu pour principaux objectifs : (1) d'explorer le rôle du facteur de transcription Hnf4 α dans la physiologie et les cancers du foie, en lien avec la signalisation β -caténine ; (2) de déterminer si une imagerie tumorale par tomographie par émission de positrons (TEP) spécifique de la captation de choline pouvait prédire les CHC mutés pour la β -caténine et si le métabolisme de la choline pouvait présenter une piste thérapeutique des cancers du foie.

Pour ces deux projets, j'ai eu accès à des cohortes de patients atteints de cancers du foie, mais j'ai également pu bénéficier du modèle gain-de-fonction de la β -caténine développé au laboratoire, qui consiste en une perte du suppresseur de tumeur Apc, frein majeur de la voie β -caténine conduisant à des cancers du foie.

Grâce à un modèle d'inactivation hépato-spécifique et conditionnelle du gène Hnf4 α , j'ai pu prouver que la perte de Hnf4 α mène à une augmentation de la prolifération, du stockage des lipides dans le foie et à une désorganisation de l'architecture zonale hépatique, en particulier celle de la triade portale. J'ai aussi démontré que dans un contexte de carcinome murin invalidé par Apc, le rôle suppresseur de tumeur d'Hnf4 α était mineur.

Une approche métabolomique avait montré qu'un signal β -caténine perturbait le métabolisme des phospholipides dérivant de la choline. Grâce à une étude parallèle réalisée chez des patients porteurs de CHC d'une part, et dans nos modèles murins d'autre part, nous avons pu mettre en évidence par TEP une fixation accrue de la F-choline dans les tumeurs activées β -caténine. Ce phénotype est spécifique d'une signalisation β -caténine active puisque cette captation accrue n'était pas présente chez les patients porteurs de carcinome hépatocellulaire non mutés ou chez les souris présentant une cancérogenèse indépendante de la β -caténine (modèle DEN). J'ai ensuite étudié le devenir intracellulaire de la choline. En utilisant de la choline radiomarquée j'ai montré *in vitro* qu'un signal β -caténine aberrant accroît l'incorporation de choline dans les phospholipides, et accroît également son rôle de donneur de groupements méthyles, participant à la méthylation de

l'ADN. Cela pourrait expliquer pourquoi l'ADN est hyperméthylé chez les souris avec la perte d'Apc, puisque l'administration d'un régime sans choline et méthionine à ces souris reverse le phénotype d'hyperméthylation.

L'ensemble de ces résultats suggère que la choline pourrait jouer un rôle important dans la cancérogenèse liée à la β -caténine. Nous proposons que des TEP F-choline pourraient être utilisés pour diagnostiquer les CHC mutés β -caténine, et à terme des thérapies ciblées sur ce métabolisme pourraient être envisagées.

2) Synthèse française :

Le foie ; généralités

Le foie adulte, organe le plus volumineux du corps après la peau, est considéré, du fait de son rôle majeur dans l'homéostasie tissulaire, comme un des organes les plus complexes de l'organisme (après le cerveau).

Le foie est constitué de différents types cellulaires dont les hépatocytes, cellules majoritaires puisqu'elles constituent à elles seules 70 à 80% du poids hépatique, et les cholangiocytes.

Ces deux types cellulaires constituent ce que l'on appelle le « parenchyme hépatique » et sont responsables de la plupart des fonctions assurées par le foie, telles que la production de bile, la détoxification des substances toxiques pour l'organisme, ou encore la régulation du métabolisme des lipides, des glucides et des acides aminés. Cependant, le foie est également constitué de cellules non parenchymateuses comme les cellules de Küppfer, les macrophages, les cellules endothéliales, les cellules étoilées, assurant plus spécifiquement des fonctions immunitaires et de vascularisation ou impliquées dans la fibrose hépatique.

Les hépatocytes et les cholangiocytes dérivent d'une cellule progénitrice commune appelée « hépatoblaste ». La différenciation de cet hépatoblaste est très finement régulée par diverses voies de signalisation et différents facteurs de transcription.

En effet, une des voies de régulation majeure est la voie de signalisation WNT/ β -caténine dont l'activation est nécessaire pour favoriser un destin cholangiocytaire. Au contraire, la différenciation de l'hépatoblaste en hépatocyte nécessite non seulement l'absence de l'activation de cette voie mais également la présence d'un facteur de transcription fondamental, contrôlant l'expression de gènes spécifiques déterminants le phénotype « hépatocyte », HNF4 α .

HNF4 α , un régulateur hépatocytaire majeur

HNF4 α est un facteur de transcription exprimé dans le foie, les reins et l'intestin, agissant dans le noyau sous forme d'homodimère. Ce facteur existe sous deux isoformes P1 et P2. Alors que l'isoforme P2 est spécifique de l'hépatoblaste et que son expression diminue de façon continue tout au long du développement, l'isoforme P1 est spécifique de l'hépatocyte. L'inactivation constitutive d'*Hnf4 α* n'est pas envisageable parce que sa perte est létale pendant le développement embryonnaire.

Dans le foie adulte, HNF4 α est capable de réguler la différenciation hépatique, le stockage des lipides au sein de ce tissu, la prolifération ainsi que le maintien d'une structure tissulaire épithéliale. Ainsi, la régulation de ces différentes fonctions telles que le destin cellulaire, le métabolisme et la structure du tissu hépatique, lui confère un rôle de régulateur majeur au sein du foie.

Les premières études s'intéressant à la dérégulation de l'expression génique dans les carcinomes hépatocellulaires, ont permis de mettre en évidence une perte du facteur HNF4 α au sein des tumeurs de patients atteints par ce cancer, lui conférant alors un rôle de suppresseur de tumeur. De plus, cette perte d'expression est associée à un mauvais pronostic.

Des expériences sur des lignées de cellules humaines d'hépatocarcinomes (Hep3B et HepG2) ont montré que la restauration du facteur HNF4 α par la transduction d'un adénovirus exprimant ce facteur de transcription, annihile la capacité migratoire des cellules et favorise leur adhérence.

De plus, chez les rongeurs, d'autres expériences ont montré que la restauration de l'expression d'*Hnf4α* au cours de la cancérogenèse hépatique par administration de Diethylnitrosamine (DEN) a pour conséquence de ralentir la croissance tumorale, tandis que la suppression d'*Hnf4α* après administration de DEN aggravait le phénotype.

Le métabolisme hépatique

Le foie est la glande la plus volumineuse de l'organisme possédant des fonctions à la fois endocrine et exocrine.

Grâce à sa fonction exocrine, le foie, par l'intermédiaire des hépatocytes, va produire la bile ; cette dernière va ensuite être sécrétée et va pouvoir être collectée et concentrée au niveau de la vésicule biliaire qui, à son tour pourra la sécréter au niveau du duodénum après les repas.

En revanche, la capacité du foie d'absorber et d'élaborer par la suite les substances présentes dans la circulation sanguine est, au contraire, une activité endocrine hépatique.

Le foie est également très important dans le métabolisme des carbohydrates. En effet, en réponse aux différentes hormones (insuline et glucagon) synthétisées par le pancréas, le foie va contrôler en particulier la concentration sanguine de glucose.

A l'état nourri (concentration élevée de glucose dans le sang), l'insuline produite est capable d'induire l'expression de différents gènes, qui, dans le foie, vont être responsables de l'utilisation du glucose et aussi de la production de glycogène.

À jeun (concentration basse de glucose dans le sang), dans le foie l'utilisation des stocks de glycogène aura lieu, ainsi que la néoglucogenèse (production *de novo* de glucose).

Le foie est aussi impliqué dans le métabolisme des acides gras. La production d'acides gras est active quand le niveau de glucose sanguin est élevé. Les acides gras peuvent être stockés dans l'organe ou bien être exportés dans la circulation sanguine au sein de macromolécules, les VLDL (very low density

lipoprotéine). Outre leur production, le foie peut également utiliser les acides gras comme source d'énergie, à travers la β -oxydation ou la ω -oxydation quand cette dernière n'est pas efficace.

Le foie est constitué d'unités fonctionnelles microcirculatoires appelées « lobules » ou « *acini* hépatiques ». Le lobule est une structure hexagonale avec au centre une veine centrolobulaire et aux extrémités une triade portale, composée par une branche de la veine porte, de l'artère hépatique et un canal biliaire.

Les hépatocytes se classent en deux sous-groupes : hépatocytes périportaux et péricentraux, en fonction de leur position au sein du lobule. Chaque fonction hépatique en réalité ne peut être accomplie que par l'un des deux groupes d'hépatocytes (spécialisation des cellules).

L'environnement au niveau de l'espace porte (triade portale) est riche en oxygène, nutriments et toxines divers de l'organisme apportés par le sang de l'artère hépatique et de la veine porte, qui ensuite traverse le lobule jusqu'à la veine centrolobulaire *via* les sinusoides (capillaires entre les hépatocytes); ainsi il existe tout au long du lobule hépatique un gradient d'oxygène, de nutriments et de déchets, qui diminue de la triade portale à la veine centrolobulaire. Ce gradient a pour conséquence de restreindre certaines fonctions à un de deux cotés : par exemple la glycolyse se déroule au niveau péricentral alors que la gluconéogenèse est une fonction assurée par l'espace porte. Cette zonation métabolique est à présent bien caractérisée sur le plan moléculaire et met en jeu une voie de signalisation clé dans le métabolisme hépatique, la voie WNT/ β -caténine. En effet, cette voie de signalisation est active au niveau de l'espace péricentral et absente au niveau de l'espace porte, ceci pouvant expliquer les différents programmes métaboliques au sein du tissu hépatique.

L'hépatocarcinome ; une détection et une prise en charge difficiles

Le foie peut être sujet à différentes pathologies, pouvant affectées les cholangiocytes (ex : ductopenie, ciliopathies, cirrhose biliaire et cholangiocarcinome...) et les hépatocytes. Différents types d'affections

peuvent apparaître : un empêchement du flux sanguin ou la présence d'un ou de plusieurs facteurs étiologiques comme l'alcool, les infections par les virus de l'hépatite B ou C, ou l'exposition à l'aflatoxine-B1, pouvant donner lieu à une inflammation chronique, une lésion tissulaire conduisant à la nécrose du tissu. Malgré son caractère quiescent en situation physiologique, le foie possède néanmoins une capacité de régénération remarquable lui permettant en situation pathologique de mettre en place un mécanisme de prolifération compensatoire. Cependant, à long terme ces cycles de nécrose/régénération peuvent conduire à l'apparition de tissu cicatriciel (présence de collagène) et de nodules, ces derniers pouvant à terme se transformer en carcinome hépatocellulaire.

Au cours de ces vingt dernières années, l'incidence du cancer primitif du foie (carcinome hépatocellulaire dans 80% des cas, et cholangiocarcinome dans 10% des cas) a particulièrement augmenté dans les pays développés particulièrement en raison d'une augmentation du nombre de cas de cirrhose (due notamment aux facteurs de risque décrits précédemment).

A l'heure actuelle, les méthodes de détection trop sensibles pour envisager un diagnostic précoce et précis et le manque d'alternatives thérapeutiques pour la prise en charge des patients atteints d'un hépatocarcinome, en font un cancer de très mauvais pronostic et un problème de santé publique majeur.

Le cancer est une pathologie qui se développe dans un contexte d'accumulation de mutations génétiques et/ou des modifications épigénétiques. Son initiation et sa progression sont liées à des « caractéristiques distinctives » (Hallmarks), notamment décrites dans la littérature par *Hanahan* et *Weinberg* (2000 et 2010). Les cellules tumorales commencent notamment à être indépendantes aux facteurs de croissance, acquièrent un potentiel de réplication illimité, elles deviennent insensibles à la mort cellulaire par apoptose. Enfin, la prolifération anarchique des cellules tumorales va être à l'origine d'une adaptation de la tumeur qui devra non seulement augmenter son apport intratumoral en oxygène et en

nutriments, grâce à une néoangiogenèse, mais également adapter sa vitesse de production d'énergie en changeant son métabolisme.

Adaptation métabolique tumorale

En cancérologie, le métabolisme est de plus en plus étudié du fait de son rôle clé au cours de la progression tumorale et de l'adaptation des cellules cancéreuses à leur environnement ; le plus connu est l'effet Warburg.

Dans une cellule normale et en présence d'oxygène la phosphorylation oxydative est la voie majeure et la plus efficace pour produire de l'énergie. Au contraire, en présence de faibles taux d'oxygène (hypoxie), la glycolyse est alors la voie privilégiée. L'effet Warburg est l'utilisation de la glycolyse, même s'il y a de l'oxygène à disposition.

La choline a été décrite récemment comme un nutriment essentiel et est principalement absorbée avec l'alimentation, même si une partie peut être aussi recyclée dans l'organisme. L'apport de la choline est essentiel dans la cellule car elle possède deux rôles fondamentaux : elle peut être donneur de groupes méthyles et est également capable d'être à l'origine des phospholipides membranaires, grâce à un ensemble des réactions appelé « Kennedy pathway ».

En oncologie, un intérêt particulier s'est récemment porté sur la choline suite aux analyses de *Glunde et al.* (2011) montrant que l'expression de différentes enzymes de la voie d'utilisation de la choline est augmentée dans différents types des cancers par rapport aux tissus sains. De manière très intéressante, avec un essai clinique en cours, une de ces enzymes, la Choline Kinase α , paraît être cible thérapeutique spécifique potentielle.

Contexte et objectifs de mes travaux de thèse

Le laboratoire dispose de différents modèles murins, dont je me suis servie pendant ma thèse : deux modèles d'inactivation hépatospecifique et inducible du gène *Hnf4 α* et du gène *Apc*. APC est fondamental pour le recrutement de la β -caténine dans son complexe de dégradation, où elle va

subir une première phosphorylation par la CK1 puis des phosphorylations par la GSK3. Ce signal de phosphorylations est essentiel pour conduire à la dégradation protéasomale de la β -caténine. Si APC n'est pas fonctionnel, la β -caténine n'est plus dégradée, elle peut transloquer dans le noyau, même s'il n'y a pas des signaux WNT à la membrane, et activer la transcription de ses gènes cibles.

Mon équipe d'accueil avait montré précédemment que la voie de signalisation WNT/ β -caténine était nécessaire pour établir et maintenir la zonation métabolique, condition préalable pour la fonctionnalité de l'organe, mais qu'elle est aussi cause d'un pourcentage non négligeable (11-32%) de carcinomes hépatocellulaires (CHC), qui surviennent après mutation activatrice du gène codant la β -caténine.

Mes travaux se sont inscrits dans la continuité de travaux auxquels j'ai participé, et ont eu pour principaux objectifs : (1) d'explorer le rôle du facteur de transcription Hnf4 α dans la physiologie et les cancers du foie, en lien avec la signalisation β -caténine ; (2) de déterminer si une imagerie tumorale par tomographie par émission de positrons (TEP) spécifique de la captation de choline pouvait prédire les CHC mutés pour la β -caténine et si le métabolisme de la choline pouvait présenter une piste diagnostique et thérapeutique des cancers du foie.

Premier projet : caractériser le rôle suppresseur de tumeur de Hnf4 α in vivo dans un modèle de cancérogenèse hépatique liée à la β -caténine

J'ai utilisé le modèle d'inactivation d'Hnf4 α et les expériences effectuées ont permis de confirmer que HNF4 α dans le foie adulte contrôle négativement la prolifération et l'accumulation des lipides dans l'organe. Mais de manière intéressante, j'ai également pu montrer qu'il intervient dans la maintenance de l'architecture et de l'organisation du tissu hépatique ; en effet, dans 1/4 des souris invalidées les triades portales sont désorganisées. La veine porte présente une sténose (son lumen est fortement réduit), il y a plus des canaux biliaires et le diamètre de l'artère hépatique est augmenté, mimant une pathologie humaine : « l'hypertension portale idiopathique ».

L'invalidation d'*Hnf4α* a été aussi effectuée de manière focale pour tester son rôle dans la carcinogenèse hépatique (comme simple invalidation ou double avec la perte d'*Apc* dans les mêmes cellules), en sachant les résultats préliminaires dans la littérature précédemment énumérés dans le paragraphe « HNF4α, un régulateur hépatocytaire majeur », qui suggèrent pour ce facteur de transcription un rôle de suppresseur de tumeur dans le foie.

Ce rôle suppresseur de tumeur était également attendu des données récentes obtenues dans l'équipe, puisque une approche thérapeutique anti miR-34a réalisée *in vivo*, avait permis de montrer que l'effet anti-tumoral obtenu sur les tumeurs β-caténine activées est orchestré pour partie par la restauration d'un niveau élevé d'*Hnf4α* (qui est une cible de miR-34a). J'ai participé à ce travail publié dans *Gut* en 2015 : « Antitumour activity of an inhibitor of miR-34a in liver cancer with β-catenin-mutations » de *Gougelet et al.* présent dans la section résultats de ce manuscrit.

Cependant, dans notre modèle les résultats sont différents. Nos expériences ont montré que dans un contexte de cancérogenèse liée à la suractivation de la voie β-caténine, la perte du facteur HNF4α n'avait pas d'impact sur l'initiation ou la progression tumorale, ne les rendant pas plus précoces ou plus agressives.

Deuxième projet : Vers un diagnostic des tumeurs activées pour la β-caténine basé sur le métabolisme de la choline

Le modèle murin de cancérogenèse hépatique lié à l'invalidation d'*Apc* a également permis d'élucider certaines caractéristiques des cancers du foie activés pour la β-caténine. Dans les souris avec une invalidation totale hépatique, une dérégulation du métabolisme de lipides a été mise en évidence par une approche métabolomique. En effet, dans les foies de ces souris invalidées pour *Apc* on remarque une diminution de la quantité de triglycérides et en parallèle une augmentation des sphingolipides et des phospholipides, ces derniers lipides étant en grande majorité issus du métabolisme de la choline.

Le but de nos travaux de recherche n'est pas seulement d'acquiescer une meilleure compréhension des mécanismes impliqués dans l'initiation et la croissance tumorale hépatique mais également de contribuer à l'élaboration de nouvelles thérapies ciblées.

C'est dans ce contexte que nous nous sommes particulièrement intéressés à l'étude de la voie de la choline sur des biopsies de patients atteints de carcinomes hépatocellulaires (muté ou pas pour la β -caténine) et sur notre modèle murin pour analyser les mécanismes moléculaires mis en jeu.

Le TEP avec le F-choline est déjà utilisé en pratique clinique, comme aide au TEP plus classique avec le Fluoro-deoxyglucose (FDG), parce qu'il a été démontré que la sensibilité du TEP FDG est faible pour les cancers du foie et que l'utilisation de la F-choline permet une visualisation plus aisée des tumeurs. Nous avons entrepris, à la fois sur une cohorte de patients et sur nos modèles murins transgéniques, une analyse de TEP (pour tomographie à émission de positrons), et avons cherché à établir une corrélation entre les tumeurs avec une hyperactivation de la β -caténine et l'hyperfixation par la tumeur de la F-choline.

Dans les tumeurs humaines, le statut mutationnel de la β -caténine a été confirmé par recherche des mutations sur l'ADN et par analyse d'expression de ces gènes cibles (Glutamine Synthetase et Axin2). J'ai ainsi obtenu 6 tumeurs mutées β -caténine et six non mutées. Or, toutes les tumeurs mutées β -caténine captent activement la choline, alors que les non mutées étaient toutes négatives pour cette captation.

En ce qui concerne les modèles murins, en parallèle avec le modèle Apc mimant une cancérogenèse dépendante de la β -caténine, j'ai utilisé un modèle de cancérogenèse indépendant de la β -caténine : il s'agit de souris C57BL/6 injectées avec du Diethylnitrosamine 14 jours après la naissance qui développent des cancers du foie en six mois. Là encore, une hyperfixation de F-choline est spécifique du statut d'activation de la signalisation β -caténine. Aussi bien chez la souris que chez l'homme, cette hyperfixation s'accompagne de la surexpression d'ARNm permettant la synthèse d'enzymes et de transporteurs liés au métabolisme de la choline.

Nous avons ensuite procédé à une étude mécanistique. Nous avons effectué des expériences de flux avec la ¹⁴C-choline, sur des cultures primaires de foie et sur des explants de tumeurs. Dans le cas d'une suractivation de la voie Wnt/ β -caténine, les cultures et explants présentent une incorporation des groupements méthyles de la choline sur l'ADN, ainsi qu'une augmentation de la quantité de phospholipides, produits à partir de la choline.

Or parallèlement, la méthylation de l'ADN a été trouvée augmentée dans le foie des souris activées pour la β -caténine associée à une augmentation de la prolifération, ces deux phénomènes étant dépendants de la choline, puisqu'ils sont tous deux abolis quand les souris sont nourries avec un régime déficient en choline et méthionine.

Ces résultats sont extrêmement encourageants car ils montrent qu'un simple examen non invasif pourrait permettre un diagnostic génétique du cancer du foie (détection du CHC muté β -caténine), permettant par la suite d'établir une stratégie thérapeutique ciblée et personnalisée.

3) Article :

T-cell factor 4 and β -catenin chromatin occupancies pattern zonal liver metabolism in mice.

Gougelet, A., Torre, C., Veber, P., Sartor, C., Bachelot, L., Denechaud, P.-D., Godard, C., Moldes, M., Burnol, A.-F., Dubuquoy, C., et al. (2014). *Hepatology*. Baltimore, Md 59, 2344–2357

T-Cell Factor 4 and β -Catenin Chromatin Occupancies Pattern Zonal Liver Metabolism in Mice

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β -catenin signaling can be both a physiological and oncogenic pathway in the liver. It controls compartmentalized gene expression, allowing the liver to ensure its essential metabolic function. It is activated by mutations in 20%–40% of hepatocellular carcinomas (HCCs) with specific metabolic features. We decipher the molecular determinants of β -catenin-dependent zonal transcription using mice with β -catenin-activated or -inactivated hepatocytes, characterizing *in vivo* their chromatin occupancy by T-cell factor (Tcf)–4 and β -catenin, transcriptome, and metabolome. We find that Tcf-4 DNA bindings depend on β -catenin. Tcf-4/ β -catenin binds Wnt-responsive elements preferentially around β -catenin-induced genes. In contrast, genes repressed by β -catenin bind Tcf-4 on hepatocyte nuclear factor 4 (Hnf-4)-responsive elements. β -Catenin, Tcf-4, and Hnf-4 α interact, dictating β -catenin transcription, which is antagonistic to that elicited by Hnf-4 α . Finally, we find the drug/bile metabolism pathway to be the one most heavily targeted by β -catenin, partly through xenobiotic nuclear receptors. **Conclusions: β -catenin patterns the zonal liver together with Tcf-4, Hnf-4 α , and xenobiotic nuclear receptors. This network represses lipid metabolism and exacerbates glutamine, drug, and bile metabolism, mirroring HCCs with β -catenin mutational activation. (HEPATOLOGY 2014;59:2344–2357)**

See Editorial on Page 2080

The adult liver is a quiescent organ, fully compartmentalized to accomplish its crucial metabolic role. Its vasculature gives rise to two distinct hepatocyte populations: one located in the vicinity of the portal vein and the other around the central vein.¹ Pericentral (PC) hepatocyte metabolism is

complementary to that of periportal (PP) hepatocytes in terms of energy, ammonia, and xenobiotic metabolism. This complementarity arises as a result of the production of distinct specialized proteins in the two zones.^{1,2} It has been demonstrated that the Wnt/ β -catenin pathway is the master transcriptional regulator of this zonal metabolism, and that control is rendered by a Wnt morphogenetic concentration gradient high in PC hepatocytes and decreasing toward PP hepatocytes.^{3,4}

Abbreviations: Ab, antibody; AhR, aryl hydrocarbon receptor; Apc, adenomatous polyposis coli; Apoc3, apolipoprotein C3; CAR, constitutive androstane receptor; ChIP, chromatin immunoprecipitation; CYP, cytochrome P450; DHS, DNase1 hypersensitive sites; GSEA, gene set enrichment analysis; H3K27Ac, histone 3 lysine 27 acetylation; HCC, hepatocellular carcinoma; HMG, high-mobility group; Hnf-4, hepatocyte nuclear factor 4; HRE, Hnf4-responsive element; IgG, immunoglobulin G; kb, kilobase; Lef, lymphoid enhancer-binding factor; Lxr, liver X receptor; mRNA, messenger RNA; PC, pericentral; PP, periportal; Pol II, polymerase 2; qPCR, quantitated polymerase chain reaction; Scd1, stearoyl-CoA desaturase 1; Tcf, T-cell factor; TF, transcription factor; TGs, triglycerides; VLDL, very-low-density lipoprotein; WRE, Wnt-responsive element.

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Received August 16, 2013; accepted November 6, 2013.

This work was supported by the Ligue Nationale Contre le Cancer (Labellisation Programme 2011–2013), the Agence Nationale de la Recherche (WNT-METABOLIV, 2011–2013), and, in part, by the French Laboratory of Excellence program, Who am I (no. ANR-11-LABX-0071), included in the Investments for the Future program no. ANR-11-IDEX-0005-01. Cancercys EU FP7 programme (2008–2010) also funded experimental work and fellowships for P.V., A.G., P.D.D., and L.B. C.T. held a fellowship from the Ministère de la Recherche et de la Technologie.

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In the absence of Wnt, β -catenin is degraded in the cytosol by a complex involving the product of the adenomatous polyposis coli (*Apc*) tumor-suppressor gene, which is the main negative regulator of β -catenin signaling. With no nuclear β -catenin, lymphoid enhancer-binding factor/T-cell factor (*Lef/Tcf*) DNA-binding proteins are described constitutively bound to Wnt-responsive elements (WREs), through their high mobility group (HMG)-box motifs, to recruit transcriptional repressors (as reviewed previously⁵). In cases of Wnt activation, β -catenin, which has transactivating properties, but no DNA-binding domain, accumulates in the nucleus and binds to *Lef/Tcfs* to recruit coactivators. This leads to the transcription of a highly context-specific repertoire of target genes.⁵

We previously defined *Apc* as the liver “zonation keeper” because its PP-enriched distribution restricts β -catenin signaling to the opposite PC area.³ In PC hepatocytes, β -catenin induces a PC-specific genetic program while repressing a PP-specific one. A genetic model of hepatospecific β -catenin overactivation engineered by an inducible *Apc* loss produced an entirely PC-like liver and led to hepatocyte proliferation and lethality.³ This generates a pretumoral liver, because *Apc* loss allows quiescent hepatocytes to engage in cell division³ and *Apc* deletion in single hepatocytes leads to the development of β -catenin-activated liver tumors.⁶ The genetic program expressed in the *Apc*-null liver resembles part of the oncogenic genetic program expressed in human hepatocellular carcinomas (HCCs) harboring activating mutations in the *CTNNB1* gene.^{6,7} Conversely, β -catenin inactivation in mouse hepatocytes induces a PP liver phenotype.^{4,8}

The aim in the present study was to determine how β -catenin can define the zonal metabolic identities of hepatocytes and to what extent this metabolic program can be subverted during hepatocarcinogenesis. We used genetically modified hepatocytes isolated from *Apc* and β -catenin knockout mice to analyze the genome-wide DNA-binding properties of β -catenin/*Tcf* complexes, together with the transcript repertoire of the two different hepatocyte populations. We demonstrate strong crosstalk between β -catenin, *Tcf-4*, and the transcription factor (TF), hepatocyte nuclear factor 4 alpha (*Hnf-4 α*), defining transactivating or -repress-

ing properties. Last, our metabolomic approach shows that the transcriptional program engaged by β -catenin has a major effect on bile and lipid metabolism, and this matches well the particular metabolic morphotype of *CTNNB1*-mutated HCCs.

Materials and Methods

Animals and Reagents

Mice carrying two floxed alleles on the 14th exon of the *Apc* gene (*Apc*^{lox/lox})⁶ or a biallelic floxed β -catenin gene (β cat^{lox2-6/lox2-6}, with loxP sites located between exons 2 and 6)⁹ were interbred with TTR-Cre^{Tam} mice.¹⁰ The transgenic mice, tamoxifen injection, liver sampling, and *in situ* hybridization protocols have been previously described.^{3,8} All animal procedures were carried out according to French legal regulations and approved by an ethical committee.

Human Samples

Forty-four patients treated for liver cancer in Cochin Hospital (Paris, France) were included in this study. All tumor samples were frozen after surgery in accord with French law and ethical guidelines.

Hepatocyte Isolation

Livers of 2-month-old male mice were perfused as described previously.⁸ Liver cells were washed three times in phosphate-buffered saline supplemented with 4-(aminoethyl)-benzenesulfonyl fluoride and centrifuged at 50 \times g for 2 minutes for subsequent experiments.

Chromatin Immunoprecipitation

The chromatin immunoprecipitation (ChIP) and re-ChIP protocol was adapted from a previous work¹¹ (see Supporting Materials).

Processing Samples for Illumina Sequencing

Messenger RNA Sequencing. The integrity of total RNA extracted with Trizol (Invitrogen, Carlsbad, CA) was determined using the Agilent 2100 bioanalyzer (Agilent Technologies, Inc., Lexington, MA). For each condition, two pools of RNA from two distinct hepatocyte isolations were submitted to deep sequencing on a Genome Analyzer II Illumina platform (Fasteris SA, Plan-les-Ouates, Switzerland).

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View this article online at wileyonlinelibrary.com.

DOI 10.1002/hep.26924

Potential conflict of interest: Nothing to report.

Additional Supporting Information may be found in the online version of this article.

ChIP Sequencing. Ten nanograms of immunoprecipitated fragments from two distinct experiments were pooled for each sequencing procedure.

Data have been deposited as GSE32513 in the Gene Expression Omnibus database.

Immunoprecipitation

Nuclear extracts (500 μ g) were submitted to immunoprecipitation using 8 μ g of anti- β -catenin or non-specific immunoglobulin G (IgG) immobilized on Protein G Dynabeads (Invitrogen).

Transfection Assays

HEK293T cells were transfected using Lipofectamine 2000 (Invitrogen) with 25 ng of a firefly luciferase reporter SuperTOPflash (8 \times multimerized WREs) or SuperFOPflash (8 \times multimerized mutated WREs; Addgene, Cambridge, MA), 10 ng of a Renilla vector (Promega, Madison, WI), 5 ng of a constitutive S45Y- β -catenin vector, and 5–80 ng of HNF-4 α 2 expression vector. Luciferase activity was measured 24 hours later with the DUAL-LUC kit (Promega).

Metabolomic Profiling and Lipid Measurements

Metabolites (469) were measured from eight Apc^{ko} and eight control frozen livers (100 mg/sample) by Metabolon, Inc. (Durham, NC). Lipid quantifications are described in the Supporting Methods.

Statistical Analysis

Statistical analyses were done using “R” free software (<http://www.r-project.org/>). Shapiro-Wilk’s normality tests and Bartlett’s test of homogeneity of variances were applied. Depending on these tests, either *t* tests with Welch’s correction for unequal variance or Mann-Whitney’s nonparametric tests were done. $P < 0.05$ was considered statistically significant.

Results

In Vivo Chromatin Accessibility and Occupancy by Tcf-4/ β -Catenin in Hepatocytes Activated or Deficient for β -Catenin. Hepatocytes activated (Apc^{ko} ; Fig. 1B) or inactivated (βcat^{ko} ; Fig. 1C) for the β -catenin pathway were genetically obtained (Fig. 1A). ChIPs were performed with antibodies (Abs) against β -catenin or Tcf-4 (Fig. 1D), the main Lef/Tcf factor interacting with β -catenin in an interactome study (Supporting Fig. 1). Deep sequencing of the ChIP products and of messenger RNA (mRNA) were then performed (Fig. 1D).

A dynamic programming calling peaks identifying TF-bound regions algorithm was used (Supporting Table 1A). A set of independent ChIP experiments was performed to validate the ChIP sequence peaks by

quantitated polymerase chain reaction (qPCR; Supporting Table 2). To further confirm the accuracy of the analysis, the genomic environment of the universal β -catenin target, Axin2, was next explored. Tcf-4-binding profiles similar to previously published ones were found¹² (Fig. 2A). We focused our analysis on genes induced by β -catenin, whose expression is pericentral and enriched in the adult liver (Fig. 2B–E), because the role of β -catenin in liver patterning is not complete until maturity (Fig. 2E). The glutamine synthase gene (*Glul*) is a prototypical target of liver β -catenin activation (Fig. 2B). Tcf-4 and β -catenin occupy at least six chromatin regions within the 44-kilobase (kb) promoter region upstream of *Glul*, four of which are enriched in WREs. The nuclear constitutive androstane receptor (CAR; *Nr1i3*) and the cytochrome P450 (CYP) enzyme, Cyp1a2, are also pericentral and induced by β -catenin. Both genes have strong chromatin occupancy on WREs by Tcf-4 and β -catenin (Fig. 2C,D). These bindings were confirmed by qPCR (Fig. 2F). To understand whether these occupancies could be associated with gene expression, particularly dynamic chromatin/histone modifications, an *in silico* investigation was performed.¹³ ENCODE data for DNase1 hypersensitive sites (DHS), for histone 3 lysine 27 acetylation (H3K27Ac), which marks active, rather than poised, enhancers,^{5,14} and for RNA polymerase 2 (Pol II) binding were extracted. These data sets were obtained on liver chromatin both in the embryonic state, when β -catenin specifies a biliary fate for hepatic progenitors,¹⁵ and during adulthood, when β -catenin patterns zonation.³ For the four genes studied, Tcf-4/ β -catenin systematically occupied chromatin with DHS and H3K27Ac marks, dramatically enriched in adult liver chromatin, compared to embryonic E14.5 chromatin. This shows that Tcf-4 and β -catenin bind WRE-encompassing enhancers specifically active in adult liver chromatin, corresponding to the period when β -catenin patterns the zonal liver.

β -Catenin Specifies How Tcf-4 Occupies Chromatin. Interestingly, our ChIP sequencing profiles suggest that in cases in which Tcf-4 and β -catenin bind WREs, Tcf-4 displays an increased binding signal in Apc^{ko} chromatin, compared to βcat^{ko} chromatin (Fig. 2A–D). This was confirmed by qPCR: It was a trend for the Axin2_Intron1 region, whereas the loss of Tcf-4 DNA binding in βcat^{ko} conditions was significant for the *Glul* 36-kb and *Nr1i3* 9-kb regions.

This enhanced Tcf-4 binding to some WREs in Apc^{ko} chromatin prompted us to ask whether it occurs at a genome-wide level. In fact, chromatin is much more occupied by Tcf-4 in Apc^{ko} hepatocytes than in βcat^{ko} hepatocytes, with Tcf-4 peaks only partially overlapping between both conditions (Fig. 3A). We

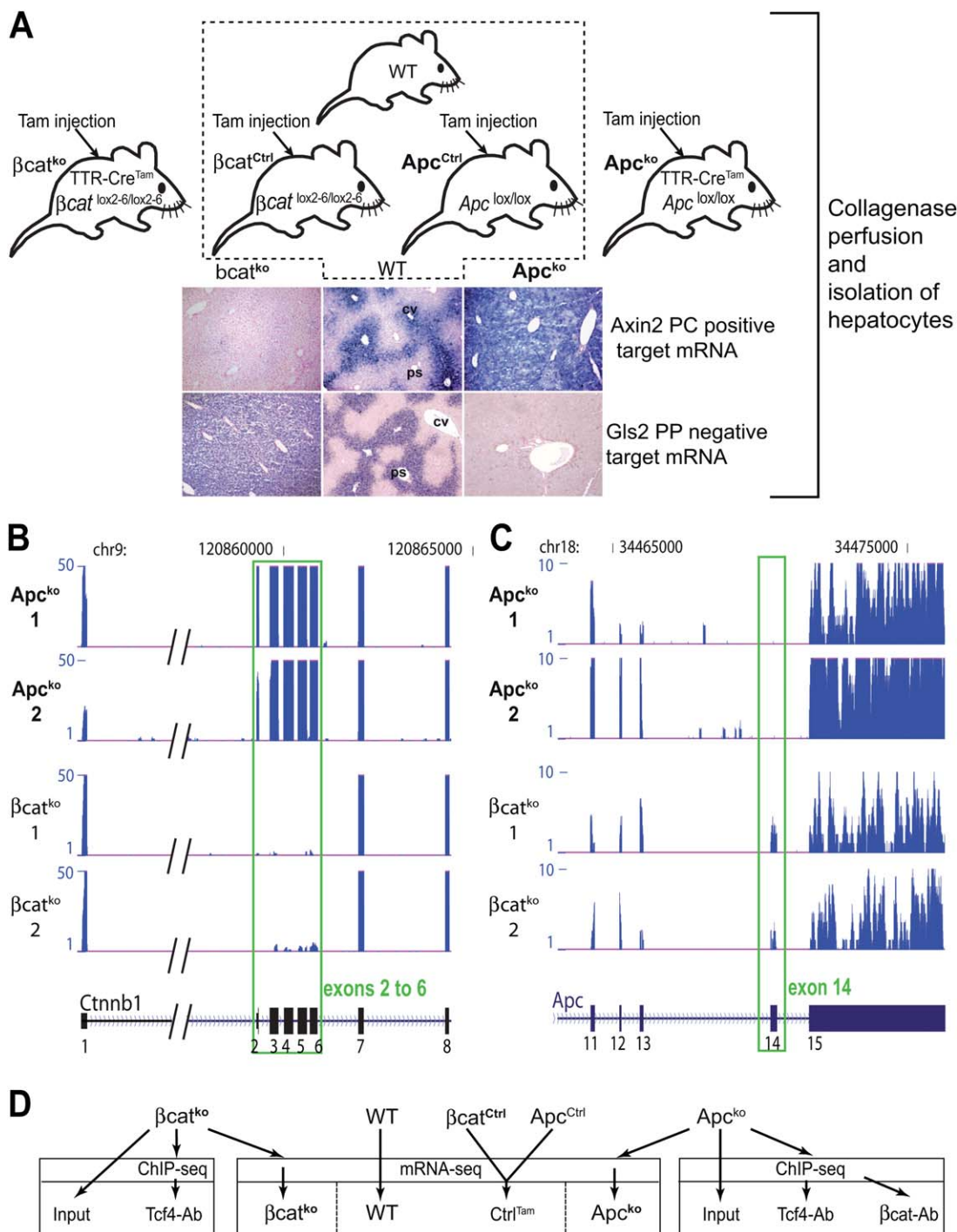


Fig. 1. Workflow used for data collection. (A) Mouse lines used and tamoxifen injection protocols. Lower: *in situ* hybridizations for Axin2 (showing β -catenin activation) and for Glis2 (showing β -catenin inactivation). Note in WT livers the Axin2 and the Glis2 stainings in the PC and PP zones, respectively. (B and C) mRNA sequencing shows (B) the excision of exons 2-6 of the *Tnnb1* in the two samples of $\beta\text{cat}^{\text{ko}}$ hepatocytes and (C) the excision of exon 14 of *Apc* in the two samples of Apc^{ko} hepatocytes. (D) Workflow for the processing of the distinct hepatocyte populations. WT, wild type; Tam, tamoxifen; cv, central vein; ps, portal space.

also observed that 90% of the β -catenin DNA-binding sites were encompassed in Tcf-4-bound chromatin (Fig. 3A), implying that Tcf-4 is a mandatory partner of chromatin-associated β -catenin in the liver. Alternatively, the lower sensitivity of the ChIP assay against

β -catenin, resulting from indirect DNA binding, could have masked any lower affinity interactions with proteins other than Tcf-4.

Thus, we hypothesized that Tcf-4 has distinct DNA-binding properties in hepatocytes, depending on

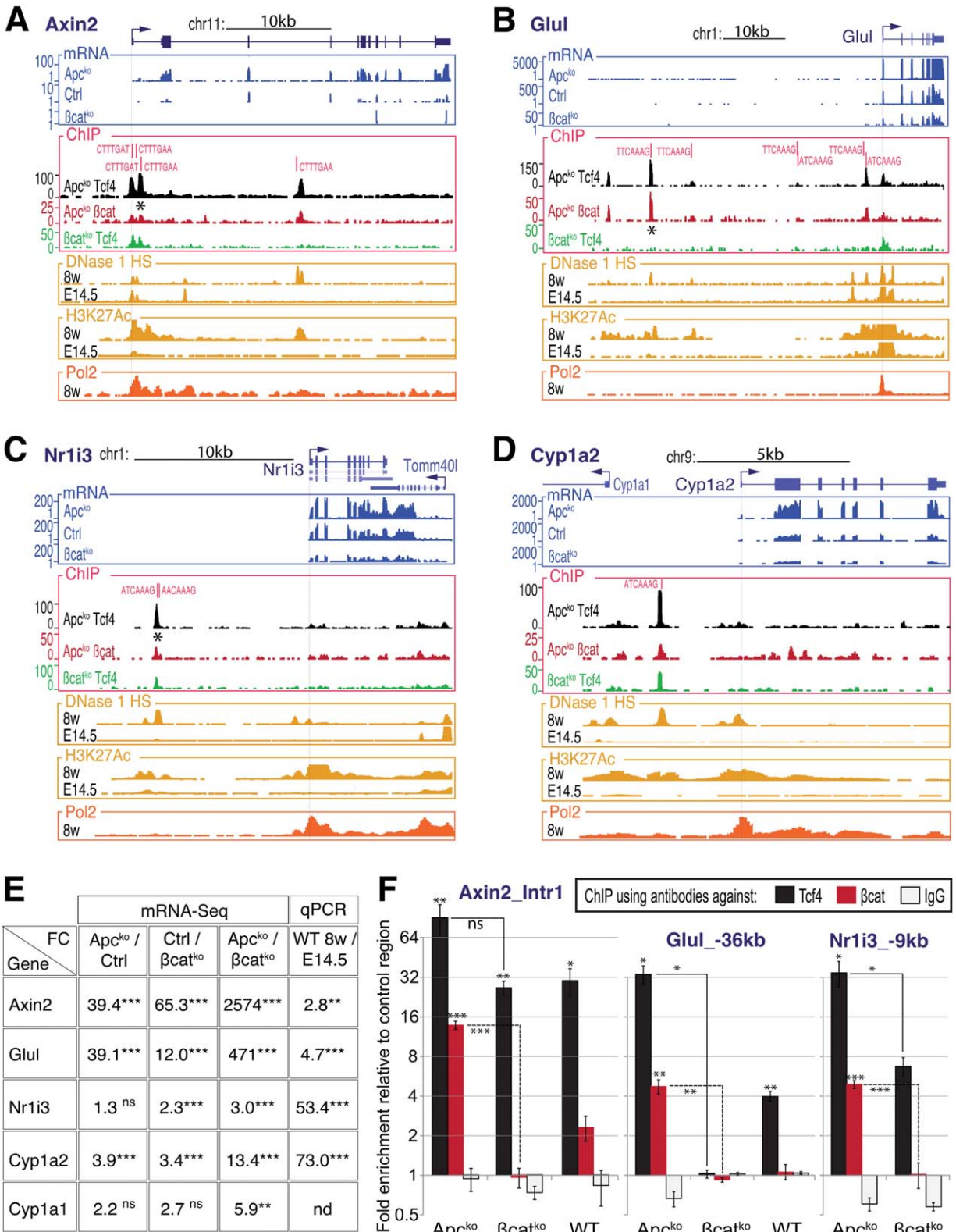


Fig. 2. Profiling targets of β -catenin. (A-D) Genomic environment of pericentral genes induced by β -catenin. Upper blue: mRNA sequencing signals. ChIP sequencing signals are shown below with the presence within peaks of WREs depicted first in red; then, in black, ChIP sequencing in Apc^{ko} conditions with an Ab against Tcf-4; in red, ChIP sequencing in Apc^{ko} conditions with an Ab against β -catenin; in green, ChIP sequencing in βcat^{ko} conditions with an Ab against Tcf-4. Below, in yellow, ENCODE data of DNase1 hypersensitivity performed in 8-week-old and in E14.5 embryonic mouse livers (DNase1 hypersensitivity by Digital DNase1 from ENCODE/University of Washington), of H3K27Ac marks in 8-week-old and in E14.5 embryonic livers (Histone Mods by ChIP sequencing from ENCODE/LICR), and, in orange, ENCODE data of ChIP sequencing against RNA polymerase II (Pol2, from ENCODE/LICR) in adult livers. (E) mRNA accumulation determined by mRNA sequencing expressed as a fold change (FC) in Apc^{ko} versus control, in control versus βcat^{ko} , or in Apc^{ko} versus βcat^{ko} hepatocytes, together with a qPCR analysis of transcript enrichment in adult versus embryonic livers. (F) Validation of ChIP sequencing experiments by qPCR on our validation set (more explanation in Supporting Table 2). The peaks tested are those with asterisks in (A, B, and C). Mann-Whitney's test has been calculated, compared to ChIP against IgG in each series, or as indicated.

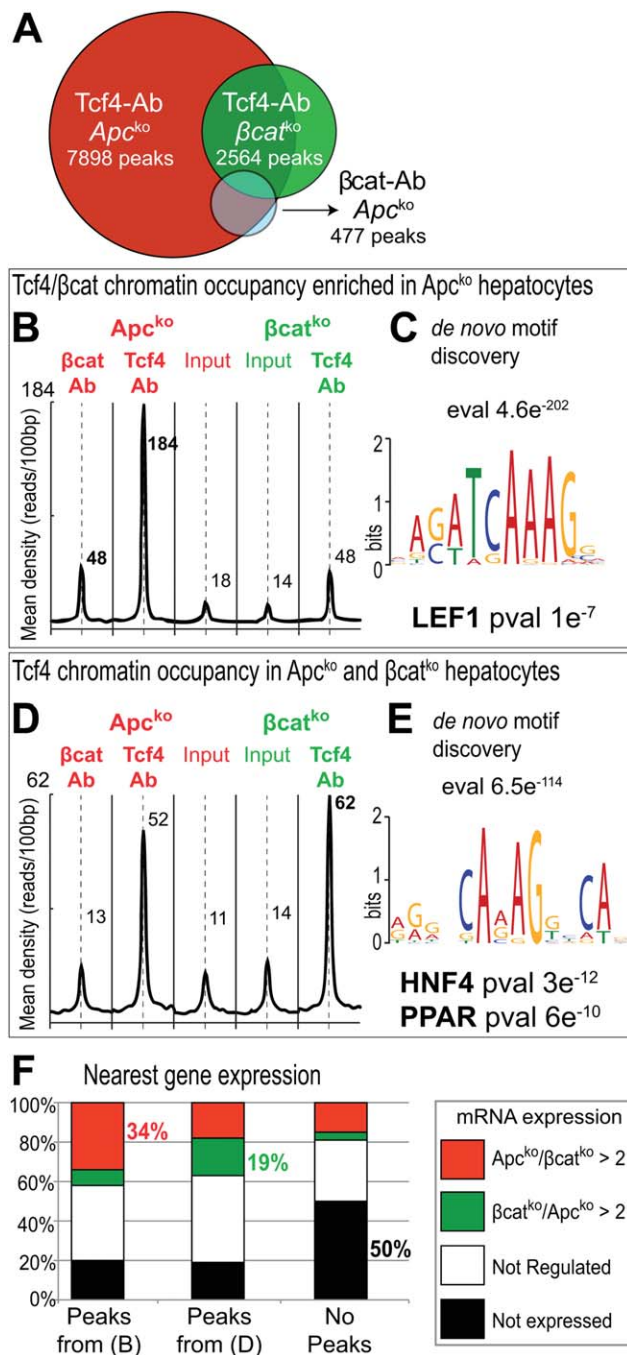


Fig. 3. Distinct chromatin occupancies by β -catenin and Tcf-4. (A) Venn diagrams representing the peak overlaps between the different data sets. (B and D) Clustering with SeqMINER (see Supporting Figs. 5 and 6 for more details) and peak distribution within each cluster. (C and E) The most enriched *de novo* motif within the peaks is shown and identified by alignment with Transfac motif database. (F) Status of the nearest gene from the peaks, regarding its transcript expression in response to β -catenin signaling.

the presence or absence of nuclear β -catenin, and asked which DNA motifs in Apc^{ko} and βcat^{ko} hepatocytes are bound by Tcf-4 alone or by β -catenin/Tcf-4 complexes. We used the seqMINER platform¹⁶ to select clusters of peaks corresponding to each binding category (Supporting Fig. 2 and Fig. 3). One cluster exhib-

ited high chromatin occupancy by β -catenin and Tcf-4 in Apc^{ko} conditions (Fig. 3B): 34% of the closest genes from these peaks were β -catenin-induced targets (Fig. 3F). A common DNA motif was enriched in 79% of these peaks and corresponded to an LEF-1 motif (WRE; Fig. 3C). Another cluster contained peaks in which Tcf-4 occupancy was nearly identical in βcat^{ko} and in Apc^{ko} chromatin and was not associated with β -catenin DNA binding (Fig. 3D). The closest genes from these peaks were enriched in β -catenin-repressed genes (19%; Fig. 3F). We *de novo* identified, in 51% of the peaks corresponding to this cluster, a motif close to an Hnf-4 or a peroxisome proliferator-activated receptor motif (Hnf-4-responsive element; HRE). We found that β -catenin/Tcf-4 transcriptional complexes bound to WREs are associated with gene induction. Independently of β -catenin, Tcf-4 also binds to HREs and this occupancy is enriched in the vicinity of β -catenin-repressed genes.

The β -Catenin/Tcf-4-Dependent Transcriptional Network Produces a Major Metabolic Output. To characterize the β -catenin/Tcf-4-dependent transcriptional network, mRNA sequencing was combined with ChIP sequencing data (Supporting Fig. 3). We identified distinct categories of genes based on their liver transcription and on their regulation by β -catenin (Supporting Table 1B; Supporting Fig. 3). From the ChIP sequencing analyses, normalized scores were extracted for peaks either within the 100 kb upstream of transcriptional start sites or within intragenic regions. The combination of mRNA sequencing with ChIP sequencing data confirm that Tcf-4, together with β -catenin chromatin occupancies, characterize genes induced by β -catenin, whereas Tcf-4 binding alone is widely represented over the whole genome (Supporting Fig. 3B).

To further investigate the direct targets of β -catenin/Tcf-4 in the liver, based both on the $Apc^{ko}/\beta cat^{ko}$ ratio of transcript abundance and on scores of the ChIP sequencing peaks (see explanations of Supporting Tables 3 and 4), a pathway analysis strategy was employed (Fig. 4A,B). The genes deregulated in βcat^{ko} hepatocytes probably reflect a physiological role for β -catenin in the liver. In contrast, the gene expression differences between Apc^{ko} and control livers reflect the pathological “pretumoral” effects of an overactivation of β -catenin signaling. We found that physiological β -catenin signaling directly controls the expression of 308 genes, mostly involved in metabolic functions (Fig. 4A): 84.6% of them are modulated in response to overactivated β -catenin (Fig. 4B). Moreover, overactivation of β -catenin signaling modulates an additional subset of 581 direct targets related to proliferation, apoptosis, and carcinogenic processes, as well as to

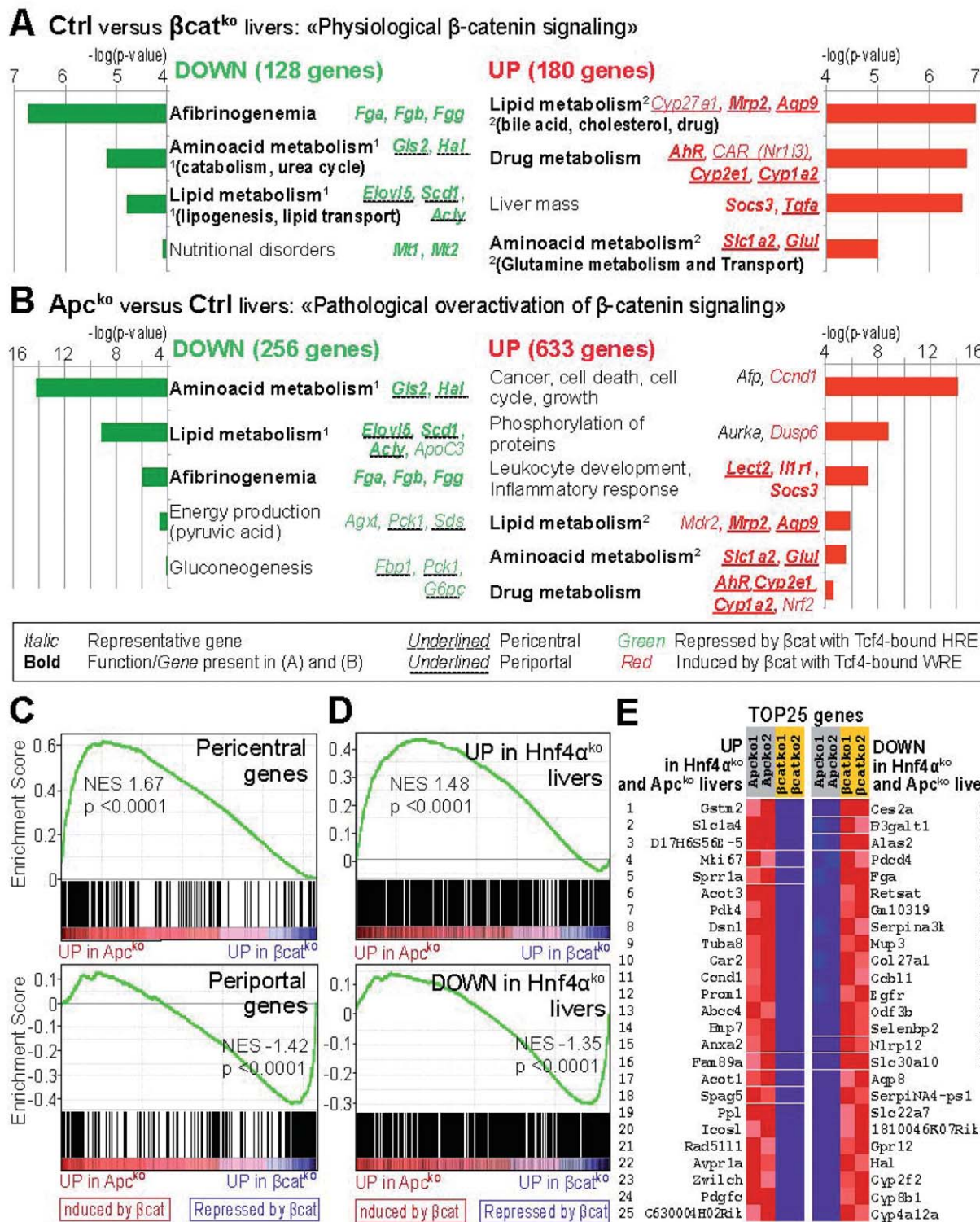


Fig. 4. Physiological and oncogenic responses induced by β -catenin in hepatocytes. (A and B) Functions modulated by genes repressed (DOWN) or activated (UP) by β -catenin (Ingenuity Pathway Analysis) either in a physiological (A) or in a pathological context (B). Representative genes are depicted in green or red italics (see legend). (C and D) GSEA in (C) with pericentral and periportal genes and in (D) with genes up- or down-regulated in $Hnf-4\alpha^{ko}$ livers. In (E), the top 25 genes more regulated by both β -catenin and $Hnf-4\alpha$. NES, Normalized Enrichment Score.

inflammatory response, as recently reported¹⁷ (Fig. 4B). The most dramatic effect of Wnt physiological signaling acts positively on bile acid, cholesterol, and

drug metabolic pathways (Supporting Table 3; Fig. 4A). The expression of aryl hydrocarbon receptor (AhR) and CAR xenobiotic receptor genes is induced by

β -catenin, and their genes have nearby WREs that are occupied by β -catenin and Tcf-4 (Fig. 2C; Supporting Table 3). This is also the case for genes encoding a number of drug-converting enzymes and transporters (e.g., multidrug resistance-associated protein 2, Cyp1a2, glutathione *S*-transferases), which are also known to be transcriptionally controlled by both receptors. Thus, β -catenin controls the expression of drug-converting enzymes and transporters both directly and indirectly through AhR and CAR (Supporting Fig. 4; Supporting Table 3). In contrast, in overactivation conditions, as in normal liver, the genes directly repressed by β -catenin are related to fibrinogen synthesis, urea cycle, and lipogenesis.

A gene set enrichment analysis (GSEA) was performed (Fig. 4C) from microarrays depicting PP versus PC gene expressions obtained after a differential isolation of periportal and -central hepatocytes.¹⁸ It was found that the direct β -catenin-induced targets are greatly enriched in PC-specific mRNAs, whereas they are PP for the β -catenin repressed. Thus, liver metabolic zonation is largely dependent on β -catenin-coordinated gene transcription. Last, having identified an Hnf-4 motif, a possible relationship between β -catenin and Hnf-4 α was examined. β -catenin-repressed targets were strongly associated in the literature with Hnf-4 α (Supporting Fig. 5). Therefore, our mRNA sequencing data were compared with published microarrays of Hnf-4 α targets characterized in adult liver after hepatospecific postnatal inactivation of Hnf-4 α ¹⁹ (Fig. 4D,E). Using GSEA, it was found that the genes repressed by β -catenin were enriched in Hnf-4 α -induced transcripts and inversely (Fig. 4D,E).

These results show that β -catenin has a major effect on the transcription of metabolic genes. This could occur through a positive cooperation with xenobiotic nuclear receptors and/or through negative cross-talk with Hnf-4 α .

β -Catenin-Repressed Target Genes Bind Tcf-4 on HRE. We then focused on the particular Tcf-4-binding profile of genes repressed by β -catenin. From Supporting Table 4, genes encoding stearyl-CoA desaturase 1 (Scd1), apolipoprotein C3 (ApoC3), and fibrinogen alpha were selected, on the basis of their chromatin occupancy by Tcf-4 on a consensual HRE according to the Transfac motif database (Fig. 5A-D). All these genes were positive Hnf-4 α targets (Fig. 5D). It was found that Tcf-4 occupied chromatin on Hnf-4 motifs, which are effectively bound by Hnf-4 α , as assessed from CHIP sequencing assays performed on adult liver chromatin.²⁰ This occurs on enhancers active in adult liver chromatin, as assessed by DHS and H3K27Ac marks (Fig. 5A-C). We confirmed these bindings by qPCR (Fig. 5E,F): Interestingly, Tcf-4 and Hnf-4 α bindings occur independently of β -catenin status and also without a concomitant chromatin occupancy by β -catenin.

Hnf-4 α /Tcf-4/ β -Catenin Functional Antagonism Involves DNA- and Protein-Protein Interactions.

Binding of Tcf-4 on HREs led us to explore the potential links between Hnf-4 α , β -catenin, and Tcf-4. The WRE motif presents strong similarities with the HRE motif (Fig. 6A). Indeed, the HRE consists of two poorly conserved AGGTCA hemisites separated by one base, whose central region is similar to the core region of the WRE. We investigated whether Tcf-4 was able to bind the HRE in a sequence-specific manner. By a re-ChIP experiment, we found that Tcf-4 binds to Hnf-4 α -bound ApoC3 HRE *in vivo* (Fig. 6B). Hnf-4 α reciprocally bound Tcf-4-bound Axin2 WRE (Fig. 6C).

We investigated the functional consequences of these interactions (Fig. 6D,E). We first assessed WRE-dependent reporter activity using TOP-Flash plasmid. Cotransfection of increasing amounts of a plasmid encoding Hnf-4 α with the TOP (WRE) plasmid decreases the responsiveness of the WRE reporter to β -catenin. We also found that the mutated WRE of the FOP-flash plasmid behaves as a true HRE because of its specific DNA mutations, which conferred reporter inductiveness by Hnf-4 α (Fig. 6E). Cotransfection of a plasmid encoding an active β -catenin mutant with the FOP plasmid was able to decrease Hnf-4 α -dependent luciferase production. These results show that β -catenin-dependent transcription and Hnf-4 α -dependent transcription are antagonistic. To understand how β -catenin can abolish Hnf-4 α DNA binding, we determined whether Hnf-4 α interacts with β -catenin. Hnf-4 α immunoprecipitates with Tcf-4 *in vitro*, as reported previously (Supporting Fig. 6A).²¹ Significantly, Hnf-4 α also coimmunoprecipitates *in vitro* with β -catenin (Supporting Fig. 6B), validating an interaction between β -catenin and Hnf-4 α *in vivo* in nuclear extracts from Apc^{Ko} hepatocytes (Fig. 6F). Altogether, these results show that Tcf-4 and Hnf-4 α are able to bind reciprocally their respective motifs, and that Hnf-4 α interacts with Tcf-4 and β -catenin. As a consequence, Hnf-4 α represses β -catenin-dependent transcription, whereas β -catenin represses Hnf-4 α -dependent transcription.

Impact of β -Catenin-Dependent Metabolism on Liver Carcinogenesis.

We hypothesized that the profound effect that β -catenin exerts on liver zonal metabolism could also affect liver carcinogenesis. It has been suggested that CTNNB1-mutated HCCs have a metabolism distinct from nonmutated ones.⁷ In line with this, we found a set of genes commonly up- or down-regulated in Apc^{Ko} hepatocytes and CTNNB1-mutated HCCs (Fig. 7A). This regulation was significant for up-regulated genes and encompassed typical Wnt targets as well as genes involved in glutamine and bile/drug metabolism and transport (Fig. 7B). Moreover, genes involved in amino acid catabolism were

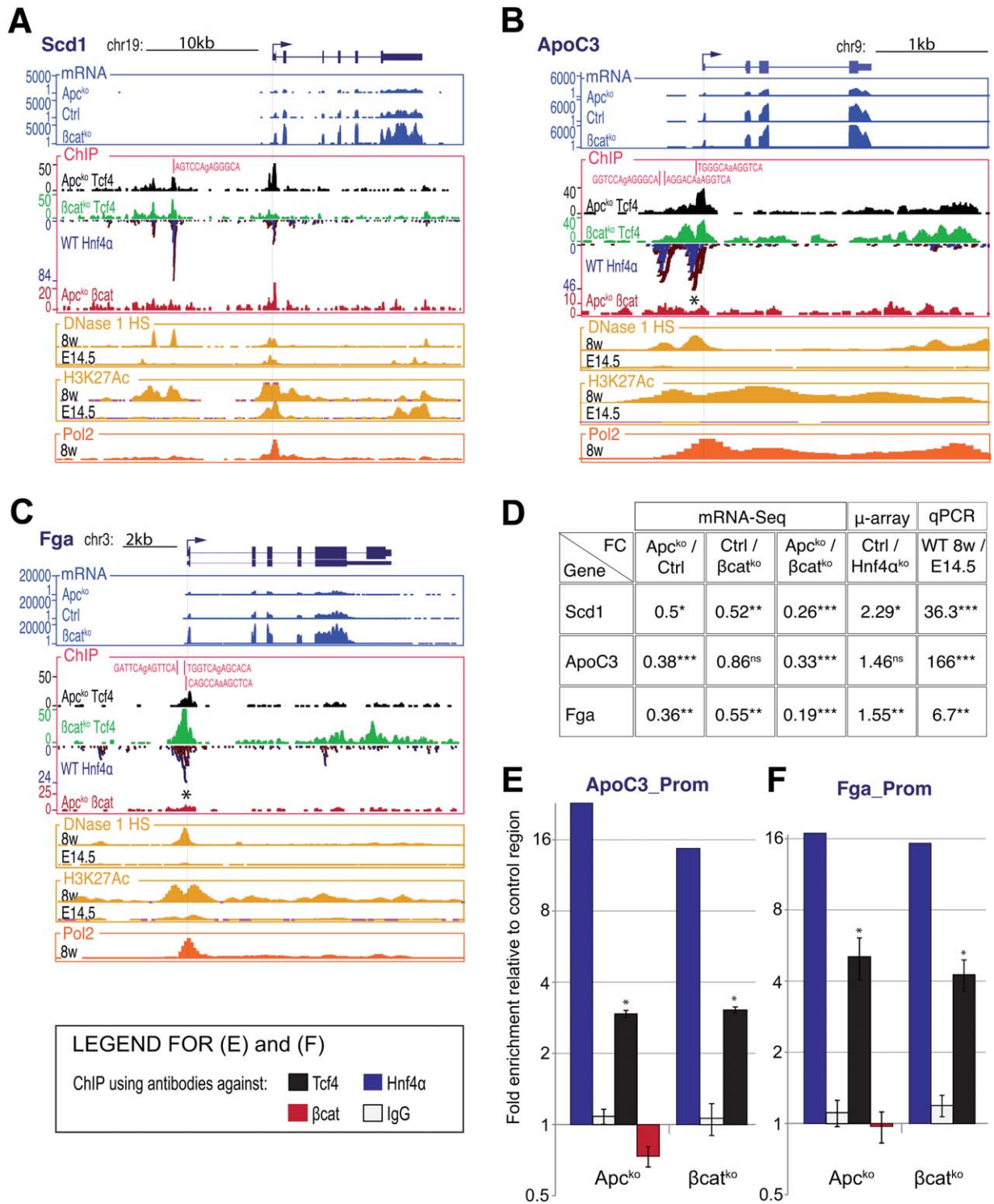


Fig. 5. Genes repressed by β -catenin and their relationship to Hnf-4 α . (A-C) Genomic environment of genes repressed by β -catenin. Upper blue: mRNA sequencing signals. ChIP sequence signals are shown below with the presence within peaks of HREs depicted first in red; then, in black, ChIP sequencing in Apc^{ko} conditions with an Ab against Tcf-4; in green, ChIP sequencing in β cat^{ko} conditions with an Ab against Tcf-4; in blue/red, the crude reads of ChIP sequencing data performed in adult livers against Hnf-4 α ²⁰; in red, ChIP sequencing in Apc^{ko} conditions with an Ab against β -catenin. Below, in yellow, ENCODE data of DHS, H3K27Ac, and in orange of polymerase 2 (Pol2; same as in Fig. 2). (D) mRNA accumulation determined by mRNA sequencing expressed as a fold change (FC) in Apc^{ko} versus control, in control versus β cat^{ko}, or in Apc^{ko} versus β cat^{ko} hepatocytes, together with microarray data performed in Hnf-4 α ^{ko} livers¹⁹ and a qPCR analysis of transcript enrichment in adult versus embryonic livers. (E and F) qPCR on ChIP validation set against Tcf-4, β cat, Hnf-4 α , or IgG in both Apc^{ko} or β cat^{ko} conditions: The regions analyzed are the peaks with asterisks in B and C. Data represent the mean \pm standard error of the mean (for ChIP Hnf-4 α in Apc^{ko} and β cat^{ko}: n = 1; for the other ChIPs, see Supporting Table 2).

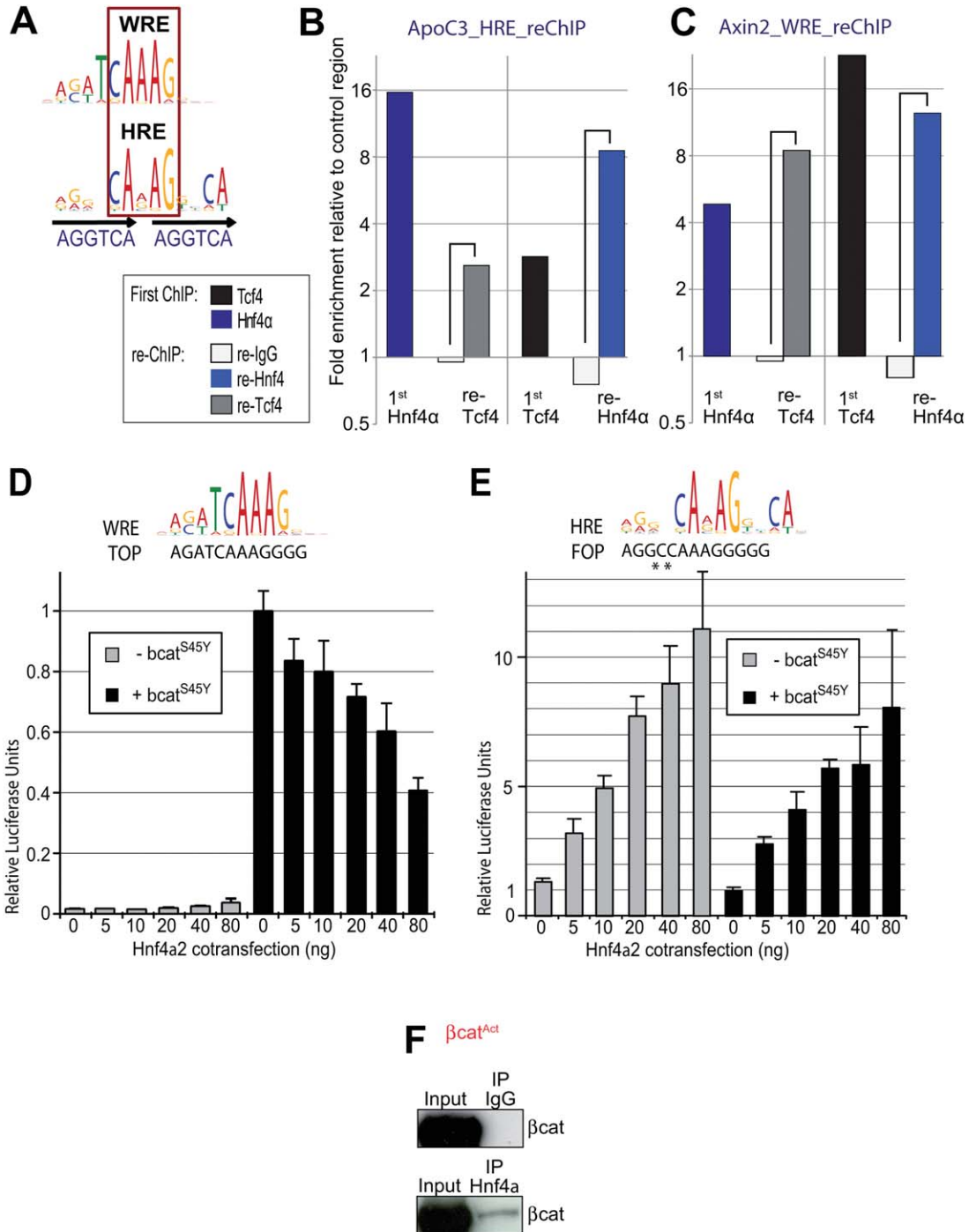


Fig. 6. Interactions between Hnf4- α , Tcf-4, and β -catenin drive the functional antagonism between Hnf-4 α and β -catenin. (A) Consensus WRE and HRE. (B and C) Re-ChIP assays on WT hepatocytes: legend below (A). One mouse was used on which Hnf-4 first ChIP was performed on the whole preparation of hepatocytes; then, the two re-ChIPs against IgG and Tcf-4 were performed from the first ChIP. Another mouse was used for Tcf-4 in the first ChIP, and then Hnf-4 and IgG in the re-ChIP. (D and E) Luciferase reporter assays: Cotransfection of β -catenin represses Hnf-4 α -induced luciferase activity from an HRE-containing plasmid (FOP reporter); cotransfection of Hnf-4 α represses β -catenin-induced luciferase from a WRE-containing plasmid (TOP reporter). Data represent the mean \pm standard error of the mean (n = 3). (F) Co-IP of β -catenin with Hnf-4 α and not with IgG in β -catenin-activated hepatocyte nuclear extracts. Blottings shown are representative of two separate experiments. WT, wild type.

commonly down-regulated in *Apc*^{ko} livers and CTNNB1-mutated HCCs (Fig. 7B).

We asked to what extent gene deregulations could have metabolic consequences. We performed a metabo-

mic experiment to measure metabolite concentrations in *Apc*^{ko}, compared to control, livers. We found that several metabolic pathways were deregulated. From our previous results, the striking up-regulation of the

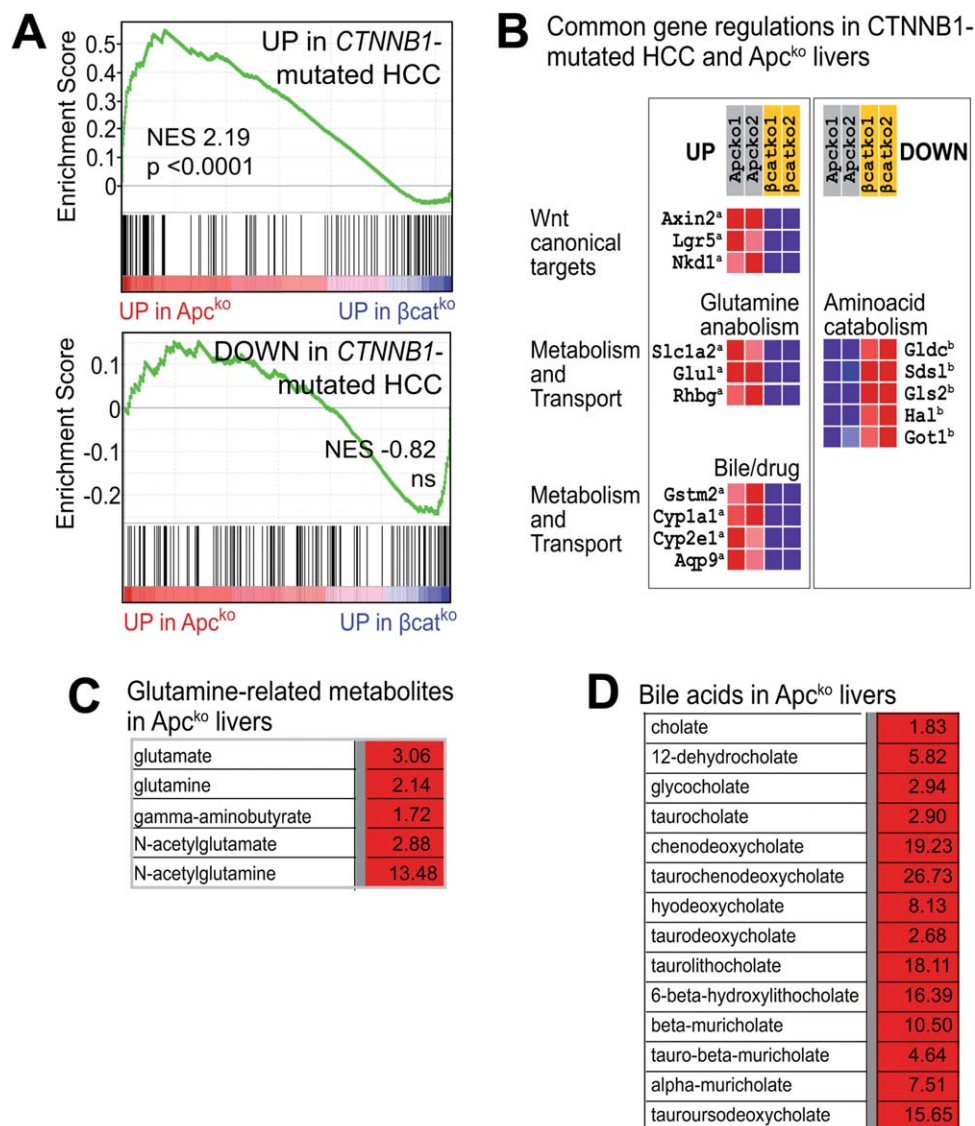


Fig. 7. Common metabolic and genic regulations in CTNNB1-mutated HCCs and *Apc*^{ko} livers. (A) GSEA: Genes up- or down-regulated in both *Apc*^{ko} hepatocytes and in CTNNB1-mutated HCCs. (B) Extraction from (A) of the most informative common gene regulations (in red, up-regulations; in blue, down-regulations). The letter in superscript in (B) corresponds to the following: a, direct positive target of β -catenin; b, direct negative target. (C and D) Amount of metabolites in *Apc*^{ko} livers, expressed as a ratio relative to control livers (in red, significant accumulation with $P < 0.001$; in green, significant decrease with $P < 0.001$).

glutamine pathway was expected (Fig. 7C).³ More interesting, a dramatic accumulation of bile acids in *Apc*^{ko} livers was found (Fig. 7D). There was also a significant decrease in long-chain fatty acids (Fig. 8C), associated with a lower level of triglycerides (TGs), and a lower quantity of lipid droplets (Fig. 8A,B). These features correlate with the decreased expression of the lipogenic adenosine triphosphate citrate lyase and *Scd* genes (Figs. 4A,B and 5D). There was also less very-low-density lipoprotein (VLDL) secretion (Fig. 8B), correlating with the lower level of *Apoc3* transcription (Figs. 4B and 5D), suggesting a diminished transfer of TGs from the liver. This would mean that the lack of steatosis is not the result of an

increased export of lipids, but rather to decreased lipogenesis, together with an enhanced commitment of cholesterol toward bile acid synthesis. The fact that *Apc*^{ko} liver accumulates bile acids and is less prone to store TGs is reminiscent of CTNNB1-mutated HCCs, being cholestatic and never steatotic. We found, in both *Apc*^{ko} liver and CTNNB1-mutated human HCCs, a common up-regulation of two master genes for bile acid synthesis (*Cyp7a1* and *Cyp27a1*) and of the CAR, known for its involvement in bile acid synthesis²² (Fig. 8D,E). It appears that *Cyp27a1* is a direct target of β -catenin (Supporting Table 3), whereas *Cyp7a1* is not. Because the main transcriptional regulator of *Cyp7a1* expression is a Rev-erb/Lxr (liver X receptor)

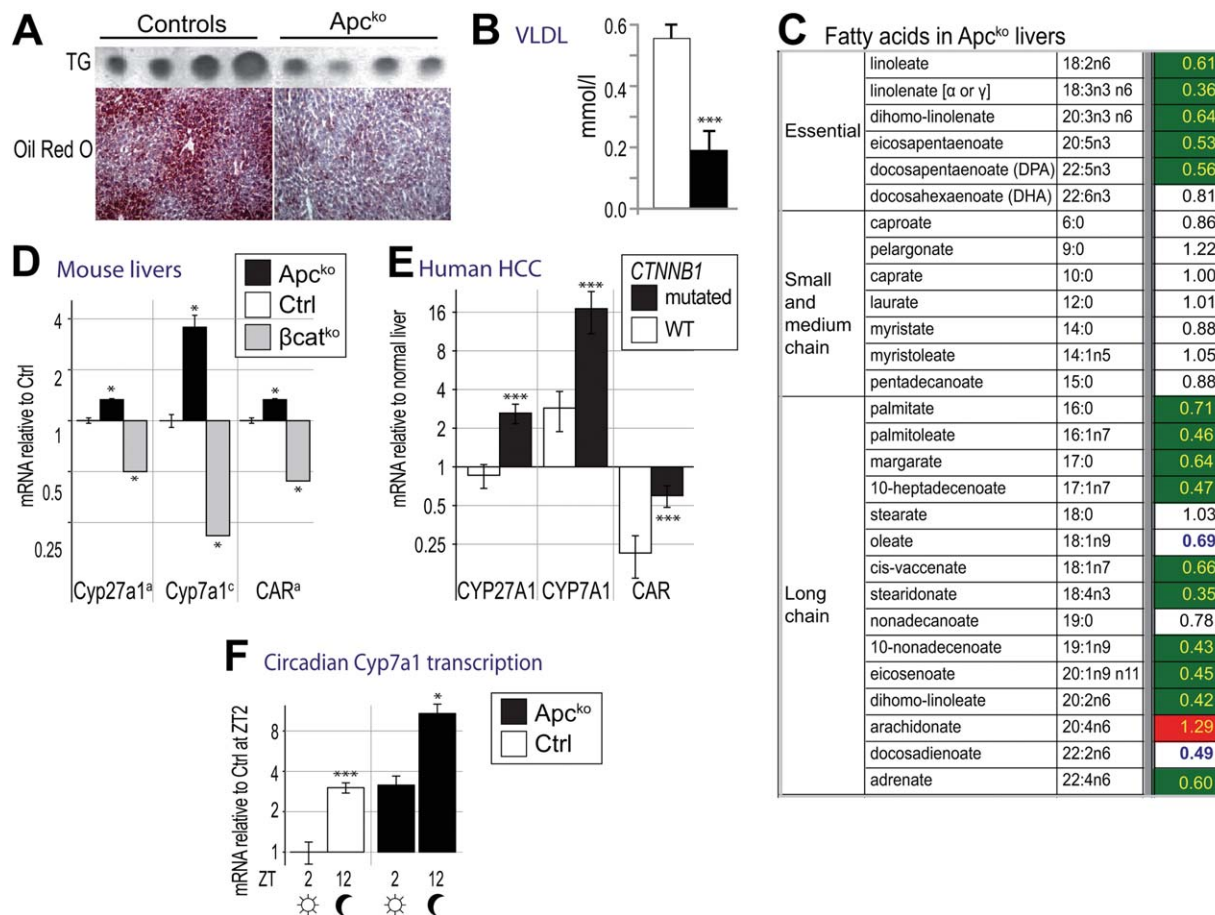


Fig. 8. Common regulations of lipid metabolism in CTNNB1-mutated HCCs and Apc^{ko} livers. (A) Chromatography of lipids in Apc^{ko} livers, showing a decrease in TG storage. Below, Oil Red O staining on a representative liver section (from four experiments each in Apc^{ko} and control conditions). (B) Serum VLDL from Apc^{ko} mice. Data represent the mean \pm standard error of the mean (SEM; $n = 6$ for each group of mice). (C) Amount of metabolites in Apc^{ko} livers, expressed as a ratio relative to control livers (in red, significant accumulation with $P < 0.001$; in green, significant decrease with $P < 0.001$; in bold blue, significant decrease with $P < 0.01$). Data represent the mean \pm SEM ($n = 6$ for each group of mice). (D-F) qPCR analysis of mRNA expression. The letter in superscript in (D) corresponds to the following: a, direct positive target of β -catenin; b, direct negative target; c, indirect positive target. ZT, Zeitgeber time; ZT2 corresponds to 2 hours after the light was turned on, and ZT12 corresponds to the time at which the light was switched off. Data represent the mean \pm SEM ($n = 6$ for each group of mice, $n = 20$ for CTNNB1-WT, and $n = 26$ for CTNNB1-mutated human HCCs, with two pools of normal livers for normalization). (A-D) Experiments were performed in refed conditions, in which lipogenesis is stimulated (24-hour fasting followed by 18-hour feeding with high glucose).

cascade that occurs in a circadian-dependent manner, we wondered whether the control of Cyp7a1 by β -catenin was dependent on the circadian clock.²³ We found that β -catenin up-regulates Cyp7a1 independently of the light/dark cycle and thus does not implicate the Rev-erb/LXR cascade (Fig. 8F). Thus, oncogenic β -catenin has a dramatic effect on bile acid synthesis and accumulation, at least through its direct control over Cyp27a1 and CAR transcription and indirectly over Cyp7a1 transcription.

Discussion

Through our genome-wide studies, we propose that β -catenin imposes unique transcriptional properties on Tcf-4 that pattern liver zonation (Supporting Fig. 7). In the presence of β -catenin, Tcf-4 binds to WREs,

whereas independently of the former it is recruited to HREs. Tcf-4 was thought to bind to WREs, even in the absence of β -catenin, recruiting corepressors such as members of the groucho/transducin-like enhancer of split family (as reviewed previously⁵). However, there had been no ChIP sequencing study performed both *in vivo* and comparing a state of β -catenin activation to one of β -catenin inactivation to confirm this model. Our ChIP sequencing study suggests that β -catenin should be able to unlock chromatin over WREs and make them accessible to Tcf-4. Many studies have shown the recruitment of β -catenin in chromatin remodeling complexes (as reviewed previously²⁴), such as histone acetylases (CREB-binding protein/p300), histone-remodeling factors (Brahma-related gene 1 and ISW1), or the histone methyltransferase, SET1.

Therefore, we can hypothesize that β -catenin, thanks to these different complexes, could create a chromatin context favorable or not to TCF-4 recruitment. These complexes remain to be identified in the liver to investigate which one is a prerequisite for DNA occupancy by Tcf-4. Our results also show that Tcf-4 is an opportunistic transcriptional partner that shifts between β -catenin and Hnf-4 α , a capacity that has been proposed for Tcf-3 in embryonic stem cells.²⁵ Independently of β -catenin, Tcf-3, together with Nanog, SRY (sex determining region Y)-box 2, and octamer-binding transcription factor 4, co-occupies gene promoters that lack WREs.²⁵ We propose such a mechanism that ensures cell-context specificity, with Tcf-4 mediating the repressor or activator effects of β -catenin through its interactions either with β -catenin or with Hnf-4 α in the absence of β -catenin. In this model, we propose a new way by which β -catenin signaling could direct both hepatospecific transcription and gene repression, still poorly understood even if considered as a prerequisite for development.²⁶

Hnf-4 α in β -Catenin-Dependent Zonal Metabolism. Hnf-4 α is a master liver TF because it binds to 12% of hepatic promoters.²⁷ Moreover, it can be considered as a transactivator of PP genes because the Hnf-4 α -null liver loses some PP markers and adopts a discrete PC phenotype.²⁸ Last, Hnf-4 α has been linked molecularly with the β -catenin pathway in intestine and liver.^{21,29} However, no mechanistic studies have explained how Hnf-4 α exerts its PP-specific effects while antagonizing those of β -catenin.^{30,31} Because we found no zone-specific differences in Hnf-4 α immunolocalization (data not shown), the zonal effect of Hnf-4 α is not the result of zone-specific distribution. Instead, the equilibrium that we propose here for Hnf-4 α , Tcf-4, and β -catenin, which involves DNA-binding and protein-protein interactions, explains how Hnf-4 α acts as a PP gene transactivator and a PC gene transrepressor.

Tcf-4/TCF7L2 and Liver Metabolism. The strong effect that Tcf4/ β -catenin exerts on metabolic genes can partly explain why Tcf-4 (*TCF7L2* gene) is a type 2 diabetes gene candidate in humans.³² Recently, it has been shown that this role for *TCF7L2* is related to its hepatic expression.¹² *HNF4A* is also an MODY1 diabetes susceptibility gene in humans and strongly controls both glucose and lipid metabolism.³³ Therefore, it is not surprising that the β -catenin pathway has also been implicated in the hepatic production of glucose,^{34,35} which is consistent with our findings. Here, we propose that a Tcf-4/Hnf-4 α association antagonized by β -catenin dictates PP gene expression to allow not only hepatic glucose production, but also

lipogenesis through the activation of several key genes. These genes include *ACLY* and *SCD1*, which are involved in critical steps of fatty acid synthesis. This makes both *TCF7L2* and β -catenin highly relevant to the study of diabetes and obesity.

Consequences on Liver Carcinogenesis. Human *CTNNB1*-mutated HCCs are typically characterized by the absence of steatosis and the presence of cholestasis.⁷ We also previously showed that β -catenin could elicit an energetic shift toward aerobic glycolysis (i.e., the so-called “Warburg” effect³⁴). Accordingly, we found an increase in *Pkm2* gene transcription in *Apc*^{ko} livers, but this effect is not specific for β -catenin pathway because HCC did not differ in glycolytic gene expression, depending on *CTNNB1* mutations (data not shown). The cholestatic and nonsteatotic phenotypes can be linked to β -catenin negative effect on lipogenesis and to its positive effect on bile acid and cholesterol metabolism, as supported by our present findings and by those of others.³⁶ However, a previous study showed that the loss of liver β -catenin induced bile canalicular abnormalities associated with a higher hepatic total bile acid level as a result of alterations in bile secretion.³⁷ This phenotype is likely to be the result of a developmental defect in hepatic architecture because this knockout was performed perinatally, whereas bile synthesis is a transcriptional consequence of β -catenin signaling. We have identified two cascades that could shed light on these β -catenin-mediated effects. First, the β -catenin/Tcf-4/Hnf-4 α connection makes sense with the recent realization of Hnf-4 α as a tumor suppressor in the liver.³⁸ Second, AhR and CAR xenobiotic nuclear receptor genes are direct positive targets of β -catenin, corroborating previous reports,³⁹ and this indicates that bile acid/drug/stress metabolisms are major downstream targets that are activated by β -catenin in the liver. Thus, our results strongly support a prominent role for β -catenin in the metabolic reprogramming of HCC and support further investigation of this relationship.

Acknowledgment: The authors warmly thank Pascale Bossard for her critical reading of the manuscript. The authors thank Dr A. Ribeiro for the gift of Hnf-4 α 2 expression vector and for helpful discussion. The authors are grateful to I. Lagoutte and the staff of Cochin’s animal housing facility. ENCODE H3K27Ac and Pol II data were generated and analyzed in Bing Ren’s laboratory at the Ludwig Institute for Cancer Research (University of California San Diego, La Jolla, CA) and the DHS at the University of Washington (UW ENCODE group; Seattle, WA).

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4) Article :

Antitumour activity of an inhibitor of miR-34a in liver cancer with β -catenin-mutations.

Gougelet, A., Sartor, C., Bachelot, L., Godard, C., Marchiol C, Renault G, Tores F, Nitschke P, Cavard C, Terris B, Perret C, Colnot S. (2015). Gut

ORIGINAL ARTICLE

Antitumour activity of an inhibitor of miR-34a in liver cancer with β -catenin-mutations

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► Additional material is published online only. To view please visit the journal online (<http://dx.doi.org/10.1136/gutjnl-2014-308969>).

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Received 5 December 2014
Revised 16 February 2015
Accepted 18 February 2015

ABSTRACT

Objective Hepatocellular carcinoma (HCC) is the most prevalent primary tumour of the liver. About a third of these tumours presents activating mutations of the β -catenin gene. The molecular pathogenesis of HCC has been elucidated, but mortality remains high, and new therapeutic approaches, including treatments based on microRNAs, are required. We aimed to identify candidate microRNAs, regulated by β -catenin, potentially involved in liver tumorigenesis.

Design We used a mouse model, in which β -catenin signalling was overactivated exclusively in the liver by the tamoxifen-inducible and Cre-Lox-mediated inactivation of the *Apc* gene. This model develops tumours with properties similar to human HCC.

Results We found that miR-34a was regulated by β -catenin, and significantly induced by the overactivation of β -catenin signalling in mouse tumours and in patients with HCC. An inhibitor of miR-34a (locked nucleic acid, LNA-34a) exerted antiproliferative activity in primary cultures of hepatocyte. This inhibition of proliferation was associated with a decrease in cyclin D1 levels, orchestrated principally by HNF-4 α , a target of miR-34a considered to act as a tumour suppressor in the liver. In vivo, LNA-34a approximately halved progression rates for tumours displaying β -catenin activation together with an activation of caspases 2 and 3.

Conclusions This work demonstrates the key oncogenic role of miR-34a in liver tumours with β -catenin gene mutations. We suggest that patients diagnosed with HCC with β -catenin mutations could be treated with an inhibitor of miR-34a. The potential value of this strategy lies in the modulation of the tumour suppressor HNF-4 α , which targets cyclin D1, and the induction of a proapoptotic programme.

INTRODUCTION

Liver cancer is the third leading cause of cancer-related death worldwide. Hepatocellular carcinoma (HCC) is the most frequent form of primary liver cancer, which mostly affects men with cirrhosis. HCC frequently appears in a context of chronic liver disease caused by infection with the hepatitis B and C viruses (HBV and HCV, respectively), alcohol consumption, obesity or genotoxic exposure (ref. 1 for a review). We, and others, have shown that mutations of the *CTNNB1* gene, encoding β -catenin, are observed in 15%–40% of

Significance of this study**What is already known on this subject?**

Hepatocellular carcinoma (HCC) is the most common form of primary liver cancer and the third leading cause of death by cancer worldwide, due to the lack of effective treatments. We, and others, have shown that mutations of the *CTNNB1* gene, encoding β -catenin, are observed in 15%–40% of HCCs. HCCs with β -catenin mutation constitute one of the two subclasses of HCC, and are thought to follow a particular pattern of pathogenesis.

What are the new findings?

Using a transgenic mouse model developing tumours with β -catenin activation, we provide the first evidence of an oncogenic role of miR-34a in liver tumours with β -catenin mutations. An inhibitor against miR-34a slows tumour progression, and inhibits the development of new tumours in this model.

How might it impact on clinical practice in the foreseeable future?

These findings call into question the preclinical trial designed by miRNA therapeutics, using miR-34a mimics to combat liver tumour growth. Instead, we provide here proof-of-concept that a locked nucleic acid-based inhibitor of miR-34a may be useful for treatment exclusively in HCCs with β -catenin mutation.

HCCs.^{2,3} HCCs with β -catenin mutation constitute one of the two subclasses of HCC^{4,5} and are thought to follow a particular pattern of pathogenesis, as most are well differentiated, cholestatic, and with a better prognosis.⁶ Several years ago, we engineered a mouse model in which β -catenin signalling was overactivated exclusively in the liver by the tamoxifen-inducible and Cre-Lox-mediated inactivation of the *Apc* (*Apc*^{KO}) gene encoding a tumour suppressor, which is an inhibitor of the Wnt/ β -catenin pathway, and the liver 'zonation-keeper'.^{7,8} In this model, the absence of *Apc* in single hepatocytes results in the development of β -catenin-activated liver tumours with properties

To cite: Gougelet A, Sartor C, Bachelot L, et al. Gut Published Online First: [please include Day Month Year] doi:10.1136/gutjnl-2014-308969

similar to those observed in human patients.⁸ If *Apc* is deleted in all hepatocytes, the β -catenin signalling in hepatocytes leads to the induction of a transcriptional programme very similar to that of human HCC with β -catenin mutation.^{8,9}

The molecular pathogenesis of HCC has been elucidated,¹⁰ but the incidence and mortality of HCC remain high, despite the use of the tyrosine kinase inhibitor sorafenib. Different therapeutic strategies could, therefore, be used to target tumours in accordance with their pathogenic pathways. MicroRNAs (miRNAs) have recently emerged as potent diagnostic and prognostic biomarkers, and numbers of reports devoted to miRNAs have shown that these molecules act as oncogenes or tumour suppressors.¹¹ The global analysis of the miRNAs expressed in liver tumours highlighted a miRNA signature common to all types of HCC.^{12–14} A loss of miR-122,¹³ miR-29,¹⁵ and miR-124¹⁶ has been described. Inversely, some oncogenic miRNAs have been identified: miR-21,¹⁷ miR-221/miR-222,¹⁸ and miR-224.¹⁹ Despite the number of global analyses, the role of miR-34a in liver tumorigenesis remains unclear. Even if this miRNA is currently described as a tumour suppressor in various cancers,²⁰ two studies suggested that miR-34a levels decrease in HCCs, mainly HBV+,^{21,22} while other studies reported an induction of miR-34a, particularly in HCC with β -catenin mutations.^{17,18}

We aimed to identify candidate miRNAs, regulated by β -catenin, potentially involved in liver tumorigenesis. We observed that miR-34a was overexpressed in response to β -catenin overactivation; the levels of miR-34a being inversely correlated with those of HNF-4 α (hepatocyte nuclear receptor α), another key factor for hepatocyte differentiation which we have shown to antagonise β -catenin function.⁹ MiR-34a induction was observed specifically in mouse and human liver tumours with β -catenin mutations, and miR-34a was found to play an oncogenic role in the liver. These findings call into question the systematic use of miR-34a mimics to combat liver tumour growth (<http://www.mirnarx.com/pipeline/mirna-MRX34.html>). Instead, we provide here proof-of-concept that a locked nucleic acid (LNA)-based inhibitor of miR-34a may be useful for treatment exclusively in HCCs with β -catenin mutation.

METHODS

Animals and reagents

Transgenic mice, liver sampling and immunostaining have been described elsewhere^{7,9,23} and have also been detailed in the supplementary materials.

Hepatocyte isolation

To directly lyse hepatocytes after collagenase perfusion, that is, for RNA or protein extraction, either transgenic or wild-type (wt) livers were perfused 6 days after tamoxifen injection (1.5 mg) as previously described.^{9,23} Non-parenchymal cells were excluded in the supernatant, and hepatocytes preparations were used for subsequent experiments. Periportal (PP) and pericentral (PC) subpopulations of wild-type (wt) hepatocytes were isolated by perfusion with digitonin and collagenase, as previously reported.²⁴

Cell culture

For primary culture of hepatocytes, livers were perfused 3 days after tamoxifen injection to *Apc*^{lox/lox} mice, the cells were dispersed in William's medium (supplemented with 10% fetal bovine serum, penicillin-streptomycin, fungizone, 25 nM dexamethasone, insulin 4 μ g/mL, and 1% Bovine Serum Albumin), and plated in wells coated with rat-tail collagen I (Gibco).

Transfection assays

The cells were transfected 4 h after plating, as described in the supplementary materials.

xCELLigence assay

Hepatocyte adhesion and cell cycle progression were measured with the xCELLigence system (Roche). Cells were transfected with 100 nM LNA or mimic 4 h after plating, as described above (see supplementary materials). The impedance was then measured for 48 h.

Western blotting

The membrane with 50 μ g proteins was probed overnight with a primary antibody against the protein of interest (see online supplementary materials), and then incubated with a HRP-conjugated secondary antibody (Cell Signaling) for detection with the Enhanced ChemiLuminescence system (Biorad).

In situ hybridisation

Sections were deparaffinised and treated with 0.1 mg/mL proteinase K, then hybridised for 1 h at 55°C with 100 nmol digoxigenin-labeled-LNA-scramble or LNA-34a (Exiqon). Signals were detected with anti-digoxigenin (Roche) and Nitro Blue Tetrazolium/5Bromo-4Chloro-3Indolyl phosphate (Roche).

Samples processing for miRNA-seq

Total RNA was extracted from purified hepatocytes six days after tamoxifen injection with Trizol reagent (Life Technologies). For each set of conditions, we analysed four distinct hepatocyte preparations by deep sequencing on an Illumina platform (Fasteris, Switzerland). The reads generated by the HiSeq were analysed with miRDeep2 and miRAnalyzer software (see online supplementary materials).

Chromatin sonication and immunoprecipitation

The protocol for Chromatin Immunoprecipitation (ChIP) was adapted from that of Nelson,²⁵ as previously described⁹ (see online supplementary materials).

Human samples

In this study, we included 44 patients treated for liver cancer at Cochin hospital (see online supplementary table S3). All tumour samples were frozen after surgery.

RNA extraction and RT-qPCR

Total RNA was extracted from purified hepatocytes with Trizol reagent (Life Technologies). Levels of miRNA were determined on 2 ng total mRNA, with a specific Taqman miRNA assay (Applied Biosystems). Levels of cyclin D1 and HNF-4 α were determined on 100 ng total mRNA relative to 18S RNA (see online supplementary materials).

Statistical analysis

We assessed the significance of differences between two groups of samples in Wilcoxon tests if $n < 30$, and Student's *t* tests if $n > 30$. Analysis of variance was used to compare three groups of samples. $p < 0.05$ Was considered statistically significant.

RESULTS

MiR-34a is induced following β -catenin activation

We searched for candidate miRNAs regulated by β -catenin in hepatocytes by deep-sequencing the miRNAs expressed in the pretumoral *Apc* deletion (*Apc*^{KO}) model in which there is pan-

lobular β -catenin overactivation, comparing the results with those for wt hepatocytes. A statistical analysis showed that 123 miRNAs discriminated between Apc^{KO} and wt hepatocytes (see online supplementary tables S1 and S2), and that these miRNAs mostly targeted the Wnt/ β -catenin pathway (see online supplementary figure S1). We confirmed that miR-375 levels were significantly decreased by β -catenin activation (see online supplementary table S2), as reported in HCCs with β -catenin mutation.¹³ We also identified 54 miRNAs generated from the imprinted *Dlk1/Gtl2* locus as positive targets of β -catenin (see online supplementary table S1). We discarded this locus for further studies as this is not a relevant target of β -catenin in human HCCs (see online supplementary materials, tables S1, S3 and figure S2). We thus focused on miR-34a which was induced

by a factor of about 3 after β -catenin activation, with respect to wt hepatocytes in the miRNA-seq analysis (see online supplementary table S1) and in qPCR (figure 1A). This miRNA displayed the most significant induction following β -catenin activation after the miRNAs from the *Dlk1/Gtl2* locus, and its overexpression in HCC with β -catenin mutation has already been reported.¹⁸ We collected 24 human tumours with a mutation of *CTNNB1* leading to β -catenin activation, and 20 tumours without *CTNNB1* mutation. This cohort covered the major aetiologies observed for HCC (HBV, HCV, alcohol abuse) (see online supplementary table S3). In our cohort of patients, miR-34a levels were higher in tumours than in normal tissue (by a factor of 2 in the absence of *CTNNB1* mutation, and 5 in the presence of *CTNNB1* mutation, figure 1B); this induction was

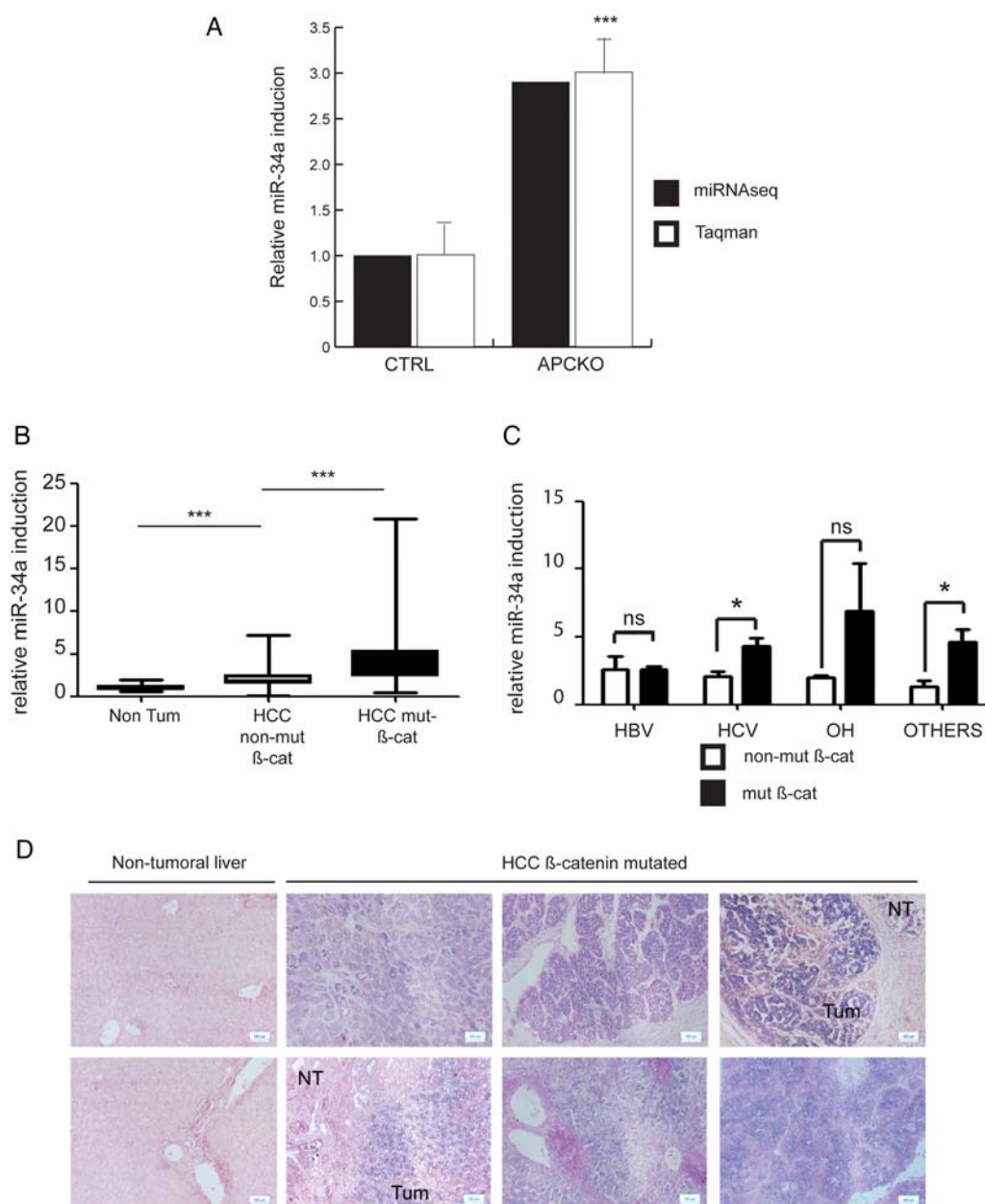
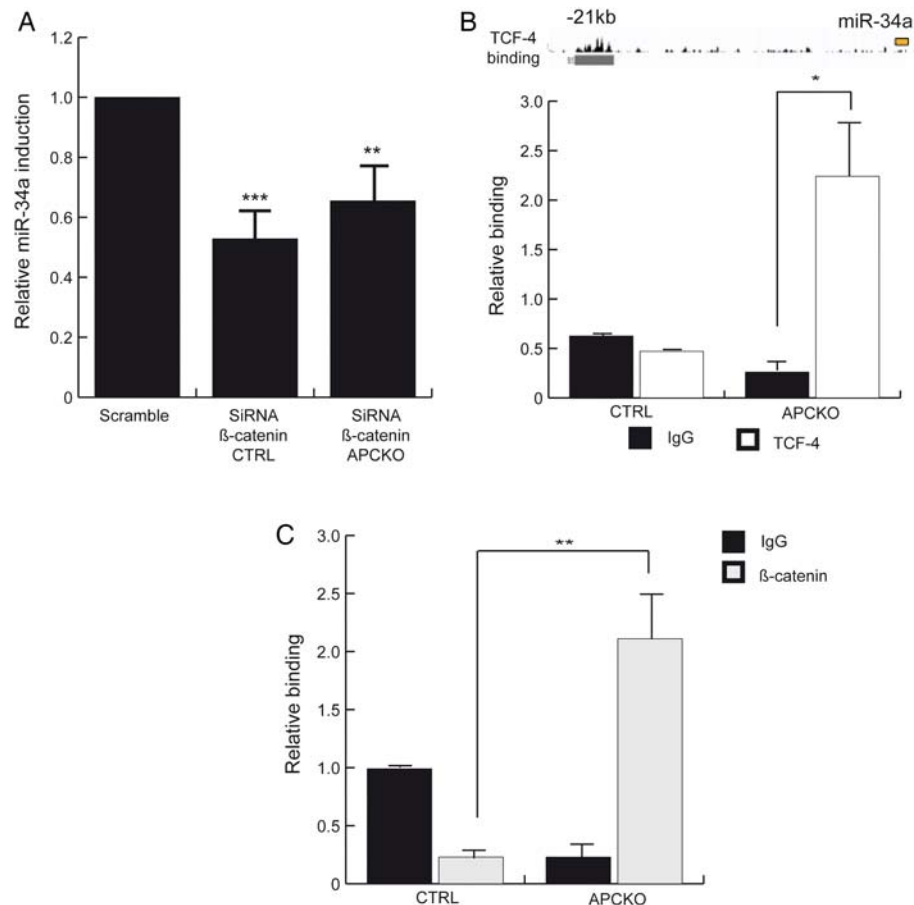


Figure 1 miR-34a is induced following β -catenin activation in mouse hepatocytes and in hepatocellular carcinoma (HCC) samples from patients. (A) miR-34a induction in Apc^{KO} hepatocytes isolated at day 6 after tamoxifen injection in miRNA-seq was confirmed in 15 samples by RT-qPCR relative to snoRNA135. (B and D) miR-34a levels, relative to RNU24 and RNU49 levels, in 24 samples of HCC with β -catenin mutation (mut- β -cat) and 20 samples of non-mutated (non-mut- β -cat) HCC, as compared with normal tissues. OH, alcohol exposure; others, no specific aetiology, ns, non-significant. (C) Detection of miR-34a in HCC samples with β -catenin mutation by in situ hybridisation.

Figure 2 miR-34a is induced following β -catenin activation, through the direct binding of β -catenin. (A) Hepatocytes from wt mice injected with Tamoxifen were used as controls (CTRL). CTRL and *Apc*^{KO} hepatocytes were transfected 3 days after tamoxifen injection with 100 nM of a siRNA against β -catenin for 24 h. Results are shown as the mean RQ (relative quantity) values for miR-34a for five independent experiments \pm SEM, as compared with scramble siRNA. (B and C): T-cell factor 4 (TCF-4) (B) and β -catenin (C) binding to a site 21 kb upstream from the miR-34a promoter in CTRL and *Apc*^{KO} hepatocytes at day 6 after tamoxifen injection, as compared with the isotype control (IgG). Results are expressed as the binding value \pm SEM normalised with respect to three control sites in the *myc* and *Alb* genes. The means of at least three independent experiments are shown.



significantly stronger in tumours with β -catenin mutations ($p < 0.005$). The overexpression of miR-34a in tumours with mutations of β -catenin was correlated with a context of HCV infection or no particular risk factors (figure 1C). The sample size was too small to obtain a significant difference in miR-34a overexpression as a function of alcohol abuse status (figure 1C). The overexpression of miR-34a was confirmed by in situ hybridisation in another panel of tumours with β -catenin mutation (figure 1D). Thus, miR-34a is overexpressed in human HCCs with *CTNNB1* mutations, and could constitute a major mediator of the oncogenic signal initiated by β -catenin activation in hepatocytes.

β -catenin directly regulates miR-34a expression

For confirmation of miR-34a regulation by β -catenin, we transfected isolated wt and *Apc*^{KO} hepatocytes with a siRNA against β -catenin. β -catenin silencing decreased miR-34a levels by about 47% for wt hepatocytes and 33% for *Apc*^{KO} hepatocytes (figure 2A). We thus investigated whether β -catenin could directly control miR-34a expression by ChIP experiments, with an antibody against TCF-4 (T-cell factor 4), the main LEF/TCF factor associated with β -catenin in liver,⁹ and an antibody against β -catenin. We measured the binding of TCF-4 and β -catenin to sites upstream from miR-34a identified as a TCF-4 binding site in our ChIP-seq data.⁹ β -catenin activation led to a significant increase in the binding of TCF-4 (figure 2B), and β -catenin (figure 2C) to a site 21 kb upstream from miR-34a. β -catenin binding was also significantly increased following *Apc* depletion on a more proximal TCF-4 site (see online supplementary figure S3). Overall, our data suggest that an excess of β -catenin could significantly increase miR-34a transcription in hepatocytes.

MiR-34a zonal redistribution after β -catenin overactivation

We then assessed the hepatic distribution of miR-34a by in situ hybridisation. Exceptions, such as cyclin D1,²³ are known, but the direct targets of β -catenin are generally expressed in PC hepatocytes, in which Wnt/ β -catenin signalling is activated.⁷ Counterintuitively, in normal livers, miR-34a was found to be preferentially expressed in hepatocytes around the portal vein and, more particularly, in bile duct cells (figure 3A). For confirmation of this prevalence of miR-34a in the PP subpopulation, we isolated PP hepatocytes and PC hepatocytes separately by injecting digitonin into the central vein and the portal vein, respectively. This differential perfusion also showed that PP samples were enriched in miR-34a (figure 3B). The results were non-significant, since using this technique, biliary cells are mainly lost during washing steps. Additionally, β -catenin overactivation led to a loss of miR-34a zonation, and to miR-34a expression in all hepatocytes (figure 3A). In conclusion, miR-34a appears to be expressed in hepatocytes throughout the liver only when β -catenin is overactivated.

We then searched the DIANA database (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/index), a database with experimentally validated targets for miRNAs, for potential targets of miR-34a. A number of potent targets have been identified (see online supplementary table S4), but we focused on three interesting proteins: LEF-1, a transcription factor associated with β -catenin, caspase 2, recently identified as a direct target of miR-34a in the liver,²⁶ and HNF-4 α , which we recently showed to be an antagonist of β -catenin in the liver.⁹ These opposing activities result in a reciprocal physiological distribution in the liver, with miR-34a found in biliary cells (figure 3A) and HNF-4 α in hepatocytes, consistent with

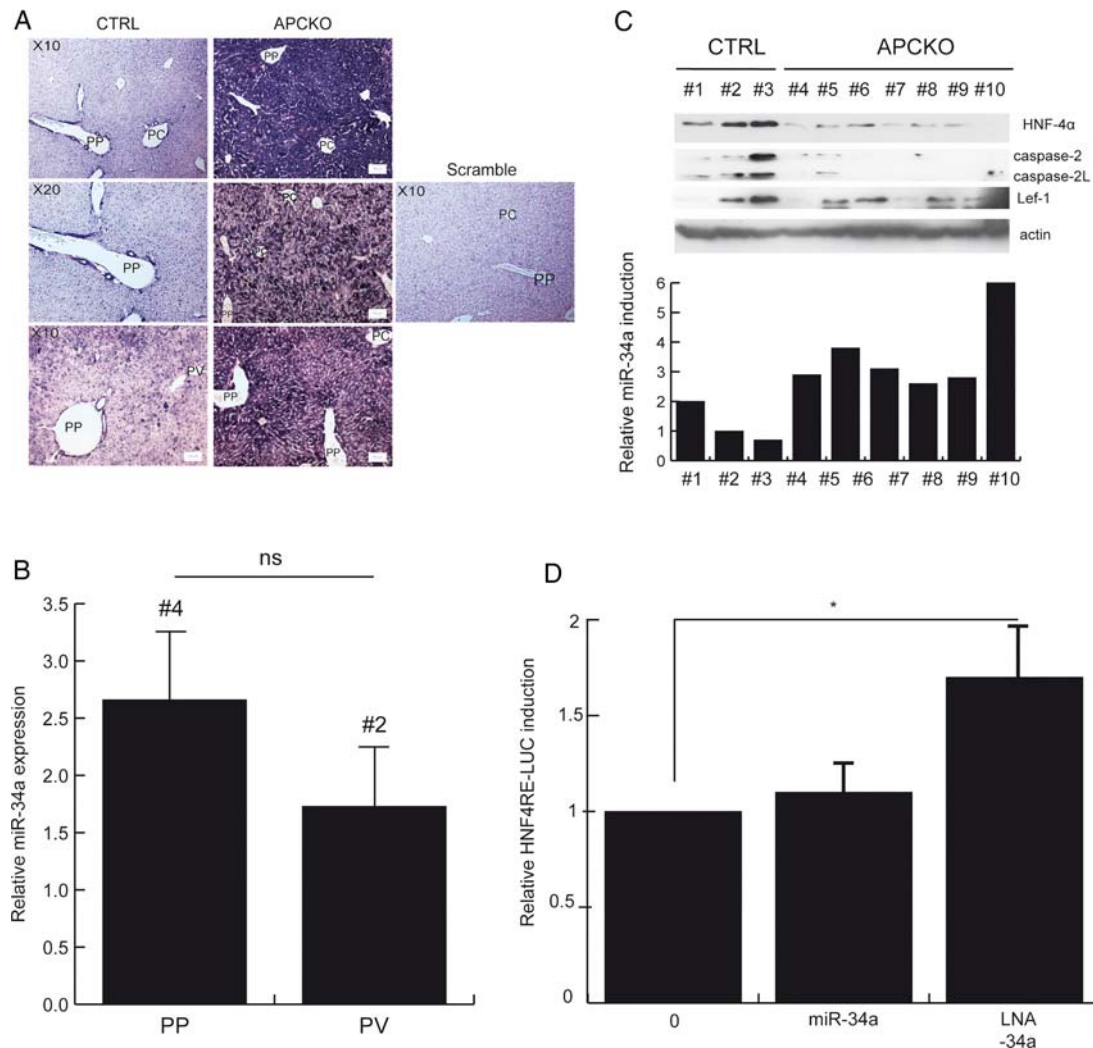


Figure 3 (A and B) miR-34a induction following β -catenin activation is associated with lower HNF-4 α level and activity. A: miR-34a localisation by in situ hybridisation with locked nucleic acid (LNA)-34a in comparison with a scramble LNA. PP, periportal zone; PC, pericentral zone. (B) miR-34a Level assessed by RT-qPCR on wt hepatocytes isolated by digitonin/collagenase perfusion, and expressed as previously. ns, non-significant. # Represents the number of samples tested. (C) HNF4 α protein expression in comparison with miR-34a levels in Apc^{KO} hepatocytes, by comparison with CTRL hepatocytes. # Represents the sample number. (D) Apc^{KO} hepatocytes transfected with the FOP construct (HNF-4 α response element), with LNA-scramble or LNA-34a or a mimic of miR-34a. Results are expressed as the mean luciferase induction of three independent experiments carried out in duplicate.

the hypothesis that miR-34a could repress HNF-4 α expression in biliary cells.²⁷

MiR-34a inhibition improves HNF-4 α transactivation capacity in hepatocytes displaying β -catenin-activation

We confirmed by western blotting that the levels of HNF-4 α , caspase 2, and LEF-1 proteins (figure 3C) were inversely correlated with miR-34a levels in Apc^{KO} mice. Similarly, the overexpression of miR-34a in human HCCs with *CTNNB1* mutations was also associated with weaker immunostaining for HNF-4 α in these tumours (see online supplementary figure S4). In a previous study, we showed that β -catenin impaired the transcriptional activity of HNF-4 α on a luciferase reporter gene under the control of an HNF-4 α -response element (FOP for Far-from-Optimal Tcf-binding site).⁹ Having shown that β -catenin induces miR-34a, which could, in turn, regulate HNF-4 α levels, we investigated the involvement of miR-34a in the antagonism between HNF-4 α and β -catenin in Apc^{KO} hepatocytes. An inhibitor of miR-34a based on LNA technology (LNA-34a), which consists of the complementary sequence for miR-34a

with a methylene group in the ribose, significantly activated the FOP reporter (figure 3D). A mimic of miR-34a had no effect, probably due to the maximal miR-34a overexpression in Apc^{KO} hepatocytes (figure 3C). In conclusion, miR-34a represses HNF-4 α transcriptional activity, and appears to be involved in the β -catenin/HNF-4 α antagonism in our model of β -catenin overactivation.

MiR-34a is induced in tumours displaying β -catenin activation

We then assessed the level of miR-34a in liver tumours mutated for β -catenin in mice. We compared 35 tumours with seven normal tissues obtained from wt mice that had received tamoxifen injections. MiR-34a levels in these tumours were twice those in normal tissues (figure 4A). HNF-4 α mRNA levels in these tumours were also half those in normal tissues (figure 4B), confirming its tumour suppressor role in the liver.^{28 29} Interestingly, the tumours with the highest miR-34a levels also had the lowest HNF-4 α levels (figure 4C, D). The deregulation of miR-34a in these tumours was independent of p53 mRNA levels (see online

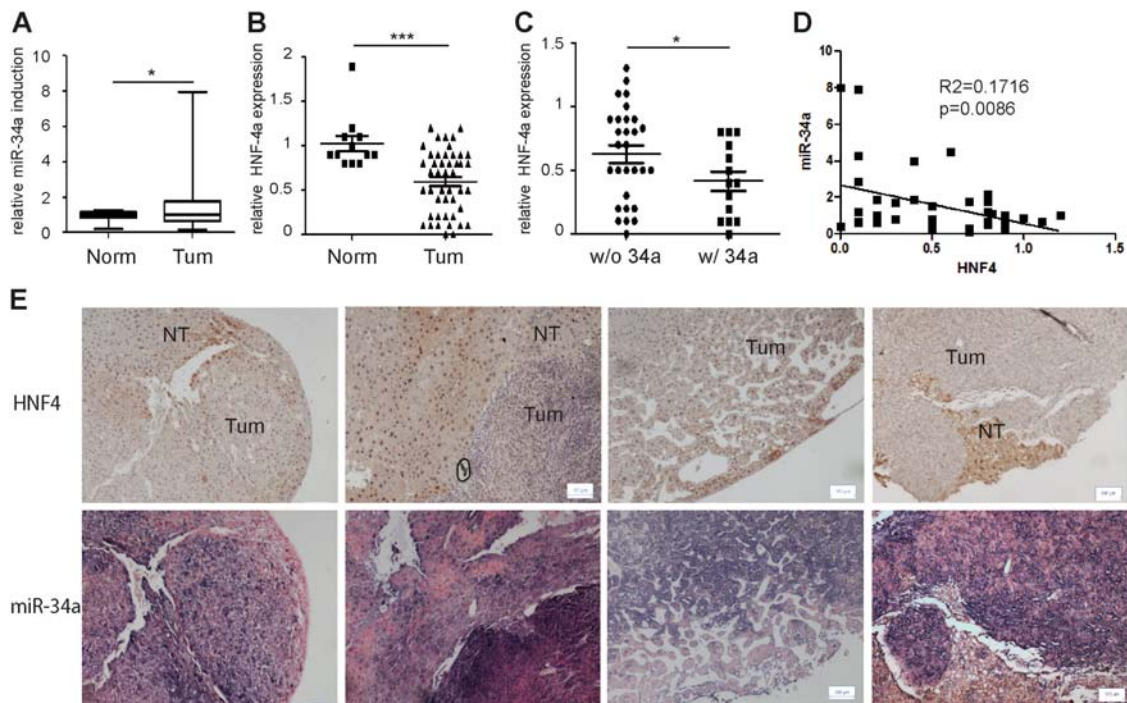


Figure 4 miR-34a is induced in tumours from *Apc*^{KO} mice. (A) miR-34a levels in 42 tumours with β -catenin mutations are compared with those for normal tissues, and are expressed as previously described. (B–D) HNF-4 α levels, relative to 18S, are determined by qPCR, and represented as previously, as compared with non-tumour tissues. (D) Correlation between miR-34a and HNF-4 α RNA levels. (E) Correlation between miR-34a by in situ hybridisation and HNF-4 α by immunohistochemistry in *Apc*^{KO} tumours. NT, non-tumoural tissue; Tum, tumour.

supplementary figure S5), despite miR-34a being a target of p53.³⁰ Finally, we confirmed that overexpression of miR-34a in tumours with β -catenin overactivation by in situ hybridisation was associated to a loss of HNF-4 α (figure 4E).

MiR-34a inhibition by a LNA

These data highlight the unique oncogenic role of miR-34a in a context of β -catenin overactivation. This led us to try to block miR-34a activity with a LNA, which has proved promising in human liver disease.³¹ We assessed the efficacy of this approach in cultures of hepatocytes isolated from wt and *Apc*^{KO} mice. No change in the morphology of hepatocytes transfected with LNAs was observed on microscopy 72 h after transfection (data not shown). The efficacy of LNA-34a was assessed by cotransfection with a luciferase reporter construct with a miR-34a binding site in its 3'UnTranslated Region (UTR). As expected, following cotransfection with LNA-34a, miR-34a activity was impaired, resulting in an increase in luciferase activity (about three times higher than that obtained after cotransfection with LNA-scramble, figure 5A). The LNA efficacy was also confirmed by western blotting for HNF-4 α : HNF-4 α levels were higher in the presence of LNA-34a (figure 5B).

LNA-34a impairs hepatocyte proliferation after β -catenin overactivation

We then investigated the effect of LNA-34a on the proliferation of hepatocytes. We used the xCELLigence system which measures cell impedance in real time through the use of gold microelectrodes placed under each well. WT hepatocytes underwent one round of cell division, due to the proliferative effect of tamoxifen and collagenase perfusion (see online supplementary figure S6A). WT hepatocytes had a lower cell proliferation index than *Apc*^{KO} hepatocytes (see online supplementary figure S6A), confirming that an absence of *Apc*

leads to hepatocyte hyperproliferation.⁸ Transfection with LNA-34a significantly impaired *Apc*^{KO} hepatocyte proliferation (35% inhibition), but had no effect on wt hepatocytes (figure 5C). Similar results were obtained with the Cell Titer assay, measuring the reduction of tetrazolium in formazan, a process dependent on NADPH (data not shown). The activity of lactate dehydrogenase in cell supernatant was not altered following LNA-34a transfection, suggesting an absence of LNA-34a toxicity (see online supplementary figure S6B). This suggests that LNA-34a has a specific antiproliferative effect on hepatocytes displaying β -catenin overactivation. Additionally, the LNA-34a transfected over 3 days successively increased the amount of caspase 2 propeptide, as well as caspase 2 L, its proapoptotic isoform, and also decreased the level of full-length PARP-1, suggesting its increased cleavage (figure 5D). This was not observed for wt hepatocytes (see online supplementary figure S6C). In vivo, the LNA-34a injected in mice only increased caspase 2 L level in *Apc*^{KO} mice (see online supplementary figure S6D). In conclusion, LNA-34a promotes a proapoptotic signal in the *Apc*^{KO} hepatocytes.

Cyclin D1 has been identified as a target of miR-34a.³² We therefore assessed cyclin D1 levels after transfection with LNA-34a. Contrary to expectations, cyclin D1 protein and mRNA levels at a lower extent were decreased in the presence of LNA-34a (figure 5E). However, this is consistent with the antiproliferative effects of LNA-34a on *Apc*^{KO} hepatocytes (figure 5C). The loss of cyclin D1 may be a consequence of the increase in HNF-4 α amount in the presence of LNA-34a (figure 5B), as HNF-4 α has been shown to repress cyclin D1³³ (figure 6E). In agreement with this hypothesis, no change in cyclin D1 protein and mRNA levels was observed when *Apc*^{KO} hepatocytes also lacking *hnf-4a*, were transfected with LNA-34a (figure 6A). LNA-34a had also a weaker antiproliferative effect on these hepatocytes isolated from a

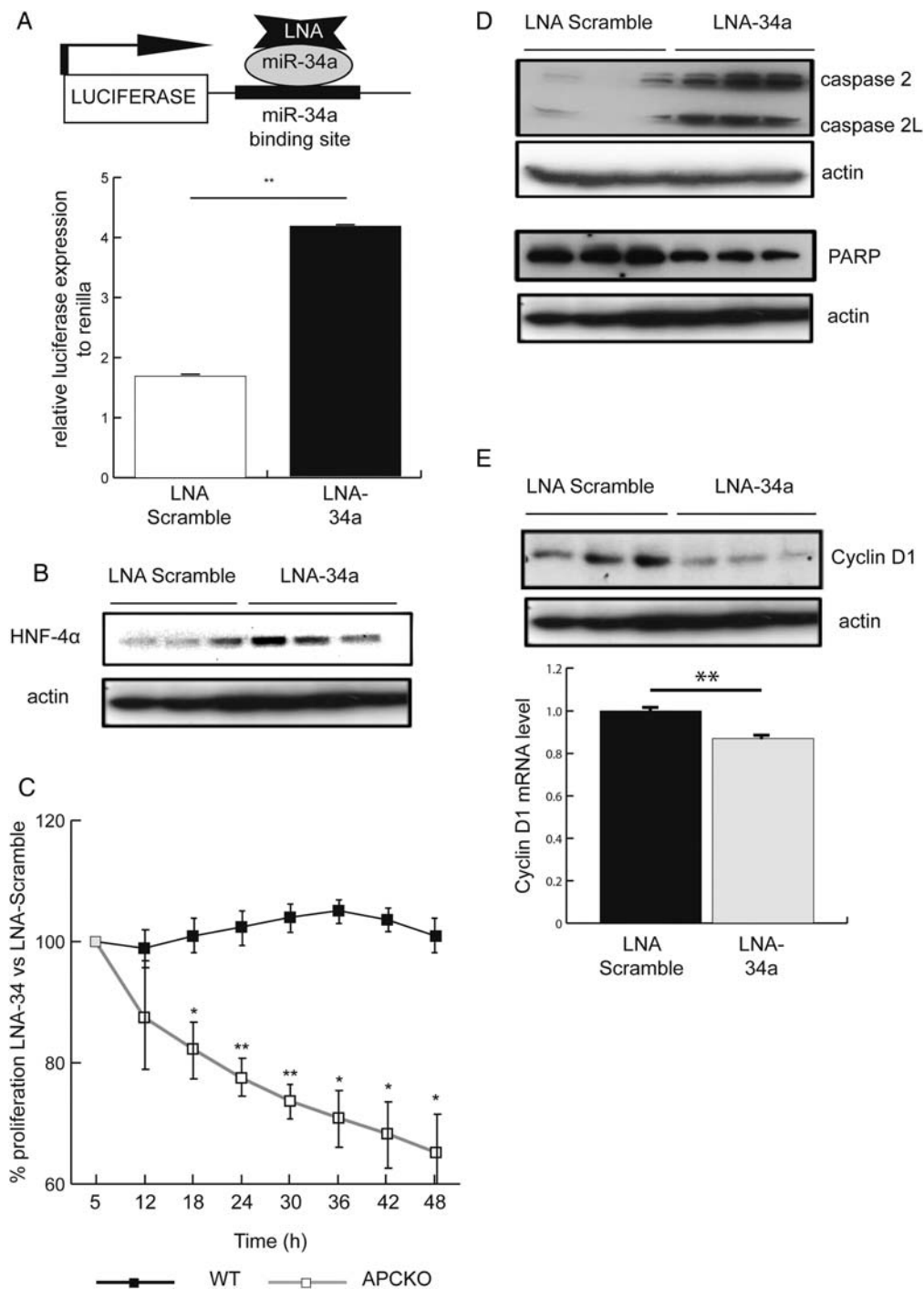
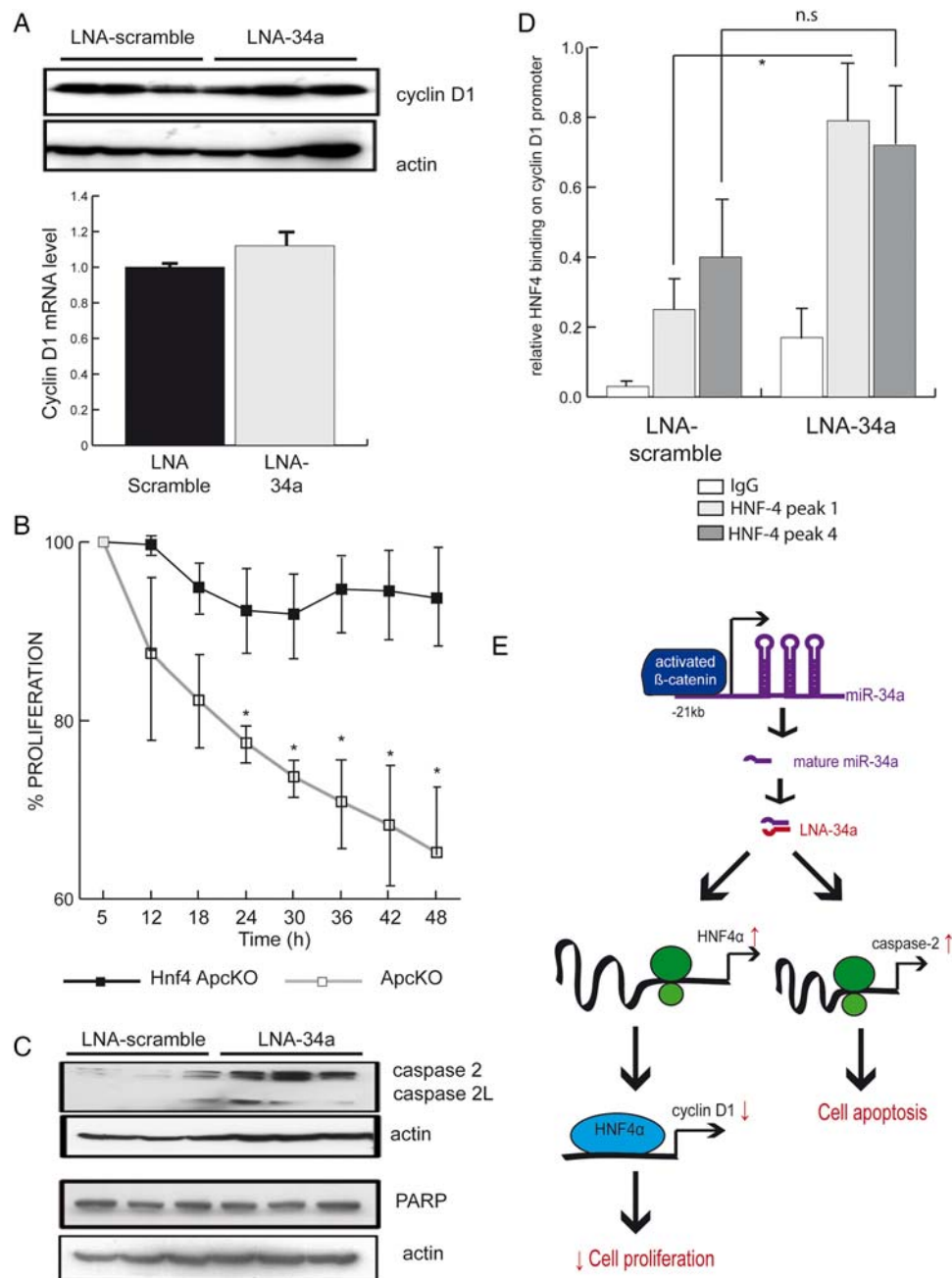


Figure 5 Locked nucleic acid (LNA)-34a inhibits *Apc*^{KO} hepatocyte proliferation, decreases cyclin D1 levels, and promotes apoptosis. (A) Isolated *Apc*^{KO} hepatocytes were transfected with a luciferase construct with a miR-34a binding site in 3'UTR, and with LNA-scramble or LNA-34a. Results are expressed as the mean luciferase activity in three experiments performed in duplicate. (B) Confirmation of LNA efficacy on HNF-4 α protein levels. Isolated *Apc*^{KO} hepatocytes were transfected with LNA-scramble or LNA-34a for 48 h. (C) Cell proliferation was assessed, for 48 h on wild-type (wt) or *Apc*^{KO} cells, transfected with LNA-scramble or LNA-34a. Results are expressed as the mean % proliferation normalised against cells treated with LNA-scramble at time t in three independent experiments carried out in quadruplicate \pm SEM. (D) Caspase 2 and PARP protein level (D), and Cyclin D1 protein and mRNA levels (E) in isolated *Apc*^{KO} hepatocytes transfected with LNA-scramble or LNA-34a for 48 h.

transgenic mouse model, in which both *Apc* and *hnf4a* had been deleted by Cre-Lox strategy (figure 6B). The higher proliferation rate observed for *hnf4/Apc*^{KO} hepatocytes in the presence of LNA-34a was associated with a minor induction of caspase 2L level, even if caspase 2 propeptide was efficiently increased (figure 6C); PARP amount was also not altered in this case (figure 6C). Finally, we realised ChIP

experiments with an antibody against HNF-4 α in *Apc*^{KO} hepatocytes, and analysed the binding of HNF-4 α in the presence of LNA-34a on two binding sites identified in published ChIP-seq experiments done in HepG2 cells and in murine livers (see online supplementary figure S7). We showed that the binding of HNF-4 α to cyclin D1 promoter tended to increase following exposure to LNA-34a, in particular for

Figure 6 Locked nucleic acid (LNA)-34a impact depends on HNF4 α expression. (A) Cyclin D1 mRNA and protein level in isolated hnf-4 α ^{KO}/Apc^{KO} hepatocytes transfected with LNA-scramble or LNA-34a for 48 h. (B) Cell proliferation of Apc^{KO} or Apc/hnf-4 α ^{KO} cells, transfected with LNA-scramble or LNA-34a for 48 h. Results are expressed as in [figure 5](#). (C) Caspase 2 and PARP protein levels in isolated hnf4/Apc^{KO} hepatocytes transfected with LNA-scramble or LNA-34a for 48 h. (D) HNF4 α binding to the cyclin D1 promoter in Apc^{KO} hepatocytes, as compared with the isotype control. Results are expressed as in [figure 1](#). They represent the means of at least three independent experiments. (E) The cascade modified by the LNA-34a.



peak 1 ([figure 6D](#)). This finding corroborates that HNF-4 α mediates the decrease in cyclin D1 levels in response to LNA-34a. In conclusion, following its overactivation, β -catenin binds to the miR-34a promoter, thereby increasing its expression. MiR-34a, in turn, decreases the amount of HNF-4 α leading to increases in cyclin D1 transcription and hepatocyte proliferation ([figure 6E](#)).

MiR-34a inhibition reduces tumour progression in Apc^{KO} mice

We thus developed a tumour-suppressive strategy based on miR-34a inhibition, which could constitute the first targeted therapy for liver tumours displaying β -catenin activation. We assessed the impact of LNA-34a on liver tumour progression in an Apc^{KO} context. Once the tumours became detectable on ultrasound scans, we injected LNA-34a (10 mg/kg) once weekly, and followed tumour development twice monthly by 2D-ultrasound (see online supplementary figure S8). We found

that LNA-34a slowed the progression of tumours displaying β -catenin activation to rates about half those for untreated tumours (see online supplementary figure 7A and S8). This treatment also inhibited the development of new tumours, with only one new tumour, on average, occurring on LNA-34a treatment, versus four on LNA-scramble treatment ([figure 7B](#)). As expected, HNF-4 α levels increased and cyclin D1 levels decreased in tumours treated with LNA-34a (see online supplementary figure S9A). Even if cyclin D1 level was impaired, Ki-67 labelling was similar in tumours treated with LNA-34a, and in those treated with the scramble LNA (see online supplementary figure S9B). Similar results were obtained with western blots for phospho-Histone H3 and cyclin A labelling (data not shown). However, LNA-34a promoted caspases 2 and 3 activation in tumours, in both western blot and immunohistochemistry experiments, confirming its proapoptotic effect ([figure 7C, E](#)), with minor effects on non-tumoural liver ([figure 7D](#)). Thus, treatment based on the use of LNA-34a could reduce tumour

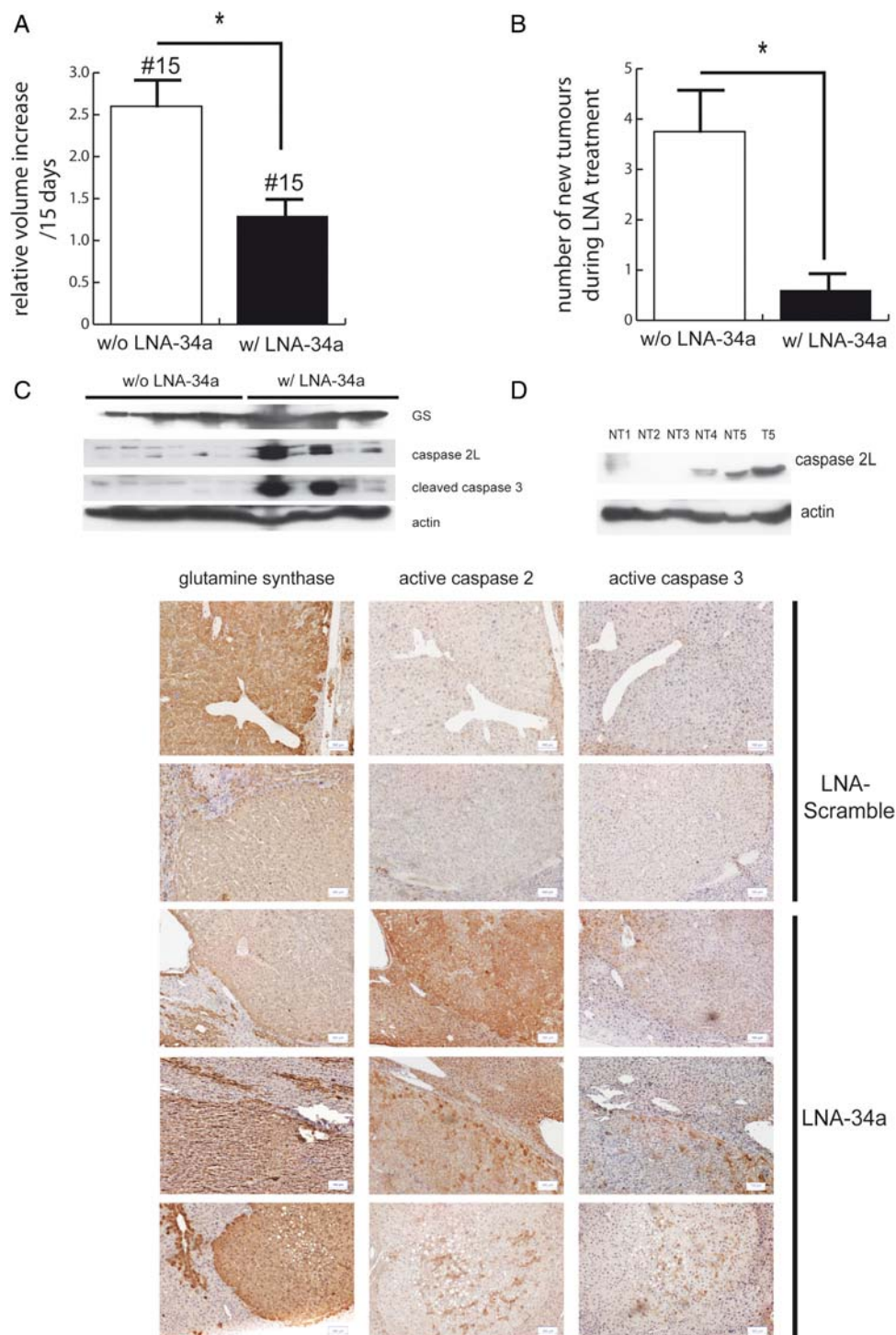


Figure 7 Locked nucleic acid (LNA)-34a slows the development of tumours from APC^{KO} mice, by activating caspases 2 and 3. (A) Tumour development was followed by 2D ultrasound. The results are expressed as the fold-change in volume over 15 days±SEM normalised with respect to t0, for mice with and without LNA-34a treatment (10 mg/kg). (B) Numbers of new tumours detected during the treatment of mice with LNA-scramble or LNA-34a. (C–E) Caspases 2 and 3 expression. Immunohistochemistry experiments have been performed on parallel sections.

development through an increase in HNF-4 α levels, leading to a loss of cyclin D1, and through a proapoptotic programme associated with the induction of caspase 2 and caspase 3 cleavage.

DISCUSSION

The miRNA miR-34a has been widely described as a tumour suppressor. Indeed, its expression is frequently impaired in various cancers following a hypermethylation of its promoter²⁰ including

breast and prostate cancers.^{34 35} A preclinical trial is currently underway with a mimic of miR-34a encapsulated into liposomes in various murine models of solid tumours (miRNATherapeutics, <http://www.mirnarx.com/pipeline/mirna-MRX34.html>). However, the role of miR-34a in liver tumorigenesis remains unclear. One study suggested that miR-34a level decreases in HCC²² and is associated with migration and invasion,²¹ but two other studies reported the induction of miR-34a in HCC

samples,^{17 18} in HCV+ sera,³⁶ and in HCC cells in response to alcohol.^{2 6} Our findings suggest that miR-34a is an oncogenic mediator in tumours displaying β -catenin activation both in mice and humans, confirming the data reported by Pineau *et al.*¹⁸ Our results could reconcile the data of Lou *et al* and Li *et al*, which suggest that miR-34a was lost in two-thirds of the HCCs studied.^{21 22} These samples corresponded mostly to HBV+ tumours, a subgroup with a lower frequency of *CTNNB1* mutations.¹⁰ The other third of patients in their cohort, with HCV infection, displayed an increase in miR-34a expression, and this group is likely to be enriched in *CTNNB1* mutations, according to transcriptomic classifications.^{4 5} In our cohort, tumours without β -catenin mutations displayed heterogeneity in terms of miR-34a amount. A greater understanding of this variability would be very useful.

In our study, we used a LNA approach against miR-34a to affect the cell viability of hepatocytes isolated from *Apc*^{KO} mice (figure 5C). The reduction of the proliferation rate was mainly associated with a decrease in cyclin D1 protein and mRNA levels (figure 5E), and with caspase 2 activation (figure 5D). The decrease in cyclin D1 protein levels was actually due to HNF-4 α , because cyclin D1 levels were not altered if *hnf4a* was invalidated in the *Apc*^{KO} model (figure 6A). Additionally, in the absence of HNF-4 α , the cell proliferation of hepatocytes depleted for *Apc* was weakly impaired (figure 6B) in the presence of LNA-34a. LNA-34a increased HNF-4 α protein level (figure 5B), and also its transcriptional activity on a luciferase construct under the control of a HNF-4 responsive element (figure 3D), and on the cyclin D1 promoter (figure 6D). Thus, HNF-4 α , a tumour suppressor in the liver,²⁸ appears to orchestrate some of the effects of miR-34a in the *Apc*^{KO} model (figure 6E). More importantly, miR-34a emerges as a crucial component of the oncogenic signal induced by β -catenin in hepatocytes.

In normal livers, miR-34a was particularly expressed in bile duct cells (figure 3A, B). This specific pattern of expression may hinder the accumulation of HNF-4 α protein in the biliary cells, which is required for bile cell lineage patterning²⁷ probably independently of β -catenin, which is not activated in normal bile duct cells.³⁷ The pattern of miR-34a expression is not consistent with the usual model in which β -catenin targets are located near the central vein. Cyclin D1 is one exception to this model; its pattern of expression is not restricted to the PC zone.²³ Interestingly, the region bound to TCF-4/ β -catenin located in miR-34a promoter contains no classical Wnt-responsive element, suggesting that the control on miR-34a is complex (figure 2B). This complexity is lost when β -catenin is overactivated, as in HCCs.

The key finding of this study was the decrease in liver tumour growth afforded by the delivery of LNA-34a (see online supplementary figures S7A and S8). Only a few studies to date have demonstrated the potency of miRNA-based approaches to cancer treatment. However, the great advantage of this approach for HCC is that these molecules are preferentially delivered to the liver.³⁸ The first authorisation for clinical trials with miRNA-based therapies concerns the LNA against miR-122 (miravirsin) for HCV treatment.³¹ The use of these modulators poses a number of challenges, particularly for their specificity. Our therapeutic approach, involving the inhibition of miR-34a, displays no toxicity (see online supplementary figure S6B), and has the great advantage of restoring the tumour suppressor HNF-4 α , targeting cyclin D1 and the metabolic pathways regulated by HNF-4 α (see online supplementary figure S9). Additionally, this approach may also alter the anti-inflammatory

loop orchestrated by HNF-4 α , controlling interleukin-6 production by hepatocytes.^{39 40} More importantly, the LNA-34a promotes a proapoptotic signal orchestrated by caspases 2 and 3 (figure 7C–E). In conclusion, our data reveal that miR-34a inhibition mainly induces a pro-apoptotic programme centred onto caspase 2, and could affect cell proliferation through cyclin D1 inhibition. However, the fact that Ki-67 and cyclin A labellings were not affected by LNA-34a treatment, suggests that miR-34a effects on cell proliferation are more complex.

In conclusion, despite the current preclinical trials being run by miRNA therapeutic, our findings suggest that the precise differential diagnosis of HCC, including an assessment of the mutational status of β -catenin, for which miR-34a inhibitors would be more appropriate than miR-34a mimics, is vital. Using this approach, we hope to affect the tumour itself, and also its environment, through the first effective targeted therapy for one-third of HCCs worldwide.

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Acknowledgements We thank Dr F Gonzalez for the *hnf4*^{-/-} mice. We are grateful to Dr P Bossard and M Caüzac for LDH protocol, and to Dr A-F Burnol for the gift of PARP-1 antibody.

Contributors AG: study concept and design and drafting of the manuscript; CS, LB and CG: technical support; CM and GR: acquisition of ultrasound data; FT and PN: analysis of miRNA-seq data; BT and CC: gift and genotyping of patient tumours; CP and SC: funding, study supervision and critical revision of the manuscript.

Funding This work was supported by the *Ligue Nationale Contre le Cancer (Labellisation Program 2011–2013 and 2014–2016)*, the *Agence Nationale de la Recherche (WNT-METABOLIV. 2010–2014)*, the *Institut National du Cancer (Epigenetics and Liver Cancer. 2014–2016)*. The *Association Française pour l'Etude du Foie* also funded experimental work and fellowships for AG. This work was supported by the SESAME funding program from Region Ile de France.

Competing interests None.

Patient consent Obtained.

Ethics approval All procedures were carried out according to the French guidelines for the care and use of experimental animals. All animal studies were approved by the regional veterinary services of the Paris police headquarters (agreement no. 75-1306) and by the Comité d'Ethique pour l'Expérimentation Animale Paris Descartes and its registered number is CEEA34.SC.047.12. For patients with HCC, samples were obtained with written informed consent, and the study protocol was approved by the ethics committee of the Cochin Hospital.

Provenance and peer review Not commissioned; externally peer reviewed.

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Gut published online March 19, 2015

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