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Apc Tumor Suppressor Gene Is the "Zonation-Keeper" of Mouse Liver

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Summary

The molecular mechanisms by which liver genes are differentially expressed along a portocentral axis, allowing for metabolic zonation, are poorly understood. We provide here compelling evidence that the Wnt/β-catenin pathway plays a key role in liver zonation. First, we show the complementary localization of activated β-catenin in the perivenous area and the negative regulator Apc in periportal hepatocytes. We then analyzed the immediate consequences of either a liver-inducible *Apc* disruption or a blockade of Wnt signaling after infection with an adenovirus encoding Dkk1, and we show that Wnt/β-catenin signaling inversely controls the perivenous and periportal genetic programs. Finally, we show that genes involved in the periportal urea cycle and the perivenous glutamine synthesis systems are critical targets of β-catenin signaling, and that perturbations to ammonia metabolism are likely responsible for the death of mice with liver-targeted *Apc* loss. From our results, we propose that *Apc* is the liver "zonation-keeper" gene.

Introduction

The liver is organized to fulfill its metabolic purpose, which is to receive and remove raw materials from the intestine, and to biotransform, synthesize, and secrete manufactured products that are delivered to the systemic circulation via the hepatic vein and to the intestine via the bile ducts. Consequently, liver functions are compartmentalized in the hepatic lobule within the liver cell plate. This functional unit of hepatic parenchyma is the area of tissue (15–25 hepatocytes) in which all liver cells receive blood from a common final vessel. The first row of periportal (PP) hepatocytes, surrounding the portal space (ps), receives a mixture of blood from both the hepatic artery and the portal vein and is in contact with the bile ducts. On the opposite side of the liver cell plate, the centrilobular hepatocytes surround a hepatic centrilobular vein (cv) and are called perivenous (PV) hepatocytes. This portocentral axis is the basis for the metabolic liver zonation.

Carbohydrate metabolism, the removal of ammonia, bile formation, transport and secretion, and drug biotransformation are the classical zonated functions of the liver. The proteins involved in these functions are also compartmentalized, and in view of evidence of the localization of some key enzymes in the lobule, the PP and the PV areas should each be further subdivided into a proximal and a distal part (Jungermann and Kietzmann, 1996, 2000). The molecular basis for these zonations remains unclear: some zonated protein expression is static and is only under transcriptional control, such as that of glutamine synthetase (GS) related to ammonia detoxification, whereas zonated protein expression related to carbohydrate and xenobiotic metabolisms is dynamic and depends on environmental factors such as oxygen or hormone or nutrient gradients and can be controlled at a transcriptional or posttranscriptional level (Gebhardt and Gaunitz, 1997; Oinonen and Lindros, 1998). Even when static transcriptional control of compartmentalized expression has been proposed, no major transcription factor was implicated; thus, the molecular pathways involved in liver zonation still need to be identified (Lindros et al., 1997).

Inappropriate β-catenin signaling has been found in 30%–40% of human hepatocellular carcinomas (HCCs) (see de La Coste et al., 1998 and Giles et al., 2003 for a review). The general signaling scheme for this is the following: in the absence of Wnt signals and without any β-catenin-dependent oncogenic process, a degradation complex containing the products of the tumor suppressor genes *APC* (adenomatous polyposis coli) as well as axins and the kinases GSK-3β and CK1 allows β-catenin to be phosphorylated and tagged for degradation. In this case, β-catenin remains at the membrane of the hepatocyte and participates in an adhesion complex with cadherins. Wnt/β-catenin signaling may be activated either by Wnt-soluble proteins binding to frizzled receptors or by oncogenic activation due primarily to β-catenin mutations in human HCCs, which leads to the β-catenin degradation complex being disrupted and the unphosphorylated β-catenin being stabilized. This stable β-catenin binds to members of the Tcf-Lef family of transcription factors and activates Wnt-dependent gene transcription.

In previous studies, we have shown that activating a β-catenin signal in mouse liver is oncogenic (Colnot

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et al., 2004a). We also showed that a massive activation of the β-catenin pathway in more than 70% of hepatocytes in mice, either by expressing a constitutively oncogenic form of β-catenin (Cadoret et al., 2002; Ovejero et al., 2004) or by disrupting a negative regulator of the pathway, that is, the *Apc* gene (Colnot et al., 2004a), killed the mice within 2 weeks, after a dramatic hepatomegaly. We identified several positive β-catenin liver-specific target genes (Cadoret et al., 2002; Ovejero et al., 2004). These target genes, which encode glutamine synthetase (GS), transporter-1 of glutamate (Glt1), ornithine aminotransferase (Oat), and leukocyte cell-derived chemotaxin-2 (Lect2), had all been described as being exclusively physiologically expressed by the proximal PV hepatocytes (Cadoret et al., 2002; Kuo et al., 1991; Moorman et al., 1988; Ovejero et al., 2004), whereas they were expressed in the whole lobule of β-catenin-activated livers. If the GS, Glt1, and Oat proteins belong to the same glutamine metabolic pathway, Lect2 does not.

Consequently, we wondered whether β-catenin signaling could be a pathway involved in liver zonation. We show that Apc plays a key role in this zonation by spatially controlling the activation level of the β-catenin pathway in the liver. We also demonstrate that activating β-catenin signaling positively controls the PV gene expression program and simultaneously inhibits the PP gene expression program. We also show that expression of the PP program requires Wnt signaling inhibition. Finally, we show that β-catenin signaling is the master pathway that controls nitrogen metabolism in the liver, and that after *Apc* loss, the forced expression of the PV program and the deficient PP program caused severe metabolic perturbation, which kills the mice.

Results

Zonation of Key Molecules Participating in the Wnt/β-Catenin Pathway in the Liver

We first investigated whether a physiological Wnt/β-catenin signaling was involved in liver zonation by analyzing by immunohistochemistry the expression of two partners of this signaling pathway: β-catenin and the product of the tumor suppressor gene *Apc* (Figure 1). As expected, we observed staining for β-catenin at the hepatocyte membrane. This is consistent with β-catenin being part of the adhesion complex in every hepatocyte (Figure 1A). We specifically observed stronger β-catenin membrane staining in the entire PV compartment, with a small amount of β-catenin accumulating in the cytosol in some cells (Figure 1A, PV and PP). We next investigated whether this increased β-catenin staining was due to activation of the Wnt/β-catenin pathway by using an antibody directed against the unphosphorylated form of β-catenin (Figures 1B and 1C). This antibody reveals the stabilized form of β-catenin, which plays a role in signaling (Murtaugh et al., 2005; van Noort et al., 2002). We found active β-catenin signaling in all of the PV hepatocytes, both in the GS-positive proximal PV area and in the distal PV part (Figures 1B and 1C).

We then looked for the negative regulator Apc. As Apc immunostaining is difficult to achieve (Brocardo et al., 2005), we used a new antibody (Spring Bioscience) directed against the C-terminal part of the protein and checked its staining specificity in the liver (Figure S1; see the Supplemental Data available with this article online). We detected Apc only in the complementary PP compartment of the liver lobule opposite the PV compartment (Figure 1D).

Therefore, this complementary distribution of activated β-catenin in the PV area and Apc in the PP zone strongly suggests that the β-catenin pathway may oppositely affect the genetic program of these two domains.

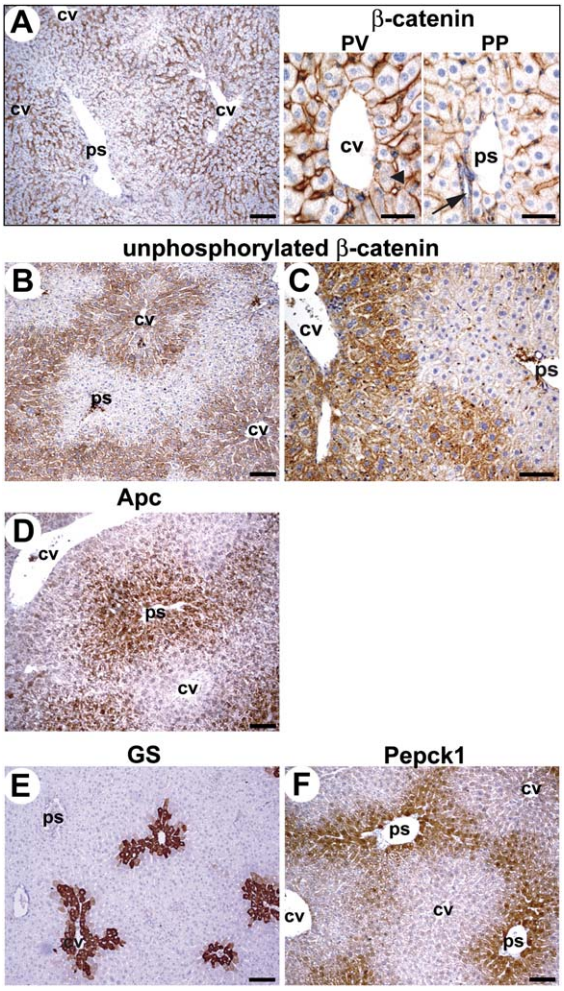


Figure 1. Complementary Immunolocalizations for Activated β-Catenin and Apc Proteins in Perivenous and Periportal Hepatocytes

(A–F) (A) β-catenin immunostaining at the membranes of hepatocytes from the whole hepatic lobule. On the left: this staining increased in hepatocytes from the PV half of the lobule, surrounding the centrilobular vein (cv). On the right: the inset with an enlarged view of PV hepatocytes shows increased staining and a faint cytosolic accumulation of the protein (arrowhead); the inset with an enlarged view of PP hepatocytes shows a bile duct (arrow) and weak staining in hepatocyte membranes. (B, C, and E) PV staining for (B and C) unphosphorylated β-catenin and (E) GS, which is a marker of the proximal PV hepatocytes. Note that the staining for unphosphorylated β-catenin is mainly cytoplasmic, is not clearly detected in the nucleus, and that it encompasses the entire PV compartment, extending from the GS-positive area. (D and F) PP staining for (D) Apc protein and (F) Pepck1 protein, which is the most commonly used marker for proximal PP hepatocytes. Note the overlap between Apc and Pepck1 protein staining. Cv = centrilobular vein; ps = portal space. The scale bars are 200 μm and 50 μm for PV and PP insets, respectively, and 100 μm for (C).

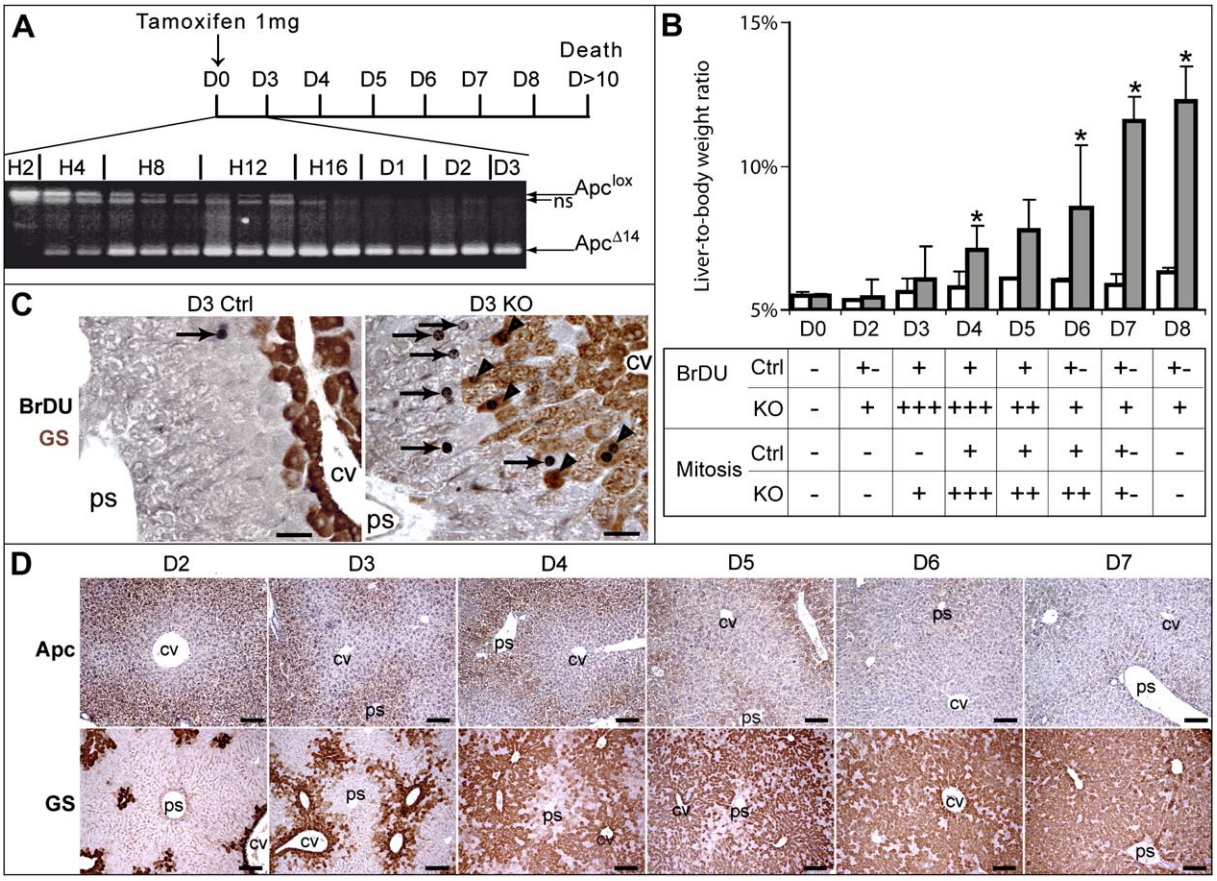


Figure 2. Tamoxifen-Inducible Liver-Targeted *Apc* Knockout, *Apc* ko^{liv} Mice

(A) PCR analysis of liver DNA from double transgenic *Apc*$^{lox/lox}$/TTR-Cre^{Tam} mice: time course of loss of the 14th exon of *Apc* (*Apc*$^{\Delta 14}$) from the *Apc* floxed allele (*Apc*^{lox}) 2, 4, 8, 12, and 16 hr (H2, H4, H8, H12, H16) and 1, 2, and 3 days (D1, D2, D3) after intraperitoneal injection of 1 mg Tamoxifen. Note the nonspecific band (ns) below that of the *Apc*^{lox} allele (at 1500 nt). The *Apc*$^{\Delta 14}$ band is at 400 nt. Note the intensity of the PCR band for the *Apc*$^{\Delta 14}$ allele and the absence the *Apc*^{lox} allele from H16, demonstrating that excision of the 14th exon of *Apc* 14th is completed by this time. Days 4, 5, 6, 7, and 8 and after day 10 (D4, D5, D6, D7, D8, D > 10) are the different time points at which the phenotype was studied.

(B) Hyperproliferative hepatomegaly in *Apc*$^{lox/lox}$/TTR-Cre^{Tam} mice: the gray bars represent Tam-injected *Apc*$^{lox/lox}$/TTR-Cre^{Tam} mice (KO), whereas the white bars represent either Tam-injected *Apc*$^{lox/+}$/TTR-Cre^{Tam} or Tam-injected *Apc*$^{lox/lox}$ control mice (Ctrl) presenting an indistinguishable phenotype. Note the mild proliferation detected in controls injected with Tam due to the high dose of Tam used, which is negligible compared to the intense proliferation seen after *Apc* knockout. The semiquantitative score of BrdU-positive hepatocytes is as follows: −, less than 0.01%; +−, from 0.01% to 0.2%; +, from 0.2% to 1%; ++, from 1% to 5%; +++, from 5% to 15%. The semiquantitative mitotic index is as follows: −, less than 0.01%; +−, from 0.01% to 0.2%; +, from 0.2% to 1%; ++, from 1% to 3%; +++, from 3% to 6%. *p < 0.05.

(C) Costainings for BrdU (black staining, DAB+Nickel) and GS (brown staining, DAB substrate) on D3 Apc ko^{liv} and control livers; arrows indicate BrdU-stained hepatocytes, and arrowheads indicate hepatocytes costained for BrdU and GS. The scale bars are 50 μm.

(D) Immunostaining for Apc and GS after *Apc* loss in the liver. GS and Apc immunostaining in Tam-injected control mice at each time point was indistinguishable from day 0 and day 2 *Apc* ko^{liv} mice (data not shown). The scale bars are 200 μm.

Error bars represent standard deviations.

Immediate Effects of Apc Loss in a Cre-LoxP Tamoxifen-Inducible Mouse Model

We then designed a model to control both the spatial and temporal inactivation of the *Apc* gene. For this, we crossed conditional mutant mice for *Apc* (Colnot et al., 2004a) with TTR-Cre^{Tam} mice that specifically express an inducible Cre recombinase in the liver (Tannour-Louet et al., 2002). The *Apc*$^{lox/lox}$ mice transgenic for TTR-Cre^{Tam} were injected with a single dose of 1 mg Tamoxifen (Tam) (Figure 2A). PCR analysis of liver DNA showed that recombination began 4 hr after the Tam injection and was complete after 16 hr. These mice will hereafter be called *Apc* ko^{liv}. Consistent with our previous model of Cre adenovirus-mediated invalidation of *Apc* (Colnot et al., 2004a), 95% of the *Apc* ko^{liv} animals died between 10 and 15 days after Tam injection, after developing an intense and progressive hepatomegaly (Figure 2B). We demonstrated a link between this hepatomegaly and hyperproliferation: we detected a significant increase in the hepatocellular proliferation rate from day 3 in *Apc* ko^{liv} livers, after which we detected numerous mitosis and BrDU-stained cells (Figure 2B). This proliferation rate was maximal at day 4, and it persisted to a lesser extent until day 8.

This time-controlled *Apc* inactivation system allowed us to follow the progressive disappearance of Apc protein in *Apc* ko^{liv} livers (Figure 2D). On day 3 and day 4, we observed strong staining in the PP area for the Apc protein. This staining decreased from day 5 onward and was almost completely abolished by day 6. However,

at the same time that the staining for Apc was being lost, we observed an increase in staining for GS, a marker of the proximal PV compartment, which is also a transcriptional target of β-catenin signaling (Cadoret et al., 2002; Kruithof-de Julio et al., 2005; Loeppen et al., 2002). GS expression, seen in the proximal PV area at day 0 (not shown) and day 2 (Figure 2D), was also seen in the distal PV area at day 3. We detected extension into the PP compartment at day 4 and day 5, and all of the hepatocytes of the lobule were stained for GS by day 6. This increased area of staining for GS was not due to a proliferative expansion of the PV compartment, as proliferation occurred in the whole liver parenchyma, in GS-expressing PV cells and GS-deprived PP cells (Figure 2C). Our data suggest that there is an Apc expression gradient in the normal liver, and that expression is not detectable in the PV area and becomes stronger in the entire PP area. This expression can negatively control the expression of β-catenin-responsive genes, suggesting that the portocentral axis is under the control of the β-catenin pathway.

Activating the β-Catenin Pathway in the Liver Imposes a Perivenous Genetic Program to the Whole Lobule

We analyzed the role of β-catenin signaling on both the PV and PP genetic program with microarrays containing 24,100 cDNAs. We disrupted *Apc* expression by an intravenous injection of 10^9 pfu of Cre adenovirus in both $Apc^{lox/lox}$ and $Apc^{+/lox}$ mice, and we compared the transcriptome of $Apc^{-/-}$ hepatomegalic livers to $Apc^{+/-}$ livers from control mice (Table S1). The complete data sets are available in Table S2. We applied two criteria to the array data set: at least a 2-fold differential expression and a p value < 0.001. This defined a small set of genes, corresponding to 63 distinct known genes (Table S1). Among the 32 induced genes, 4 were known to be expressed in the PV compartment and encoded the proteins Glt1, GS, Oat, and the arginine vasopressin receptor 1A (Avpr1A) (Cadoret et al., 2002; Kuo et al., 1991; Moorman et al., 1988; Tordjmann et al., 1998). Among the 31 suppressed genes, 2 had been described as being expressed in the PP compartment and encoded the Glutaminase2 (Gls2) and Arginase1 (Arg1) proteins (Haussinger, 1983; Yu et al., 2003). For the remaining 57 genes, there have been no descriptions of their localization in the liver.

We extracted these zonated genes from the microarray list and used real-time quantitative RT-PCR (qPCR) to validate them in Tam-injected *Apc* ko^{liv} livers (Table 1; Figure S2). All were confirmed as early targets of a β-catenin signal in Tam-injected *Apc* ko^{liv} livers: the PV genes *GS*, *Glt1*, and *Oat* were induced as early as day 3 after *Apc* invalidation, and the *Avpr1A* gene was significantly induced at day 4 (Figure S2). The PP genes *Arg1* and *Gls2* were substantially reduced as early as day 3.

The microarray analysis was unable to reveal the upregulation of several PV genes: the chemotaxin Lect2, a direct β-catenin/TCF transcriptional target (Ovejero et al., 2004) excluded by statistical tests, was significantly induced in Tam-injected *Apc* ko^{liv} livers as early as day 3 (Table 1; Figure S2). The *RhBg* gene, which encodes an ammonium transporter, was not spotted on the array but, as suggested by Schwartz's group (Stahl et al., 2005), was an early and sensitive marker of a β-catenin signal in *Apc* ko^{liv} livers (Table 1; Figure S2). There were also several genes not spotted on the microarray that are well known to be periportal: the *Cps1* and *Pepck1* genes were significantly repressed as early as day 4 in *Apc* ko^{liv} livers (Table 1; Figure S2).

Finally, we focused on two genes not known to undergo zonation but which were clearly overexpressed in the microarray analyses (Table 1). *Axin2* is a well-known direct target of β-catenin/TCF signaling in both intestinal and liver cancers (Lustig et al., 2002), and *RNase4* has been previously identified as a putative β-catenin gene in the liver (C.P., unpublished substractive hybridization data). We confirmed the induction of *axin2* and *RNase4* mRNAs by qPCR analyses (Table 1; Figure S2). In situ hybridization confirmed that these targets were expressed in the PV hepatocytes in normal livers (Figure 3A), again confirming the close link between β-catenin signaling activation and PV gene expression in the normal liver.

We investigated whether *Apc* loss had the same effect as β-catenin gain of function by analyzing the level of expression of the zonated genes identified by microarray analysis in two other mouse models expressing activated mutants of β-catenin in the liver—transgenic mice overexpressing the ΔN131-β-catenin mutant (Cadoret et al., 2002) (Table 1) and mice infected with an adenovirus encoding the S37A-β-catenin mutant (Ovejero et al., 2004) (Figures 4B and 4D; Table 1). All of the genes respond similarly to *Apc* loss and to β-catenin-activating mutations. Thus, Apc controls the zonation of gene expression through its role as a negative regulator of the β-catenin pathway.

We then analyzed in situ the expression pattern of different PV and PP genes after the gradual loss of Apc in the hepatic lobule. In situ hybridization (Figures 3A and 3C) and immunohistochemistry experiments (Figures 3B and 3D) were carried out at different time points in *Apc* ko^{liv} livers. We observed two expression patterns for PV genes and proteins (Figures 3A and 3B, at D0): *GS* as well as RhBg, Glt1, and Lect2 were expressed in the proximal PV compartment, whereas *Axin2* and *RNase4* were expressed in both the proximal and distal PV area. After *Apc* loss, the expression of all of these PV genes progressively extended toward the PP compartment. By day 4, the PP compartment was completely invaded by the more distal PV genes (*Rnase4* and *Axin2*), and by day 7, the PP compartment was completely invaded by the proximal PV genes (*GS*, RhBg, Glt1, and Lect2) (Figures 3A and 3B).

For the PP program, we observed three expression profiles in the normal liver (Figures 3C and 3D, at D0): *Gls2* and *Pepck1* were expressed in the proximal PP compartment; *Arg1* had a wider PP expression, complementary to that of the PV gene *RNase4*; and *Cps1* expression extended into the PV compartment, complementary to that of the proximal PV gene *GS*. After *Apc* loss, *Gls2*, *Arg1*, and *Cps1* were strongly repressed as early as day 3, and *Gls2* and *Arg1* mRNA were totally absent from the liver by day 7 (Figure 3C). Pepck1 and cps1 protein levels decreased from day 4 onward and had effectively disappeared by day 7 (Figure 3D).

Therefore, we showed that simultaneous induction of the PV genetic program and repression of the PP

Table 1. Zonated Liver Genes Responding to a β-Catenin Signal in Mice

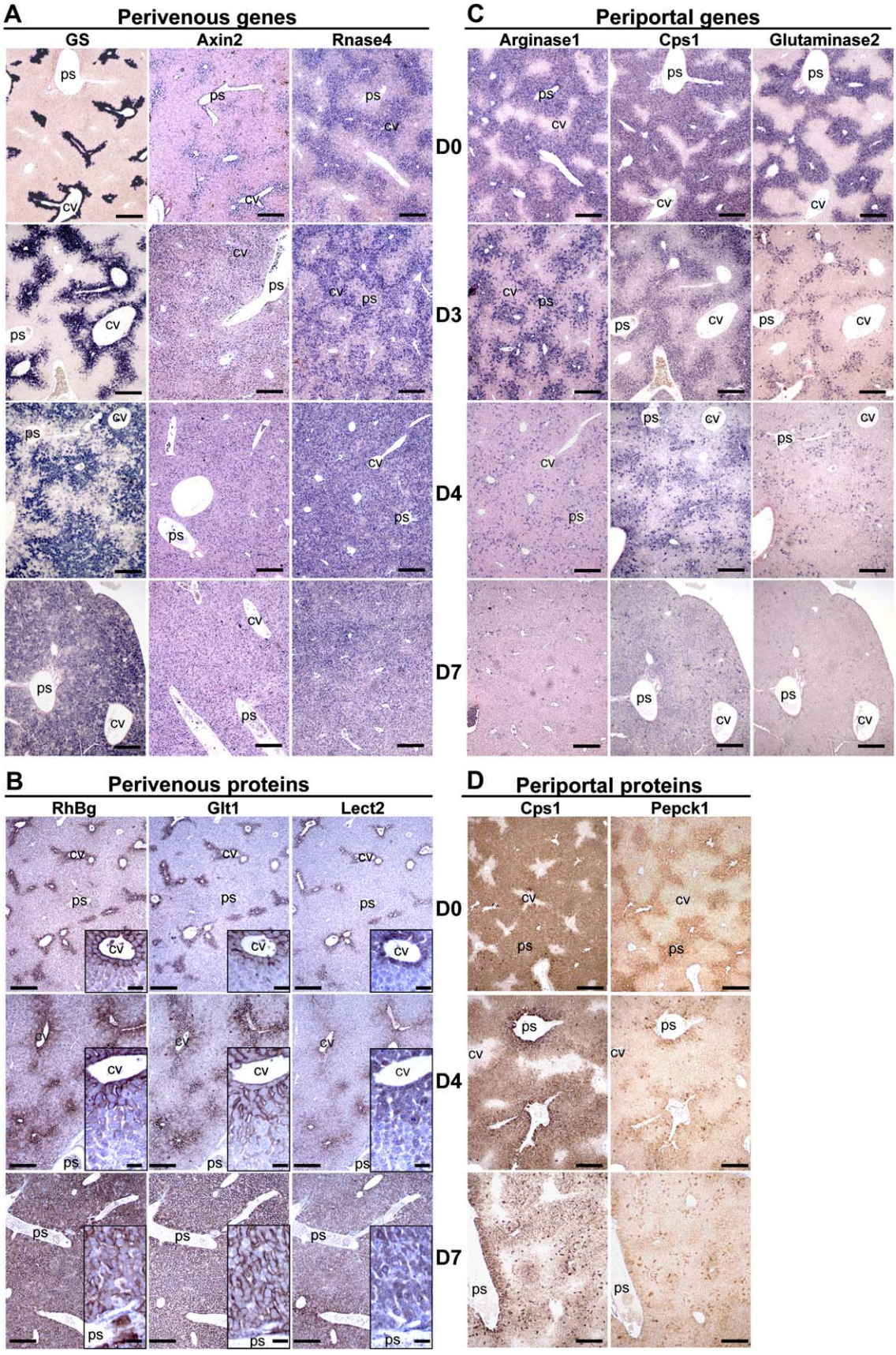
References for a Zonated Expression Pattern in the Liver	Symbol	Gene	*Apc* ko^{liv} Liver at Day 7[b]		ΔN131 or S37A β-Catenin-Expressing Liver
			AdCre Infected	Tamoxifen Injected	
			cDNA Microarray	qRT-PCR[a]	SSH
Perivenous Expression					
Ammonia metabolism and transport					
Moorman et al. (1988)	*Glul*	Glutamine synthetase (*GS*)	8.0	5.3	Induced, ref1
Cadoret et al. (2002)	*Slc1a2*	Glutamate transporter 1 (*GLT1*, *EAAT2*)	13.9	8.8	Induced, ref1
Kuo et al. (1991)	*Oat*	Ornithine aminotransferase	3.6	11.9	Induced, ref1
Weiner and Verlander (2003)	*Rhbg*	Rhesus blood group-associated B glycoprotein	Not spotted	16.7	NF
Chemotaxis					
Ovejero et al. (2004)	*Lect2*	Leukocyte cell-derived chemotaxin 2	3.7[c]	4.1	Induced, ref2
Receptor					
Tordjmann et al. (1998)	*Avpr1a*	Arginine vasopressin receptor 1A	2.7	1.22 2.14 at day 4	NF
Wnt signaling					
Figure 4	*Axin2*	Axin 2	8.6	7.7	NF
Other functions					
Figure 4	*Rnase4*	Ribonuclease 4	3.8	2.7	Induced, NP
Periportal Expression					
Ammonia and urea metabolism					
Watford and Smith (1990)	*Gls2*	Glutaminase2, liver	0.21	0.08	ND
Yu et al. (2003)	*Arg1*	Arginase1, liver	0.14	0.17	ND
Moorman et al. (1988)	*Cps1*	Carbamoyl phosphate synthetase 1	0.40[c]	0.44	ND
Carbohydrate metabolism					
Bartels et al. (1989)	*Pck1*	Phosphoenolpyruvate carboxykinase 1, cytosolic	0.62[c]	0.22	ND

NF = not found; ND = not determined; NP = not published.
[a] Detailed data for kinetics of expression in Tam-injected *Apc* ko^{liv} livers are presented in Figure 3; ref1 = Cadoret et al. (2002), ref2 = Ovejero et al. (2004).
[b] Results are expressed as fold changes, relative to AdCre-infected or Tam-injected control mice.
[c] Not significant in microarray analysis.

program are immediate consequences of Wnt/β-catenin signaling activation resulting from *Apc* loss in the liver.

Blocking β-Catenin Signaling in Normal Liver Converts Perivenous Hepatocytes to a Periportal Phenotype

We next investigated the consequences of blocking Wnt/β-catenin signaling in the liver to confirm the role of the Wnt/β-catenin pathway in liver zonation. We infected wild-type mice with AdDkk1, an adenovirus encoding the Dickkopf-1 (Dkk1) protein. Dkk proteins are well known extracellular antagonists of Wnts that act by blocking the intracellular Wnt/β-catenin signaling cascade. We checked that Dkk1 was correctly expressed in the entire liver, was secreted, and was active (Figure S4). As previously described (Kuhnert et al., 2004), mice died from major intestinal defects 7 days after AdDkk1 infection, due to defective Wnt signaling in the intestine (Figure S4). Therefore, we analyzed their livers 6 days after infection and observed no macroscopic or histological phenotype. However, major molecular hepatic changes occurred in Dkk1-expressing hepatocytes (Figure 4). For PV genes, such as *RhBg*, *Lect2*, *GS*, and *Axin2*, we observed a large decrease in the amount of staining in the PV hepatocytes (Figure 4A), which was supported by a large decrease in mRNA levels (Figure 4B). We observed the same for the other centrilobular genes, *Oat*, *Glt-1*, and *Avpr1a* (Figure 4B). By contrast, we observed staining of PP markers in the whole lobule of AdDkk1 livers, including the centrilobular area (Figure 4C). This was confirmed by an increased transcription of these PP genes, which are *Arg1*, *Gls2*,


A
Perivenous genes
GS
Axin2
Rnase4
C
Periportal genes
Arginase1
Cps1
Glutaminase2
D0
D3
D4
D7
ps
cv
B
Perivenous proteins
RhBg
Glt1
Lect2
D
Periportal proteins
Cps1
Pepck1

and *Pepck1* (Figure 4D). This was also confirmed by an increase in *Gls2*, *Pepck1*, and *Arg1* mRNA levels in AdDkk1 livers (Figure 4D).

The layer of hepatocytes in contact with the centrilobular vein still expressed GS in a few cells (Figure 4A, inset). Moreover, there was weak de novo *Arg1* expression in this area (Figure 4C, inset). This suggests that this proximal PV area is partially sensitive to Dkk1-mediated inhibition of Wnt signaling.

Taken together, these results show that Wnt signaling inhibition is required for developing the periportal program and is able to impair β-catenin signaling in the PV compartment, except for its most proximal part.

Wnt/β-Catenin Signaling Is a Master Pathway for Nitrogen Metabolism

Among the 13 zonated genes retained (Table 1) as targets of β-catenin signaling, 7 were related to nitrogen metabolism. Our results showed that the reciprocal distribution of the partners of the urea cycle in the PP area (Arg1, Gls2, Cps1) and of the glutamine scavenger system in the PV area (GS, Oat, Glt1) was, respectively, strongly dependent on negative and positive β-catenin signaling activity (Figures S2–S4). Such a metabolic zonation was proposed by Haussinger et al. (1992), who described a conserved structural and functional heterogeneity of nitrogen metabolism in the liver of most ureotelic animals. We analyzed different biological parameters to investigate whether β-catenin signaling could be the master regulator of nitrogen metabolism and whether a disorder linked to this metabolism could explain the lethality of Tam-injected *Apc* ko^{liv} animals.

We first analyzed liver dysfunction in day 10 Tam-injected *Apc* ko^{liv} mice by measuring ammonia and amino acid levels in the liver and blood (Figures 5A and 5B; Figure S3). Both glutamine and ammonia were dramatically increased in liver and blood, and uremia was decreased 2-fold (Figures 5A and 5B). These biochemical alterations could be explained by the genetic misexpressions observed in Tam-injected *Apc* ko^{liv} livers that are presented in Figure 5D. Briefly, the smaller commitment of NH_4^+ ions to the urea cycle, combined with a greater commitment of these NH_4^+ ions to glutamine synthesis, led to deficient ammonium detoxification and to glutamine accumulation.

Although we cannot exclude other metabolic defects, two critical biochemical changes could explain the death of the *Apc* ko^{liv} animals (Figure 5B): the severe hyperammonemia, being 4.4 times higher in *Apc* ko^{liv} mice than in controls, and the severe hyperglutaminemia, being 26 times higher in *Apc* ko^{liv} mice than in controls. The consequences are that NH_4^+ ions and glutamine were also found at increased levels in the brains of *Apc* ko^{liv} mice (Figure 5C): this is known to be deleterious (Butterworth, 2002) and strongly suggests that these mice developed a fatal hepatic encephalopathy, which is a well known cause of death in infants with congenital urea cycle disorders (Saudubray, 2002).

Discussion

In this study, we used the distribution of key partners of the β-catenin pathway within the liver lobule and the analysis of the immediate molecular and biochemical consequences of both β-catenin signaling activation mediated by *Apc* loss and the Dkk1-mediated Wnt signaling blockade to show that β-catenin signaling inversely controls the PV and PP genetic programs in the liver. We provide compelling evidence that the Wnt/β-catenin pathway plays a major role in the mechanisms underlying the reciprocal aspects of liver zonation along the portocentral axis. Among the different zonated functions of the liver, our results clearly show that nitrogen metabolism is under the control of the β-catenin pathway.

Apc Is the "Zonation-Keeper" Gene of the Liver

Our data show that the tumor suppressor gene *Apc* is a key partner of β-catenin signaling for regulating liver zonation. First, Apc accumulates in the PP compartment, in which there is no β-catenin signaling. Second, Apc protein could not be detected in the PV compartment, whereas the active form of β-catenin is expressed with different positive β-catenin target genes. Third, the loss of Apc from the hepatic parenchyma causes the immediate de novo expression of β-catenin-positive PV target genes and the simultaneous suppression of β-catenin-negative PP target genes. This pivotal role for Apc in controlling β-catenin signaling along the portocentral axis is similar to what occurs along the crypt-villus axis in the intestine. Indeed, Apc is not found in the proliferative crypt compartment, which is under positive control by Wnt/β-catenin signaling, whereas it is expressed at high levels in the differentiated villus compartment, in which the β-catenin/TCF program is suppressed (Smith et al., 1993; van de Wetering et al., 2002). This is supported at the molecular level in the EphB/ephrin-B system: β-catenin signaling inversely controls the expression of the EphB receptors and their ligand, ephrin-B1, along the crypt-villus axis of the small intestine, thus controlling the position of the cell populations of this epithelium (Batlle et al., 2002). *Apc* not only controls the normal homeostasis of the intestinal epithelium, but its mutation is also a key event in initiating tumorigenesis (Andreu et al., 2005; Kinzler and Vogelstein, 1996; Sansom et al., 2004). Therefore, *Apc* has been defined as the gatekeeper of the intestinal epithelium (Kinzler and Vogelstein, 1996). In the liver system, we observed that Apc similarly plays a key role in maintaining the complementary PP and PV domains of the lobule. This *Apc*-dependent metabolic zonation is crucial for normal liver homeostasis. This is supported by our data showing that a massive *Apc* loss in the liver kills the animals. As well as playing a role in the mature liver, *Apc* is a tumor suppressor gene in the liver. Indeed, our previous data showed that *Apc* can initiate liver tumor development when it is mutated in hepatocytes (Colnot et al., 2004a). Thus, analogous to the intestinal system,

Figure 3. Gradual Extension of Perivenous Gene Expression and Concomitant Loss of Periportal Gene Expression after *Apc* Inactivation

(A and C) In situ hybridization (blue) to *Apc* ko^{liv} livers for (A) PV genes and (C) PP genes; the analyses for GS, Cps1, and Glutaminase2 were carried out on serial sections and were carried out likewise for Rnase4 and Arginase1.

(B and D) Immunostaining for the PV proteins RhBg, Glt1, and Lect2, made on serial sections (B) and for the PP proteins Cps1 and Pepck1, made on serial sections (D). The scale bar is 500 μm and 100 μm for the insets.

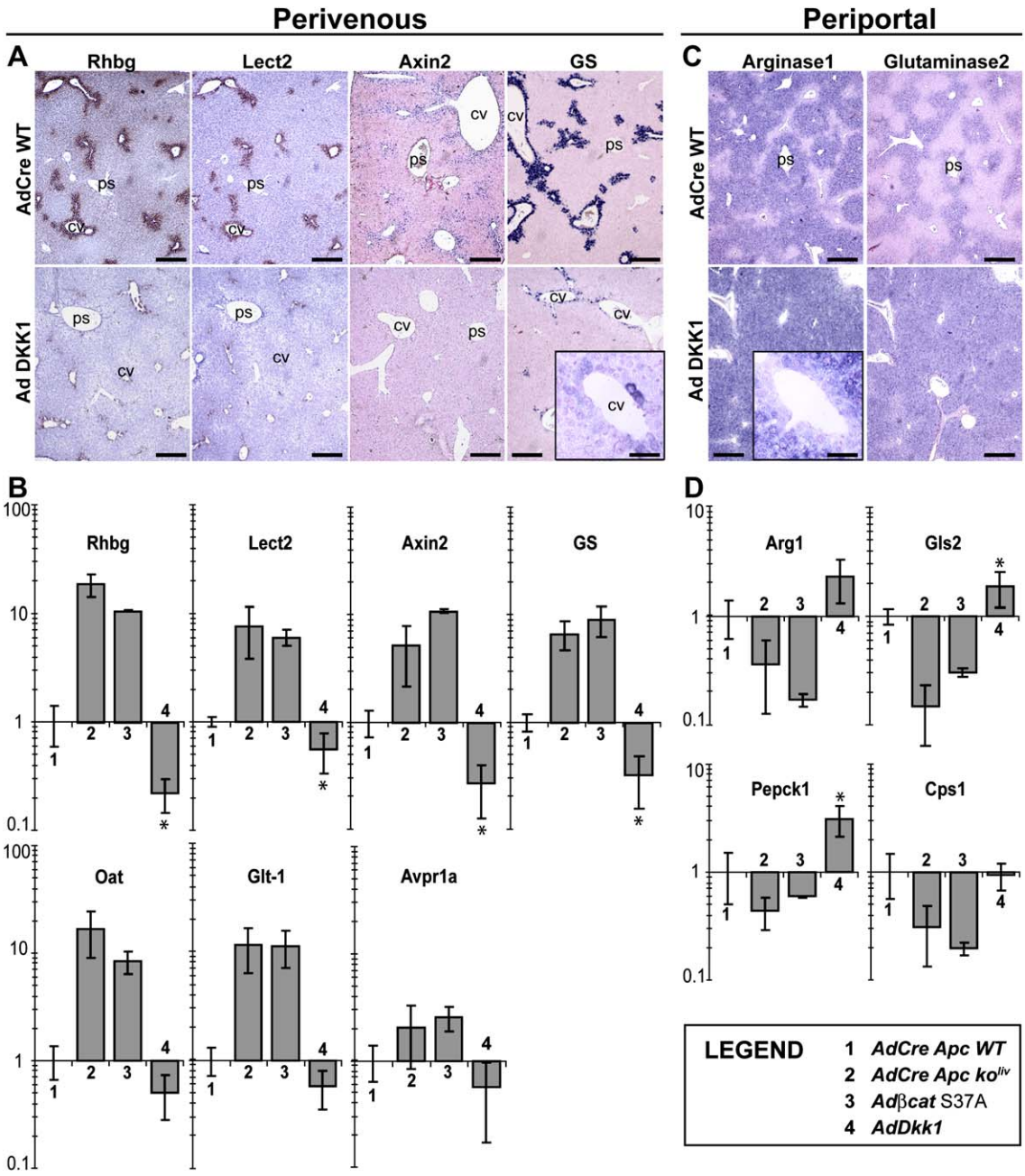


Figure 4. Dramatic Shift from a Zonated Liver to a Periportal-like Liver after Dkk1-Mediated Blockade of Wnt/β-Catenin Signaling

(A–D) (A and C) Immunostaining (for the PV proteins RhBg and Lect2, made on serial sections) or in situ hybridization (for the PV mRNA *GS*, *Axin2* and the PP mRNA *Arginase1* and *Glutaminase2*, made on serial sections) in the livers of wild-type mice infected with AdDkk1 or AdCre (for controls) at 10^9 pfu/mouse. (A) Note the near absence of lect2 and Axin2, and the net decrease of Rhbg and GS levels in the Dkk1 liver versus the control. (C) *Arginase1* and *Glutaminase2* PP mRNAs have invaded the entire lobule. (Insets in [A] and [C]): In situ hybridization (GS and Arginase1) of proximal PV hepatocytes on near-adjacent sections of AdDkk1-infected livers. (B and D) Quantitative real-time RT-PCR analyses after adenovirus-mediated activation of β-catenin signaling by either inactivating *Apc* (AdCre *Apc* ko^{liv}, lane 2) or expressing a stable mutant of β-catenin (Adβ-catS37A, lane 3), or after adenovirus-mediated blockade of the Wnt pathway by expressing Dkk1 (AdDkk1, lane 4), versus control wild-type mice infected with Cre adenovirus (AdCre *Apc* WT, lane 1). y axis values are gene expression levels relative to that of AdCre *Apc* WT control livers. (B) All the PV mRNAs tested increased in the two models of adenovirus-mediated activation of the β-catenin pathway and decreased in AdDkk1-infected livers. Only *Rnase4* expression did not decrease in AdDkk1 livers, because its expression is induced by an adenoviral infection, independent of β-catenin signaling (data not shown). (D) All of the PP mRNAs tested decreased in the two models of adenovirus-mediated activation of the β-catenin pathway and increased in AdDkk1-infected livers, except for Cps1. The numbers of animals used in this study were as follows: AdCre *Apc* WT, n = 3; AdCre *Apc* ko^{liv}, n = 4; Adβ-catS37A, n = 2; AdDkk1, n = 5. The scale bars are 500 μm and 100 μm for the insets. *$p < 0.05$. Error bars represent standard deviations.

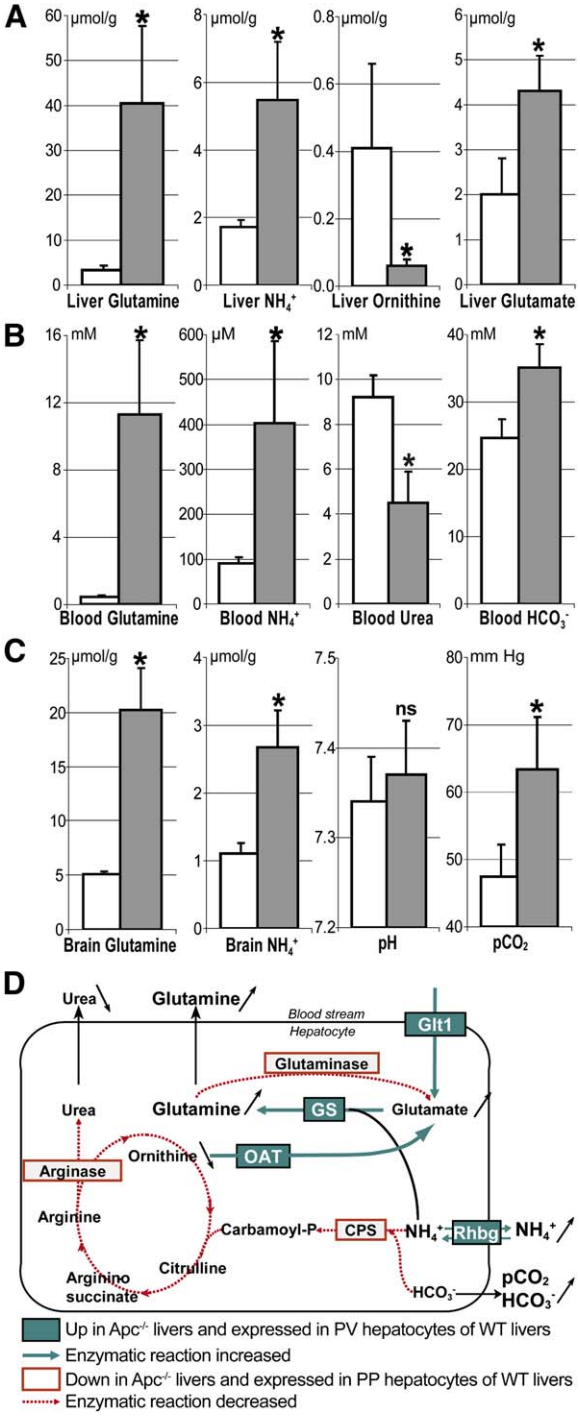


Figure 5. Perivenous-Forced Phenotype in *Apc* ko^{liv} Hepatocytes Breeds a Fatal Metabolic Disorder, Due to Ineffective Ammonium Detoxification and to Glutamine Accumulation

(A–C) Dramatic phenotypic changes after *Apc* hepatic loss. (A) Hepatic levels of glutamine, NH_4^+, ornithine, and glutamate. (B) Blood glutamine, ammonia, urea, and bicarbonate ions. (C) Brain glutamine and NH_4^+, blood pH, pCO_2. *Apc* ko^{liv} mice (n = 6 for blood NH_4^+, urea, pH, pCO_2, and HCO_3^-; n = 8 for amino acids; n = 8 for brain analyses) are depicted with gray bars, and Tam-injected control mice (n = 7, 10, and 8, respectively) are depicted with white bars. *p < 0.005; ns = not significant.

(D) Detoxifying ammonium in the liver uses two pathways. The high-capacity PP urea cycle pathway also has low specificity. NH_4^+ and HCO_3^- ions are committed to the urea cycle via Cps1 in the first step, and via Arginase1 in the final step, generating urea and ornithine. Due to a defect in the PP enzymes in *Apc* ko^{liv} mice, i.e., the effectors Cps1 and Arginase1 are strongly reduced, the mice accumulate the substrate of these reactions—NH_4^+ and HCO_3^-—whereas the final products, urea and ornithine, are decreased. The other ammonium detoxification pathway is the PV glutamine scavenger system, which is highly specific but has a low capacity. Glutamine synthetase commits NH_4^+ and glutamate toward glutamine synthesis. For this, glutamate is indirectly provided from ornithine by Oat, and its transport is ensured by Glt1. Finally, RhBg is a controversial ammonium transporter (Chambrey et al., 2005). In the *Apc* ko^{liv} mice, all of the genes involved in the metabolic process linked to glutamine are upregulated.

Error bars represent standard deviations.

we propose that *Apc* is the "zonation-keeper" gene for the liver parenchyma and is critical for the functional maturation of the liver.

Control of Gene Expression along the Portocentral Axis by Wnt/β-Catenin Signaling

The two proximal areas for each PV and PP compartment can be easily defined (Figure 6). In the proximal PV compartment around the centrilobular vein, the presence of unphosphorylated β-catenin and the strong expression of numerous positive liver β-catenin target genes support active β-catenin signaling. There is incomplete Wnt signaling in this region, as a few cells close to the central vein always seems to be unaffected by DKK1 inhibition. One hypothesis should be that in this zone the activation of β-catenin signaling may be both induced by Wnt factors and, in the one-hepatocyte layer around the central vein, partially induced by a constitutive lack or an insufficient amount of Apc protein. Conversely, in the proximal PP compartment in which Apc is present, β-catenin signaling is suppressed, as all of the identified negative targets are found in this area (Figure 6). Thus, the presence of Apc determines the absence of β-catenin signaling in this zone, as its removal leads to a rapid de novo expression of PV target genes and a disappearance of PP target genes. The intermediate distal PV and PP zones undergo more complex transcriptional regulations. For example, in the distal PV area, there is only sufficient activated β-catenin to allow for the expression of some PV target genes, *RNase4* and *Axin2* (see Figure 6). The lack of expression of the positive PV target genes *Gs*, *Lect2*, *RhBg*, and *Glt1* in this particular area may be explained by dosage effects due to different levels of β-catenin in the different compartments able to signal within the nucleus. Such dosage effects have already been proposed by Fodde's group: different *Apc* mutations in ES cells were associated with distinct amounts of active β-catenin available in the nucleus for inducing Lef-Tcf transcription (Kielman et al., 2002). Also, Fuchs's group found dosage effects in the control of Wnt signaling in skin epithelial stem cells (Lowry et al., 2005). Alternatively, a specific combination of cofactors could cooperate with β-catenin/Lef-Tcf factors to transactivate these genes in the proximal PV area or to repress them in the distal PV area.

The β-Catenin Pathway Has a Major Impact on the Control of Ammonia Metabolism

Among the different zonated functions of the liver, ammonia metabolism is the function associated with a

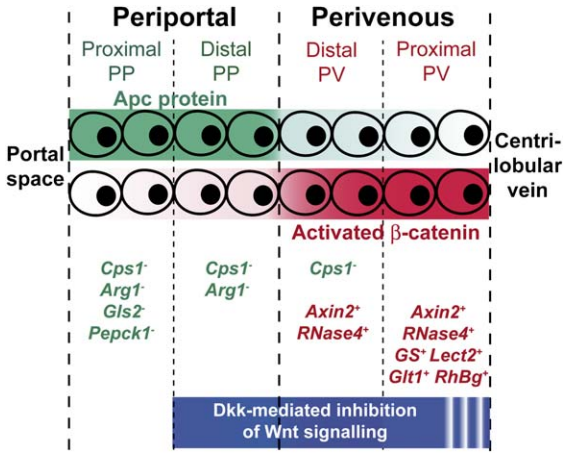


Figure 6. Organization of β-Catenin-Mediated Zonation within the Liver Cell Plate

The areas, the genes, and the proteins corresponding to: in green, the periportal zone, deprived of β-catenin signaling; and in red, the perivenous zone activated for β-catenin signaling. The positive target genes of a β-catenin signaling are followed by a "+," and the negative targets are followed by a "−." The blue area depicts the zone in which the consequences of Dkk-mediated inhibition of Wnt signaling can be seen. The stippled zone is that in which Dkk effects are incomplete.

stable gene expression zonation. Our findings show that β-catenin signaling is the determinant of this zonated function in the liver, as it spatially separates the two different ammonium detoxification systems: the periportal area devoted to urea formation and the perivenous area devoted to glutamine synthesis. For the other functions, such as carbohydrate and xenobiotic metabolisms, gene expression zonation is dynamic and depends on varying humoral and environment inputs (Jungermann and Kietzmann, 1996). We did not find a clear role of β-catenin signaling in these dynamic zonated functions in microarray analyses, and these aspects remain to be analyzed.

Our results suggest a link between a tumor suppressor gene and a metabolic pathway. Although this is a rather new suggestion, another tumor suppressor gene, the LKB1 gene, has been shown to control two disparate cellular processes—cell polarity and the AMP kinase activity that controls energy metabolism (Spicer and Ashworth, 2004). Therefore, it is important to understand whether the metabolic functions controlled by β-catenin signaling participate in liver tumorigenesis. Many of the negative or positive target genes that we have isolated are expressed in β-catenin-activated tumors in both mice and humans (Stahl et al., 2005), and a large set of these are involved in nitrogen metabolism and transport (*Gs*, *Glt1*, *Arginase1*, *RhBg*). A comprehensive study is needed to determine whether nitrogen metabolism is altered in patients or mice harboring HCCs activated for β-catenin, and to describe its impact on tumorigenesis.

To conclude, although rare *APC* mutations have been found in liver HCCs (Ishizaki et al., 2004; Legoix et al., 1999; Taniguchi et al., 2002), our findings demonstrate the critical role of *Apc* in liver homeostasis. This, together with the recent data from Jaenish's group showing that *Apc* is often lost after global hypomethylation of the genome, leading to liver tumors (Holm et al., 2005; Yamada et al., 2005), suggests that the *APC* loss of function should be investigated in a subset of human HCCs that are not mutated for β-catenin but that have an activated β-catenin pathway.

Experimental Procedures

Transgenic Mice Obtention and Care

Mice carrying the two floxed alleles on the 14th exon of the *Apc* gene (Colnot et al., 2004a) (here referred to as $Apc^{lox/lox}$) were interbred with TTR-Cre^{Tam} mice (Tannour-Louet et al., 2002). The resulting compound $Apc^{lox/+}$/TTR-Cre^{Tam} mice were interbred with $Apc^{lox/lox}$ mice to generate $Apc^{lox/lox}$/TTR-Cre^{Tam} mice. All mice had been backcrossed at least four times onto the C57Bl6N background. All reported animal procedures were carried out according to French government regulations. Mice were housed in conventional conditions.

Adenoviral Infections

Ad5-CMV-cre and Ad5-Dkk1-HA were amplified by Genethon (Evry, France) and were supplied at 10^{11} pfu/ml. Ad5β-catS37A was a gift of Dr. Jan Kitajewski (Columbia University, New York, USA). Diluted aliquots (10^9 pfu in 150 μl) were injected into the retroorbital vein of 8-week-old mice after anesthesic inhalation of Isofluran.

Tissue Processing

Unless otherwise mentioned, analyses were carried out on 2-month-old male mice. Tamoxifen (1 mg) (ICN, France) was diluted in 10 μl ethanol, adjusted to 100 μl with sunflower oil, and injected intraperitoneally (ip) at the indicated times. S phase nuclei were labeled by ip injection with BrdU (0.1 mg/g body weight, Sigma, France) 2 hrs before the mice were killed. Mice were killed at mid-day by cervical dislocation: liver samples were either snap frozen in liquid nitrogen and stored at −80ºC until molecular analyses or were fixed in 4% formol for 18 hr and then embedded in paraffin. For β-catenin and unphosphorylated β-catenin staining, ketamine-xylasine-anesthesized wild-type mice were perfused intracardiacally with a cold zinc-formaldehyde fixative. Liver slices were then postfixed with the same fixative for 3 hr at 4ºC before immediate paraffin embedding.

Immunostainings

Paraffin-embedded liver sections were treated as previously described (Colnot et al., 2004b). Antibodies are described in Supplemental Experimental Procedures.

In Situ Hybridization

Nonisotypic in situ hybridization was carried out on paraffin sections by using DIG-labeled riboprobes (see Supplemental Experimental Procedures).

RNA Extraction and Real-Time Quantitative RT-PCR

Total RNA was extracted as described elsewhere (Colnot et al., 2004b). Reverse transcription was carried out by using standard protocols (Invitrogen, Superscript II reverse transcriptase), and qPCR was done in duplicate on a LightCycler apparatus by using the LightCycler-fastStart DNA Master SYBR Green I Kit (Roche Diagnostics) and was expressed relative to rplp0 mRNA, as previously described (Cadoret et al., 2001). PCR primer sequences are available in Supplemental Experimental Procedures.

Microarray Analysis

We injected two distinct batches of Cre adenovirus into $Apc^{lox/lox}$ mice (three 2-month-old mice for the first batch, and two 5-month-old mice for the second batch) and $Apc^{lox/+}$ mice (same numbers of animals) in two separate experiments. The microarray analyses are described in Supplemental Experimental Procedures.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental Tables, and Figures S1–S4 and are available at http://www.developmentalcell.com/cgi/content/full/10/6/759/DC1/.

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Accession Numbers

Coordinates have been deposited in the EBI database with accession code E-TABM-81.