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Dual Catenin Loss in Murine Liver Causes Tight Junctional Deregulation and Progressive Intrahepatic Cholestasis

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Abstract

β -Catenin, the downstream effector of the Wnt signaling, plays important roles in hepatic development, regeneration and tumorigenesis. However, its role at hepatocyte adherens junctions (AJ) is relatively poorly understood, chiefly due to spontaneous compensation by γ -catenin. Here, we simultaneously ablate β - and γ -catenin expression in mouse liver by interbreeding β -catenin- γ -catenin double-floxed mice and albumin-cre transgenic mice. Double knockout mice (DKO) show failure to thrive, impaired hepatocyte differentiation, cholemia, ductular reaction, progressive cholestasis, inflammation, fibrosis and tumorigenesis, which was associated with deregulation of tight junctions (TJ) and bile acid transporters, leading to early morbidity and mortality, a phenotype reminiscent of Progressive Familial Intrahepatic Cholestasis (PFIC). To address the mechanism, we specifically and temporally eliminated both catenins from hepatocytes using adeno-associated virus-8 carrying cre-recombinase under the thyroid-binding globulin promoter

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(AAV8-TBG-Cre). This led to a time-dependent breach of blood bile barrier associated with sequential disruption of AJ and TJ verified by ultrastructural imaging and intravital microscopy, which revealed unique para-cellular leaks around individual hepatocytes allowing mixing of blood and bile, and leakage of blood from one sinusoid to another. Molecular analysis identified sequential losses of E-cadherin, occludin, claudin-3 and claudin-5 due to enhanced proteasomal degradation, and of claudin-2, a β -catenin transcriptional target, which was also validated in vitro. *In conclusion*, we report partially redundant function of catenins at AJ in regulating TJ and contributing to blood bile barrier. Furthermore, concomitant hepatic loss of β - and γ -catenin disrupts structural and functional integrity of AJ and TJ via transcriptional and posttranslational mechanisms. Mice with dual catenin loss develop progressive intrahepatic cholestasis, and hence provides a unique model to study diseases like PFIC.

Keywords

Wnt signaling; adherens junctions; cholestasis; E-cadherin; PFIC; Ultrastructure

Introduction

β -Catenin signaling is critical in liver health where it has been implicated in an array of roles in hepatic development, metabolic zonation, liver regeneration and xenobiotic metabolism (1). Its aberrant activation is also linked to a subset of hepatocellular cancers (HCC) (1). β -Catenin is also well-known component of adherens junctions (AJ) (2), where it bridges E-cadherin to α -catenin and actin cytoskeleton and facilitates intercellular adhesion (3,4). While its contribution to hepatic pathophysiology as a component of the Wnt signaling is relatively well understood, its role as a component of AJ has remained ambiguous. Liver-specific ablation of β -catenin in mice led to loss of the Wnt target genes like glutamine synthetase (GS), cytochrome P450 2E1 (cyp2e1), cyp1a2, lect2 and claudin-2 among others (5,6). However, there was maintenance of AJ due to compensatory increase in γ -catenin, a normal component of desmosomes, which associated with E-cadherin instead (7,8). Liver-specific γ -catenin knockout also showed increased β -catenin levels, suggesting a mutually compensatory role of the two catenins at hepatocyte junctions (9).

To conclusively address the role of β -catenin and in its absence γ -catenin in the liver, we generated β -; γ -catenin-double knockout (DKO) by interbreeding β -; γ -catenin-double floxed and Alb-cre transgenic mice. The DKO mice showed notable mortality and morbidity associated with failure to thrive, impaired hepatocyte differentiation, cholestasis, cholemia, portal hypertension, ductular reaction, fibrosis, increased serum alkaline phosphatase (ALP), elevated serum bile acid (BA) and bilirubin (BR) levels, all reminiscent of early childhood cholestatic liver diseases (CLD) like Progressive Familial Intrahepatic Cholestasis (PFIC) (10,11). To carefully delineate the mechanism, we next performed temporal and hepatocyte-specific elimination of the two catenins using hepatocyte-specific adeno-associated virus-8 carrying cre-recombinase expressed under hepatocyte-specific thyroid hormone-binding globulin promoter (AAV8-TBG-Cre). Dual catenin loss from hepatocytes also acutely also led to increased serum BR and ALP, allowing us the opportunity to carefully dissect the molecular basis of observed cholemia. Using intravital microscopy, we for the first time

show unique para-hepatocyte leaks leading to mixing of blood and bile as well as spillage of blood from one sinusoid to another. Such disruption of hepatic tissue barrier was confirmed by loss of TJ integrity as seen by transmission electron microscopy (TEM), lanthanum oxide exclusion and Evan's blue dye leakage into bile after intravenous injection. There was an associated loss of AJ assembly seen by loss of catenin-cadherin complex in DKO that led to enhanced proteasomal degradation of TJ proteins including the two major tetraspanins, occludin and plaque-forming claudins, which was further aggravated by loss of claudin-2, a Wnt/ β -catenin transcriptional target, important in biliary canaliculi formation. These findings identify important role of β -catenin in regulating TJ function.

Experimental Procedures

Animals

Previously reported homozygous β -catenin-floxed mice and γ -catenin-floxed mice were interbred to generate double floxed mice (6,9). Conditional deletion of both β - and γ -catenin is described in online methods. All animal experiments and procedures were performed according to the NIH guidelines under an animal protocol approved by the Institutional Animal Use and Care Committee at University of Pittsburgh.

Viruses and infections

AAV8-TBG-Cre or AAV8-TBG-GFP were obtained from Penn Vector Core at the University of Pennsylvania, Philadelphia. Four week or older β -; γ -double-floxed mice were given a single intraperitoneal injection of 2.5×10^{11} genome copies of AAV8-TBG-Cre or AAV8-TBG-GFP. At least 3 mice per time point ranging from 7d-14d were used for the studies.

Evan's blue vascular permeability assay

0.5% sterile solution of Evans blue was prepared in PBS. 200 μ l of Evans blue was injected in the inferior vena cava (IVC) of dWT or DKO2 mice at 10d or 12d post injection. Bile was collected from the gall bladder 15m after IVC injection to analyze the presence of Evans blue dye using fluorometric measurement (12). All experiments were done in triplicates.

Transmission electron microscopy (TEM)

For TEM, whole liver was perfused fixed in glutaraldehyde. The surgical and perfusion techniques have been described previously (5,9). Slides were examined in a JEM 1011 transmission electron microscope at 80kV. For lanthanum staining tissues were incubated with Lanthanum hydroxide (pH 7.8) for 3h at room temperature (13,14). Lanthanum hydroxide was prepared by slowly adding drops of 0.01N NaOH to 2% lanthanum nitrate until the solution reached pH 7.8.

Intravital imaging of liver

Intravital imaging of liver is described in detail elsewhere (15). Briefly, mice were anesthetized and placed on a heated stage. A catheter was placed into the right carotid artery to enable intravenous delivery of intravascular fluorochromes. Gentle vacuum suction was

applied to the abdominal imaging window device to immobilize. FITC dextran (MW: 70,000) or Texas red dextran (MW:70,000) was used to visualize the blood flow through liver sinusoids whereas carboxyfluorescein (CFDA; MW:367.82) was used to visualize biliary canaliculi (16). After being internalized by hepatocytes, CFDA is hydrolyzed by esterase into fluorogenic carboxyfluorescein (CF), which emits green fluorescence at 517nm. In WT mice, CFDA is taken up and hydrolyzed into CF in 1-5m after injection. Videos and images were recorded at 5 minutes (5'), 10' and 30' post-injection (PI). Microscopy was performed via Nikon MPE multi-photon excitation microscope at the imaging service of the Pittsburgh Liver Research Center.

TIRF imaging

The method of TIRF imaging is described elsewhere (17). Briefly, frozen section of hepatocytes was fixed on a coverslip. IF assay was performed using E-cadherin and occludin antibodies as described previously (17). Coverslips were mounted with PBS and vectashield. Microscopy was performed using a Nikon TIRF microscope.

Additional methods are available in online supplement.

Results

Simultaneous deletion of β -catenin and γ -catenin in the liver during development results in significant mortality and morbidity

Liver-specific β -catenin; γ -catenin double-knockout (DKO) mice were generated using albumin-cre as described in methods and confirmed by genomic DNA PCR analysis (Fig. 1A). When compared to WT, increased mortality was evident in DKO with >80% dying by 30 days (30d) and no survivors beyond 2.5 months (2.5m) (Fig.1B). Both male and female DKO mice were smaller in body size compared to WT (Fig.1C). Grossly, livers of DKO mice were yellow, smaller and stiffer as compared to WT at 24d, *albeit* the liver weight to body weight ratio (LW/BW) was comparable to WT (Fig.1D-1E). DKO mice that survived to 2.5m had higher LW/BW, and showed yellow and stiffer livers along with splenomegaly, a sign of portal hypertension (Fig.1D-1E). Intriguingly, serum alanine aminotransferase (ALT) levels were unchanged, although ALP and total and direct BR were significantly upregulated in DKO (Fig.1F-1G). WB and IHC confirmed loss of both β -catenin and γ -catenin in DKO livers as compared to WT (Fig.1H; Fig.2A).

DKO mice show extensive ductular reaction, liver fibrosis, inflammation, tumorigenesis and loss of E-cadherin

To further characterize the DKO phenotype displaying notable morbidity in neonatal stages, we assessed histology and performed IHC for injury and inflammation markers in DKO livers at 24d after birth. A notable increase in the presence of smaller ductular hepatocytes especially in the periportal region were evident by H&E in DKO livers (Fig.2B). Enhanced ductular reaction was also evident as seen by a notable increase in the numbers of ductules positive for epithelial cell adhesion molecule (EpCAM) and CK-19 (Fig.2C-2D). A profound increase in numbers of ductular hepatocytes was also verified by sox-9 staining, as is often seen in CLD (Fig.2D). Accompanying the ductular reaction, a noteworthy increase

in hepatic fibrosis as seen by Sirius red staining along with increased presence of activated myofibroblasts as evident by α -smooth muscle actin (α -SMA) IHC was also observed in DKO at 24d (Fig.2E).

Since 10% of mice survived to around 2.5m, we assessed the phenotype of DKO livers at this stage. A notable increase in CD45-positive inflammatory cell infiltrate was evident in DKO at this time (SFig.1A). Sirius red and α -SMA staining showed extensive collagen deposition and activated myofibroblasts in DKO, respectively (SFig.1A). Enhanced fibrosis was also confirmed by increased expression of pro-fibrogenic markers like colla1, Pdgfra and Tgfb1 by RT-PCR analysis (SFig.1B).

Any compensatory changes in ductular and hepatocyte proliferation were assessed next by IHC for Ki-67 at 24d and 2.5m (SFig.2A-2B). Careful quantification showed greater baseline Ki-67 staining in hepatocytes in WT livers at 24d due to known postnatal hepatic growth, however the DKO showed significantly fewer Ki-67-positive hepatocytes (SFig.2C). Intriguingly, a significant increase in Ki-67-positive ductular cells was evident in DKO (SFig.2C). At 2.5m, both hepatocytes and ductular cells showed significantly more Ki-67-positivity in DKO than WT (SFig.2D).

As a result of the chronic injury and regeneration, dysplastic nodules were apparent especially at appeared at 2.5m in DKO (SFig.1A). A subset of such nodules progressed to frank HCC (SFig.2E).

Loss of β - and γ -catenin leads to impaired bile flow and altered expression of several junctional proteins and biliary transporters

Elevated serum ALP and BR, extensive ductular reaction and increased biliary markers in hepatocytes, all indicated development of CLD in DKO mice. Indeed, in WT mice where gall bladders were intact and filled with bile, in DKO these structures were involuted and without bile (Fig.3A). We next assessed if cholestasis is leading to altered levels of BA in the liver and blood, to also validate presence of cholemia. DKO showed around 3-fold higher hepatic BA levels and almost 300-fold higher serum BA levels (Fig.3B-3C). Associated with increased BA levels, we observed notable ductular reaction at this stage (SFig.3A). Also, increased TUNEL-positive apoptotic nuclei were evident in DKO livers (SFig.3B).

It was intriguing that despite increased hepatocyte injury, serum ALT levels remained unchanged. We hypothesized this to be due to inappropriate hepatocyte maturation since DKO livers showed several sox-9-positive ductular hepatocytes (Fig.2D). Indeed, RT-PCR for targets of a major hepatocyte transcriptional regulator HNF4 α (18), showed significant decreases in its positive targets including Acot3, Ces3, Ugt2b1 and Dio1 and increased expression of negative targets including Akr1b7, Ect2, Egr1 and Myc, in DKO livers (SFig. 3C). Expression of Foxa2, another early hepatic transcription factor was also reduced (SFig. 3D). Likewise, expression of *alanine aminotransferase* gene was significantly reduced in DKO livers (SFig.3E) suggesting lack of increased serum ALT despite increased hepatocyte injury is due to improper hepatocyte maturation.

We next assessed localization of biliary canalicular markers to address the structure and viability of intrahepatic biliary canaliculi. IHC and IF verified decreased levels and mis-localization of CD10, occludin, claudin-2 and ZO-1 in DKO livers suggesting perturbations in biliary canaliculi (Fig.3D).

To further address any molecular aberrations that may be contributing to cholestasis and cholemia, expression of various biliary transporters, BA metabolism genes and TJ proteins were assessed. WB from whole liver lysates showed decreases in several junctional proteins like occludin, claudin-2, JAM-A and E-cadherin in DKO, while ZO-2 (TJP2) expression was increased (Fig.3E). E-Cadherin decrease was also verified by IHC in DKO (SFig.4A). BSEP protein levels were decreased (Fig.3E) while its mRNA expression was unchanged (SFig. 4B). RT-PCR also revealed decreases in the mRNA expression of NTCP, Cyp7a1 and MRP2 in DKO (SFig.4B).

Thus overall, DKO mice exhibit a phenotype that is truly reminiscent of PFIC patients showing a plethora of aberrations including defects in expression of biliary transporter BSEP and deregulation of a host of hepatic junctional proteins.

Specific deletion of β - and γ -catenin only from hepatocytes leads to time-dependent cholemia

To identify the primary molecular basis and sequence of the phenotype evident in DKO, we conditionally deleted β - and γ -catenin from the hepatocytes only by injecting AAV8-TBG-Cre in 4-week old β -; γ -catenin double-floxed mice (DKO2) and sacrificing at 7d-14d for evaluation (SFig.5A)(19). As a control, β -; γ -catenin double-floxed mice were injected with AAV8-TBG-GFP (dWT). To show the robustness and hepatocyte-selectivity, IHC for GFP was performed at 7d post-injection of AAV8-TBG-GFP. Almost 100% of hepatocytes and no cholangiocytes were GFP-positive (SFig.5B). Efficacy of AAV8-TBG-Cre was also evident by a significant and sustained decrease in the protein and mRNA expression both catenins in DKO2 livers at 7d-14d after injection as compared to dWT (SFig.5C-5E). Since, we posit that loss of both catenins simultaneously from hepatocytes affects junctional stability, we assessed serum from DKO2 mice at various times. While serum BR, both total and direct, and ALP, were unremarkable at d7 and d10, a significant and progressive increase in their levels was evident at d12-d14 (SFig.5F). An increase in ductular reaction was also observed at 14d in DKO2 livers (SFig.5G). Thus, an acute and hepatocyte-specific loss of β - and γ -catenin resulted in an acute onset of cholemia similar to DKO and provided us a unique opportunity to examine sequentially both cellular and molecular basis of the observed pathology.

Unique para-hepatocyte leaks lead to admixing of blood and bile and channeling of blood from one sinusoid to another in DKO livers as shown by multiphoton intravital microscopy

To investigate the structural basis of observed cholemia at the earliest time in DKO2, we used multi-photon-excitation (MPE)-enabled in vivo real-time fluorescence microscopy of intact liver in live mice as described elsewhere (15). FITC-dextran was intravascularly administered to visualize the blood flow in liver sinusoids. Blood flow (green) was restricted and linear within sinusoids in dWT (Mov.S1-S2; Fig.4A). However, thin conduits

reminiscent of para-hepatocyte leaks carrying cell free plasma (green) were observed connecting adjacent sinusoids outlining single hepatocytes in DKO2 livers at 12d (Mov.S3-S4; Fig.4A).

To visualize both blood and bile flow using MPE-enabled intravital microscopy, we next used Texas-Red (TXR) dextran (red) to label blood flow through liver sinusoids and carboxyfluorescein (CF) (green) to envision bile flow through biliary canaliculi (20). In dWT, the uptake of CF in hepatocytes occurred within 1-5' after injection and CF appeared as linear green streak highlighting its secretion into biliary canaliculi (Fig.4B; Mov.S5). Red fluorescence indicated normal blood flow in sinusoids. At 10', CF prominently localized to biliary canaliculi (Fig.4B; Mov. S6). By 30', CF decreased considerably in biliary canaliculi suggesting its clearance from intrahepatic biliary compartment (Fig. 4B; Mov. S7). DKO2 at 12d showed peculiar and unique phenotype in which TXR-dextran positive red spots along with CF were found in punctate patterns within sinusoids along with absence of green linear biliary canalicular presence at 5', 10' and 30' (Fig. 4B; Mov. S8-S10). Furthermore, thin conduits reminiscent of para-hepatocyte leaks, outlining hepatocytes and interconnecting sinusoids through which CF and TXR- dextran flowed freely, were evident in DKO2 at all times (Mov. S8-S10).

In vivo vascular permeability assay using the Evans blue vascular dye was performed next (12). Complete absence of Evans blue in the bile was observed after a single intravenous injection of Evans blue to DKO2 mice at 10d (Fig. 4C). However, at 12d, Evans blue was significantly increased in bile in the DKO2 but not dWT (Fig. 4C).

Altogether, these observations for the first time, allowed a direct visualization of structural perturbations in the form of para-hepatocyte leaks connecting adjacent sinusoids and sinusoids to biliary canaliculi, demonstrating the basis of cholemia in DKO2 mice at 12d.

Dual loss of β - and γ -catenin leads to the disruption of hepatocyte junctions

Since β -catenin and in its lieu γ -catenin reside as a structural component of AJ, and to understand the primary mechanism that led to disruption of the blood bile barrier following the loss of both, we next assessed the structure of AJ and the barrier forming TJ in DKO2 and dWT livers. TEM of TJ and AJ in dWT liver sections at any stage (shown 7d) revealed TJs as electron-dense structures in the space between cells near the bile canaliculi abutted by AJ, which is evident as thin double-stranded structure of defined diameter (Fig.5A). In DKO2 livers, the electron dense TJ structure appeared normal at 7d, however at 10d and 14d, TJ contained less electron-dense material resulting in a dilated structure (Fig.5A). AJ in DKO2 showed increased intercellular distance at 7d, which were further dilated by 10d, and their structure lost by 14d (Fig.5A).

TJ integrity was tested next using lanthanum hydroxide permeability (13). Intact TJs surrounding biliary canaliculi shown by lanthanum hydroxide exclusion from TJ showing only its extracellular distribution signified an intact barrier in the dWT livers (Fig.5B). In DKO2 livers, such exclusion was apparent at 7d, however by 10d and 14d, lanthanum was seen accumulating in the TJ, indicating loss of structural integrity (Fig.5B).

Dual loss of β - and γ -catenin leads to distortion of biliary canalicular network

Biliary canalicular networks, which are lined by the apical surfaces of hepatocytes and flanked by TJ proteins, allow unidirectional flow of bile secreted by hepatocytes towards the portal triad. Since the simultaneous loss of the two catenins disrupted TJ, we next investigated if this results in any disruption of the structural integrity and patency of biliary canalicular networks. We stained liver sections from DKO2 and dWT mice with TRITC-phalloidin to visualize F-actin, which normally marks the pericanalicular outline of hepatocytes. dWT livers at d12 exhibited interconnected, evenly spaced “train-track” bile canalicular structures (Fig.5C). In contrast, DKO2 livers at d12 showed pronounced distortion of bile canaliculi observed as misshapen, collapsed and incomplete network structures (Fig.5C).

Dual loss of β - and γ -catenin leads to decreased levels of key junctional proteins

To address the temporal deregulation of various junctional proteins following loss of β - and γ -catenin, we next investigated the protein and mRNA expression of these proteins in DKO2 and dWT livers. Decrease in E-cadherin protein was observed at 10d, 12d and 14d (Fig. 6A). This was also visible by IF, which not only displayed E-cadherin decrease at hepatocyte membrane but also showed its redistribution as punctate cytoplasmic staining in the DKO2 livers at 12d, suggestive of proteasomal degradation (Fig.6B). Indeed, mRNA expression of E-cadherin was unchanged until 14d (Fig.6C). Next, we assessed claudins, which are tetraspanins and key components of TJ in DKO2 liver lysates. Claudin-3 and -5, which are plaque-forming, were decreased at 12-14d (Fig. 6A). IF staining verified a notable decrease in surface localization of claudins-3 and -5 at hepatocyte surface at 12d as well (Fig. 6D). Claudin-2, a known Wnt/ β -catenin target in the liver, was significantly decreased at 7d-14d in DKO2 livers by both WB and RT-PCR (Fig. 6A, 6C). Occludin, another TJ tetraspanin protein was also decreased at 12d and 14d, while its mRNA expression was unaffected until 14d (Fig. 6A, 6C). Occludin staining at the TJ in hepatocytes was decreased in DKO2 livers as compared to dWT at 12d, while ZO-1 remained unchanged (not shown).

To unequivocally demonstrate the lack of localization of E-cadherin and occludin at cell surface in the DKO2 livers, we utilized total internal reflection fluorescence (TIRF) microscopy. TIRF revealed E-cadherin and occludin localizing at hepatocyte membrane in dWT livers, which was completely lost in DKO2 livers at 12d (Fig. 6E).

Thus, dual loss of catenins triggers deregulation of E-cadherin, along with of key TJ proteins including occludin and claudins-2, -3 and -5 in the DKO2 livers.

Absence of β - and γ -catenin leads to enhanced degradation of E-cadherin and key TJ proteins, which is alleviated by proteasome inhibitor whereas claudin-2 loss is unaffected

Next, we addressed the basis of decrease in E-cadherin, occludin and claudins following loss of both catenins, in vitro and in vivo. First, we used human Hep3B cells to query if TJ proteins like occludin co-precipitate with AJ proteins. Immunoprecipitation (IP) studies in scrambled siRNA (Sc)-transfected Hep3B cells showed association of occludin with E-cadherin, β -catenin and γ -catenin (Fig. 7A). Dual silencing of β - and γ -catenin (SiBG) led to abrogation of this association (Fig.7A). SiBG-transfection in Hep3B cells also led to

notable decreases in total E-cadherin, occludin, claudins-2 and claudin-3 (Fig. 7B, 7C). Blocking proteasomal degradation by MG132 in SiBG-transfected cultures rescued E-cadherin, occludin and claudin-3 but not claudin-2 (Fig. 7B, 7C). Similarly, increased E-cadherin ubiquitination was observed upon siBG-transfection in Hep3B cells (Fig. 7D). Blocking lysosomal degradation by cyclohexamide did not influence stabilization of any of these proteins and no advantage of combining MG132 and cyclohexamide was observed (Fig. 7B).

Since decrease in E-cadherin due to proteasomal degradation, a pivotal AJ component preceded loss of TJ proteins both in vitro and in vivo, we next silenced E-cadherin (siE) in Hep3B cells. Si-transfection in Hep3B cells led to notable decreases in occludin and claudin-3, but did not impact β - or γ -catenin levels (Fig. 7E).

To confirm if mechanism of E-cadherin and occludin loss in vivo in DKO2 livers was indeed proteasomal degradation, we immunoprecipitated E-cadherin or occludin and performed WB analysis for poly- and mono-ubiquitin. A notable and time-dependent increase in ubiquitination of both proteins was observed at d10-d12 in DKO2 livers (Fig. 7F, 7G).

Discussion

Liver-specific β -catenin KO have been instrumental in divulging its roles in development, regeneration, zonation and tumorigenesis (1,6,21). However, its relative contribution at the hepatocyte-junctions has remained poorly understood due to compensation by γ -catenin, which prompted us to generate dual KO (7,8,22). Loss of the two catenins was fatal during the first postnatal month and led to severe and progressive CLD. More controlled deletion of catenins from hepatocytes to address mechanism revealed appearance of unique paracellular leaks that disrupted blood-bile barrier and also allowed blood from one sinusoid to leak into another (Fig. 8A). At a molecular level, these phenomena were associated with disruption of catenin-cadherin complex, prompting proteasome-mediated degradation of E-cadherin and of occludin and plaque forming claudin-3 and -5. Simultaneously, decreased gene expression of claudin-2 was evident. Concomitant loss of occludin and claudins, the two major families of tetraspanins at TJ, led to disintegration of TJ, disruption of blood bile barrier and cholemia. Thus, we show that β -catenin regulates tissue barrier function through regulation of TJ components via distinct mechanisms (Fig.8B). We also show that TJ enable orderly and unidirectional flow of sinusoidal blood from portal triad towards central vein within a hepatic lobule (Fig. 8A).

CLD are a heterogeneous group of disorders characterized by impaired bile flow, biliary stasis, spillage of bile into blood and hepatic parenchyma, tissue injury, inflammation, fibrosis and eventually sequelae like biliary cirrhosis and cancer. Impaired bile flow through intrahepatic biliary canaliculi can be due to bile flow obstruction, defect in hepatocyte secretory function arising from anomalies in expression and function of various transporters, or defect in hepatocyte polarity (23-25). Loss-of-function (LOF) mutations in the components of tight junctions (TJ), the chief regulator of barrier functions in a tissue, have also been implicated in these diseases. In fact, a subset of patients with PFIC, a group of disorders responsible for 10-15% of neonatal CLD in the US and traditionally associated

with LOF mutations in transporters essential for optimal bile secretion (PFIC-1:ATP8B1; PFIC-2:BSEP/ABCB11; PFIC-3:MDR3) (26-30), have been now identified with mutations in zonula occludens-2 (ZO-2), a TJ protein (31). Based on our findings in DKO mice, we demonstrate that loss of β - and γ -catenin lead to a host of changes in levels and distribution of both bile acid transporters and tight junctional proteins to yield an early postnatal phenotype reminiscent of PFIC. Specifically, we observed a notable decrease in BSEP, ZO1, occludin, claudin-2 and JAM-A, an upregulation in ZO-2 and no change in claudin-7. All symptomatic, histological and biochemical attributes of the disease were evident in the DKO mice including failure to thrive, likely due to impaired BA secretion, failure of bile flow, and fat malabsorption; jaundice and cholemia; progressive intrahepatic cholestatic injury associated with enhanced BA accumulation, inflammation, portal fibrosis, portal hypertension and cirrhosis, and progression to HCC in a subset of animals. Thus, the disease in DKO very closely recapitulates PFIC, especially PFIC2 that is associated with loss of BSEP and could be an invaluable model to study pathogenesis and test novel therapeutics. It is important to note that targeted inactivation of *BSEP* in mice by itself did not yield a progressive cholestatic phenotype though mice showed modest but stable CLD (32). Only upon additional deletion of multidrug resistance protein 1a (*Mdr1a*) and *Mdr1b* in the BSEP null background, was there evidence of progressive cholestasis and enhanced mortality and morbidity *albeit* at around 6 months, and with distinct differences from PFIC clinically (33). Dual loss of β -catenin and γ -catenin in the liver, on the other hand, was sufficient to lead to sufficient deregulation in levels of biliary transporters like BSEP as well as additional junctional components, which led to a more profound disease reminiscent of PFIC. While our model is exciting to study disease pathogenesis, further studies are necessary to evaluate if loss of AJ catenins may itself be an important cause for subsets of CLD like PFIC or others.

The barrier function in tissues is regulated primarily by TJ (34). In liver, TJ are also thought to be the chief regulators of the blood bile barrier, although very few studies have directly demonstrated such function (35,36). These junctions prevent blood and bile from mixing, Unlike TJ, AJ are viewed primarily as facilitators of cell-cell adhesion (37). While studies have suggested AJ can influence TJ organization, the molecular basis of AJ and TJ crosstalk remains incompletely understood (38). Studies have suggested important roles of E-cadherin in the development and organization of TJ through proper localization of proteins or through regulation of claudins (39,40).

We show that β -catenin, downstream of Wnt, regulates expression of claudin-2. We have previously reported claudin-2 to be a target of β -catenin in the liver (41). While, it is a pore-forming claudin, whose loss would not make TJ more permeable, it has been shown to be pertinent in proper biliary canaliculi formation and bile flow (42,43). In fact, a basal decrease in bile flow rate in β -catenin was attributed to claudin-2 loss, while no structural defect was observed in TJ (41). Thus, one major function of β -catenin in liver that contributes to appropriate biliary homeostasis is regulating the expression of claudin-2 in hepatocytes, which is not compensated by γ -catenin in β -catenin KO (6,7).

To unequivocally address the mechanism of how dual loss of catenins in hepatocytes yielded progressive intrahepatic cholestasis, we carefully elucidated the sequence of molecular

perturbations that were evident as β - and γ -catenin were ablated. As expected, claudin-2 expression dissipated almost around the time of loss of β -catenin (and γ -catenin). The next major deregulation was that observed in E-cadherin proteins levels, which was posttranscriptional and also preceded any losses in proteins levels of occludin and plaque-forming claudins-3 and -5. At AJ, β -catenin, and in its absence γ -catenin, appears to be critical for the stability of E-cadherin. Lack of this association in both DKO, DKO2 and *in vitro*, led to proteasomal degradation of E-cadherin. Indeed β -catenin, through its physical association, is known to mask the PEST sequence motif on E-cadherin to prevent its recognition and degradation by ubiquitination (44). γ -Catenin appears to be efficiently performing this function when β -catenin was absent as seen in single β -catenin KO (7,8), and hence contributes to E-cadherin stability and maintains AJ. We also show that catenins and E-cadherin complex with TJ proteins like occludin. Loss of catenins or E-cadherin promoted degradation of occludin and claudin-3, which could be prevented *in vitro* by a proteasome inhibitor. Proteasome-dependent degradation of occludin in various cell types has also been reported, although no prior connection to AJ proteins has been shown (45,46). Thus, we divulge a complex interactome composed of key AJ and TJ proteins which help stabilize one another to eventually maintain junctional integrity. It appears that β -catenin and in its absence γ -catenin is especially critical in its role due to perhaps its dual function at junctions and as Wnt pathway component, where it additionally regulates claudin-2 mRNA expression.

Thus, loss of β -catenin alone or loss of Wnt/ β -catenin signaling via combined deletion of Wnt co-receptors LRP5-6 is well-tolerated despite lack of claudin-2 since these animals lack any overt CLD (5,6,21,41,47). This is due to the continued presence of occludin and plaque-forming claudins, the major tetraspanins at the TJ (48), due to γ -catenin, which binds and stabilizes E-cadherin. However, when two major cornerstones of TJ structural integrity, claudins and occludin, are lost simultaneously, the hepatic tissue barrier gives way and CLD ensues. Overall, our study suggests an evolutionarily conserved and higher order function of β -catenin at AJ than as a component of the Wnt signaling pathway. This can be concluded since γ -catenin is adapted to fulfill a more vital function at AJ upon β -catenin loss and this redundancy is indispensable to survival since DKO mice show severe morbidity and mortality. γ -Catenin increase that is evident in β -catenin KO is not compensating for rescue of Wnt signaling seen by continued lack of its transcriptional targets. Our study also highlights function of AJ as master regulators of TJ structure and eventually barrier function. Anomalies in the expression of key junctional proteins may thus be an important mechanism of cholemia and CLD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Conditional hepatic loss of β - and γ -catenin leads to increased mortality and morbidity due to CLD

- A.** PCR shows various genotypes to identify β -catenin^{loxp/loxp} γ -catenin^{loxp/loxp}Alb-Cre^{+/-} (DKO;2), β -catenin^{loxp/loxp} γ -catenin^{loxp/WT}Alb-Cre^{+/-} (4), GKO (β -catenin^{loxp/WT} γ -catenin^{loxp/loxp}Alb-Cre^{+/-} (1), β -catenin^{loxp/WT} γ -catenin^{loxp/WT}Alb-Cre^{+/-} (3), and β -catenin^{loxp/loxp} γ -catenin^{loxp/loxp}Alb-Cre^{-/-} (WT controls; 5)
- B.** Kaplan-Meier curve shows significant mortality in DKO mice.
- C.** Both male (M) and female (F) DKO mice show stunted growth compared to WT.
- D.** DKO livers at 24d and 2.5m exhibit yellow color and stiffness; and large spleens (2.5m).
- E.** Liver weight/body weight ratio (LW/BW) remains unchanged in 24d DKO. At 2.5m, DKO show increased LW/BW as compared to WT (p=0.0056). Spleen weight/body weight ratio (SW/BW) is also significantly increased in DKO (p=0.0022).

- F.** High serum ALP in DKO mice ($p=0.0004$ at 24d, $p=0.0039$ at 2.5m) compared to WT. No significant changes in ALT levels.
- G.** Significantly higher total ($p=0.0007$ at 24 days and $p=0.0008$ at 2.5 months) and direct ($p=0.0026$ at 24 days and $p=0.0006$ at 2.5 months) serum BR levels in DKO than WT.
- H.** Representative WB of β - and γ -catenin proteins in WT and DKO livers. GAPDH shown as loading control. Densitometry verified around 8-fold reduction in β - and γ -catenin in DKO liver.

Figure 2. DKO livers show increased ductular reaction, expression of biliary markers in hepatocytes and associated fibrosis

- A.** Liver IHC shows membranous staining of β -catenin in WT, which is lost in DKO.
- B.** H&E shows notable increase in periportal progenitors at 24d in DKO livers.
- C.** IF for EpCAM shows increased ductular cells in DKO.
- D.** IHC for Sox-9 shows localization to bile ducts in WT. In DKO, Sox-9 also localizes to periportal hepatocytes. Increased CK-19 positive ductular reaction also seen in DKO.
- E.** Sirius red staining shows increased collagen in DKO livers. Increased α -SMA-positive active-myofibroblasts also observed in DKO livers.

Scale bars: 50 μ m.

Figure 3. DKO show defect in bile flow, elevated hepatic and serum bile acids and deregulated expression of junctional proteins

- A.** Gall bladder (arrow) are involuted and lack bile in DKO but not in WT mice.
- B.** A significant increase in serum BA in DKO as compared to WT ($p=0.0001$).
- C.** A significant increase in hepatic BA in DKO as compared to WT ($p=0.0002$).
- D.** IHC on liver sections show loss of CD10, claudin-2 and occludin, and IF shows decreased ZO-1 in DKO liver. Scale bar: 50μm.
- E.** WB shows decreased protein levels of E-cadherin, JAM-A, claudin-2, occludin and BSEP and increase in GGT and ZO-2 in DKO as compared to WT liver lysates. GAPDH was loading control.

Figure 4. Dual loss of β - and γ -catenin in hepatocytes leads to leaky hepatocyte junctions, disruption of blood bile barrier and cholemia

- A.** Intravital image of dWT liver shows FITC dextran labeled liver sinusoids; DKO2 liver shows presence of thin conduits around hepatocytes (*) that connect one sinusoid to another. Scale bars: 10 μ m.
- B.** Time series images (5m, 10m and 30m) of (n=1) 12d dWT liver shows localization of CF (green) and Texas-red dextran (red) in bile canaliculi and sinusoids, respectively. Time series images of 12d DKO2 liver shows co-localization of CF and Texas-red dextran in sinusoids and failure of uptake and secretion into bile canaliculi at 5m, 10m and 30m after injection of dyes. Instead, there was appearance of thin conduits or collaterals between hepatocytes through which red and green positive aggregates move and are also seen accumulating in peri-sinusoidal areas (asterisk). Scale bars: 50 μ m.
- C.** Quantification of Evans blue in the blood and bile of 10d dWT and DKO2 mice sampled 15 minutes after Evans blue injection into the inferior vena cava shows no appearance of dye in bile. At 12d, significant increase in Evans blue dye is observed in DKO2 bile as compared to dWT (p=0.001).

Figure 5. β - and γ -catenin loss in hepatocytes leads to disruption of TJ integrity and loss of key junctional molecules

- A.** TEM of 7d dWT and DKO2 livers showed presence of electron-dense and normal appearing TJ close to biliary canaliculi (BC). Normal appearing AJ seen at 7d in dWT, while increased intercellular space (left-right arrow) evident in DKO2 livers at this time-point. TJ are less electron-dense at 10d in DKO2 while AJ continue to show loose structure. Widened TJ and AJ (left-right arrow) evident in DKO2 liver sections at 14d.
- B.** D7 dWT and DKO2 liver sections showed intact TJ devoid of lanthanum hydroxide (black particles) while AJ are positive (arrows). DKO2 liver sections at 12d and 14d show lanthanum hydroxide inside the TJ (arrows).
- C.** Phalloidin staining in DKO2 liver at 12d shows lack of classical train-track pattern of localization outlining the biliary canaliculi as seen in dWT liver (arrow) at the same time. Arrow in DKO2 points to decreased organization, collapse and incomplete of network. Scale bars: 50 μ m.

Figure 6. β - and γ -catenin loss in hepatocytes leads to deregulation of several key junctional molecules

- A.** Representative WB shows decreased E-cadherin at 10d-14d, and occludin, claudin-3 and -5 at 12d-14d in DKO2 as compared to 14d dWT. Claudin-2 was absent from 7d in DKO2 livers. GAPDH shows protein loading.
- B.** RT-PCR shows significant decrease in expression of claudin-2 ($p < 0.001$) in DKO2 at 7d onwards as compared to dWT livers. Significant decrease in occludin and E-cadherin mRNA in DKO2 livers was evident only at 14d ($p < 0.001$).
- C.** Membrane localization of E-cadherin evident in dWT livers was visibly reduced in DKO2 at 12d and appeared as punctate cytoplasmic staining by confocal microscopy.
- D.** Confocal microscopy showed membrane localization of claudin-3 and -5 in dWT livers, which was reduced in DKO2 at 12d.
- E.** TIRF imaging revealed membrane localization of E-cadherin in dWT liver sections, which was absent in DKO2 livers at 12d.

- F.** TIRF imaging revealed membrane localization of occludin in dWT liver sections, which was absent in DKO2 livers at 12d. G. Scale bars: 50 μ m.

Figure 7. β - and γ -catenin loss from liver triggers loss of junctional proteins in vitro

- A.** Immunoprecipitation (IP) of occludin from lysates of Hep3B cells transfected with scrambled (Sc) or dual siRNA against β - and γ -catenin (SiBG) showed its association with E-cadherin, β -catenin and γ -catenin in Sc only. IP of γ -catenin in these cells showed reduced association of γ -catenin with occludin in Hep3B cells transfected with siRNA against γ -catenin (SiG) and SiBG but not Sc or siRNA against β -catenin (SiB).
- B.** MG132 (MG) and cyclohexamide (C) treatment of Sc or SiBG Hep3B cells showed MG treatment alone stabilized E-cadherin and occludin in SiBG.
- C.** SiBG-transfected Hep3B cells showed decreased protein levels of occludin, claudin-3 and claudin-2. SiB-transfection alone led to decreased claudin-2 as well. MG treatment of SiBG-transfected cells rescued occludin and claudin-3 but not claudin-2 levels.
- D.** Comparable E-cadherin pull-down associated with more ubiquitin by IP in SiBG-transfected Hep3B cell lysates as compare to Sc. Densitometry showed ~1.7-fold increase in ubiquitination.

- E.** SiRNA against E-cadherin (SiE)-transfected Hep3B cells showed decreased protein levels of occludin and claudin-3 but no change in β - or γ -catenin, GAPDH verified protein loading.
- F.** Comparable E-cadherin pull-down associated with more ubiquitin by IP especially at 14d in DKO2 liver lysates. Densitometry verifies around 5-fold increase in E-cadherin ubiquitination in DKO2 livers at 14d.
- G.** IP of occludin followed by WB with ubiquitin showed increased ubiquitination of occludin in DKO2 liver samples at 7d and 14d. Densitometry showed around 2-fold and 9-fold increase in occludin ubiquitination at 7d and 14d, respectively.

Figure 8. β - and γ -catenin regulate hepatic tissue barrier through regulation of tight junctional integrity

- A.** TJ, AJ, and desmosomes exist between two adjacent hepatocytes. TJ prevent mixing of blood (red) in sinusoid and bile (green) in canaliculi, and prevent mixing of blood from adjacent sinusoids. Dual loss of catenins at AJ affects TJ to disrupt hepatic tissue barrier, resulting in para-hepatocyte leaks allowing mixing of blood and bile, and blood from one sinusoid to another.
- B.** A model depicting distinct mechanisms by which β -catenin regulates hepatic TJ integrity. At AJ, β -catenin or in its absence γ -catenin, stabilizes E-cadherin and in turn stabilizes TJ proteins occludin and plaque-forming claudins -3 and -5. As a component of the Wnt signaling pathway, β -catenin non-redundantly regulates expression of claudin-2 essential for biliary canalicular structure and bile flow. Loss of β -catenin alone affects claudin-2 expression only, but dual loss of catenins additionally affects occludin and plaque-forming claudins to affect TJ structure and barrier function.