THE STORY OF REDUNDANT CATENINS AND THEIR ROLES IN CELL-CELL ADHESION IN THE LIVER

by

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β-Catenin is important in liver homeostasis as a part of Wnt signaling and adherens junctions (AJs); however, aberrant β-catenin activation is observed in a subset of hepatocellular carcinomas (HCC). Since therapeutic targeting of β-catenin in HCC is inevitable, it is important to investigate the implications of such interventions on AJs. We address this important issue both *in vivo* and *in vitro*. We observed that hepatocyte-specific β-catenin knockout (βKO) mice have no noticeable adhesive defects. We identified an increase in γ-catenin at AJs in βKO livers. We further observed that γ-catenin was unable to translocate to the nucleus in absence of β-catenin after partial hepatectomy in βKO mice. Since γ-catenin is a desmosomal protein, we next investigated if γ-catenin changes in βKOs were at the expense of desmosomes. We did not observe any differences on desmosomal structure or composition in βKO livers. Similarly, as some tight junction components are β-catenin target genes, we observed only minor changes in tight junctions, such that the function of the junctions was not affected *in vivo*. To further determine the role and regulation of γ-catenin in absence of β-catenin we established an *in vitro* model. Hep3B human HCC cells transfected with siRNA to β-catenin led to γ-catenin increase. Using this model we showed γ-catenin was unable to rescue decreased Wnt reporter activity. Scratch-wound assays showed β- and γ-catenin single knockdowns did not affect cell migration, but double knockdown significantly increased wound closure. Centrifugal assay for cell adhesion

and hanging-drop assays, measuring hetero- and homotypic cell-cell interactions, showed significant decreases in adhesive strength with double knockdown only. Lastly, we showed the increased γ-catenin with β-catenin loss appears to be regulated by the serine/threonine phosphorylation of γ-catenin by protein kinase A, introducing the possibility of a catenin sensing mechanism. In conclusion, β-catenin loss is compensated by γ-catenin at AJs without negatively affecting other junctions; however, the function of β-catenin as part of the Wnt pathway remains unfulfilled by γ-catenin. Thus, proposed anti-β-catenin therapies for HCC may be able to target aberrant β-catenin in Wnt signaling specifically without negatively affect HCC prognosis, as long as γ-catenin is spared.

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PREFACE

First, I want to thank my husband, Greg Wickline, who has known nothing of married life but having me as a graduate student – which included lots of take-out dinners and Saturday nights alone while I was studying for tests, writing papers, working on grants, etc. I whole-heartedly appreciate all those extra hunting and fishing days that you graciously took so that I didn't feel bad working on the weekends. I could not have made it through graduate school without your grounding presence and unconditional love. Thanks for bearing with me as I decided to continue being a student after we were married. I continue to look forward to growing up and growing old with you. And, if you ever decide to pursue a crazy dream like I did, I hope that I can be as supportive and patient a partner as you have been for me during the past five years.

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I owe a huge portion of my PhD to my undergraduate research advisor and wonderful life mentor Dr. Melissa Rowland-Goldsmith at Chapman University. She was the one who enlightened me to the fact that a PhD is not a MD fallback plan, but a genuine career choice. I

hope to bring that same message to undergraduate students one day in order to motivate them to follow their love for science into a PhD career. Also, thank you for encouraging me to always think beyond the science and really care about who the research is for – the patients.

Lastly, I could not have succeeded without a wonderful dissertation mentor – Dr. Satdarshan (Paul) Singh Monga. You picked me to be a part of your lab seven years ago, and I am grateful that you did. You and your lab helped me learn what real science was. I learned at the very beginning in my SURP summer that failure is a part of science – but failure is not a disappointment, unless you do not learn from it. I appreciate your open door when graduate school seemed to get the best of me, and your generosity in bringing me back to your lab. Also, thank you for helping me get my confidence back after some trying times on my scientific path. I look forward to my future in science, and I hope my current and future success is a clear reflection of Dr. Monga's brilliant guidance.

1.0 INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide, and the severity of this disease has resulted in it becoming the third leading cause of cancer-related deaths worldwide [1, 2]. HCC commonly arises after chronic liver injury caused by diseases like hepatitis and non-alcoholic steatohepatitis (NASH), which most often lead to liver fibrosis and cirrhosis [3]. The increased incidence of Hepatitis C (HCV) in developing countries is a troubling statistic with respect to the incidence of HCC in the United States. Only about one-third of all HCCs are curable by surgical resection, and the current treatments for the majority of HCC cases involve non-targeted palliative care, such as systemic chemotherapies [4]. Therefore, understanding and treating this disease at a cellular and molecular level is imperative in order to relieve this major health burden.

Pathologically, an irregular distribution of β-catenin has been described in up to 90% of HCC cases [5-7]. β-Catenin is normally found at the hepatocyte membrane in adult livers, but in HCC there is often a decrease of membranous β-catenin and/or an increase in nuclear or cytoplasmic β-catenin localization [8]. Of the 90% of HCCs with abnormal β-catenin staining, *CTNNB1* gene mutations have also been reported in 30% of these cases [9]. Additional Wnt pathway mutations also contribute to abnormal β-catenin stability/longevity and therefore aberrant distribution. HCC mutations and inactivations have been found in *AXIN-1*, *AXIN-2*, and *Glycogen Synthase Kinase 3β* (*GSK3β*), all components of the Wnt signaling destruction

complex [10]. Based on the observation of β-catenin mutations and mislocalizations in HCC, it has the potential to be a viable therapeutic target. Our lab and others have recently proposed a number of unique anti-β-catenin therapies for the treatment of HCC [11-13].

1.1 β-CATENIN

β-Catenin is a highly conserved protein with twelve core armadillo (arm) repeats flanked by N- and C-terminal domains which are unique to each armadillo family member. β-Catenin has important roles in regulating embryogenesis, and also in maintaining normal cell homeostasis. β-Catenin classically influences the cell in two ways: 1) signal transduction via the Wnt/β-catenin pathway **(1.1.1**); and 2) cell-cell adhesion via the adherens junctions (**1.1.2**) (reviewed in [14]). The activity of β-catenin in both signaling and cell-cell adhesion reveals the dynamic properties and general importance of this protein to overall cellular function.

1.1.1 Wnt signaling

In the canonical Wnt/β-catenin pathway, the presence of the lipid-modified Wnt ligand initiates the formation of a ternary complex with the seven-transmembrane Frizzled receptor and Low-density Lipoprotein Receptor Related Protein (LRP)5/6 which starts an intracellular signaling cascade. There are 19 human Wnt isoforms and 11 Frizzled isoforms known to date [15, 16], and the large number of possible combinations of ligand and receptor results in diverse possibilities for canonical and non-canonical Wnt signaling to influence the cell. In the presence of the ligand, Dishevelled dissociates from the receptor/ligand complex to initiate the cytoplasmic

signaling cascade where GSK3β is phosphorylated while in association with Axin and Adenomatous Polyposis Coli (APC) in a multi-protein destruction complex. The phosphorylation of GSK3β leads to its inactivation as a kinase and thus results in hypophosphorylation of β-catenin, releasing the stabilized β-catenin from Axin/APC so that it can translocate to the nucleus to activate target genes (**Figure 1A**). β-Catenin cannot bind DNA directly, but acts as a co-activator by associating with T-cell Factor/Lymphoid Enhancer Factor (TCF/LEF) transcription factors. TCF/LEF binds to DNA via their High Mobility Group (HMG) domain and transactivates β-catenin/TCF/LEF target genes, including *Glutamine Synthetase* (*GS*) and *CyclinD1* which influence cell proliferation and differentiation. In the absence of ligand, at steady state, or in the presence of Wnt signaling antagonists such as Dickkopf (Dkk), β-catenin is phosphorylated at Ser45 by Casein Kinase Iα (CK1) via a similar intracellular cascade involving Dishevelled and the Axin/APC complex [17]. This phosphorylation event leads to the phosphorylation of β-catenin by GSK3β on Ser33, Ser37 and Thr41 to destabilize β-catenin and target it for degradation via the ubiquitin ligase β-Transducin Repeat-Containing Protein (βTrCP) and the proteasome (**Figure 1B**) [18].

In addition to the canonical Wnt/β-catenin signaling pathway, Wnt ligands are able to participate in non-canonical, β-catenin-independent signaling pathways. The two most well understood non-canonical pathways are the Planar Cell Polarity (PCP) and Wnt/Ca^{2+} signaling pathways, both involved in dorsal-ventral axis specification [19]. The PCP pathway regulates actin polymerization via small GTPases in a transcriptional-independent manner which influences cell polarity and migration [19]. Additionally, Wnt/Ca^{2+} pathway involves the Wnt-dependent regulation of intracellular Ca^{2+} levels via Phospholipase C, which in turn modulates

Ca^{2+}-dependent effectors: Calcineurin, Calcium/Calmodulin-dependent kinase II (CaMKII), and Protein Kinase C (PKC) [19].

Furthermore, there are also Wnt-independent, and therefore also non-canonical avenues by which β-catenin influences target gene expression. Both PKA and Akt have been shown to directly phosphorylate β-catenin without affecting canonical Wnt/β-catenin signaling. PKA phosphorylates β-catenin at Ser552 and Ser675, which facilitates TCF-dependent β-catenin transcription involving the coactivator Cyclic AMP Response Element-Binding Protein (CBP) [20]. This PKA/β-catenin signaling is independent of GSK3β-dependent β-catenin phosphorylation/stabilization, and does not use the destruction complex machinery to help induce target gene transcription [20]. Akt also phosphorylates β-catenin at Ser552 which redistributes β-catenin to the cytoplasm/nucleus and increases TCF/LEF-1 transcription in a GSK3β-independent manner [21]. Additionally a Wnt-independent regulation of β-catenin signaling via its interaction with Hepatocyte Growth Factor (HGF) receptor/tyrosine kinase c-Met has also been elucidated in hepatocytes [22]. Stimulation of hepatocytes by HGF was also shown to increase Ser654 and Ser670 phosphorylation of β-catenin causing its dissociation from c-Met and translocation to the nucleus [23]. The canonical and non-canonical pathways are not mutually exclusive within the cells, but work with one another to coordinate the efforts of the cell [20, 21].

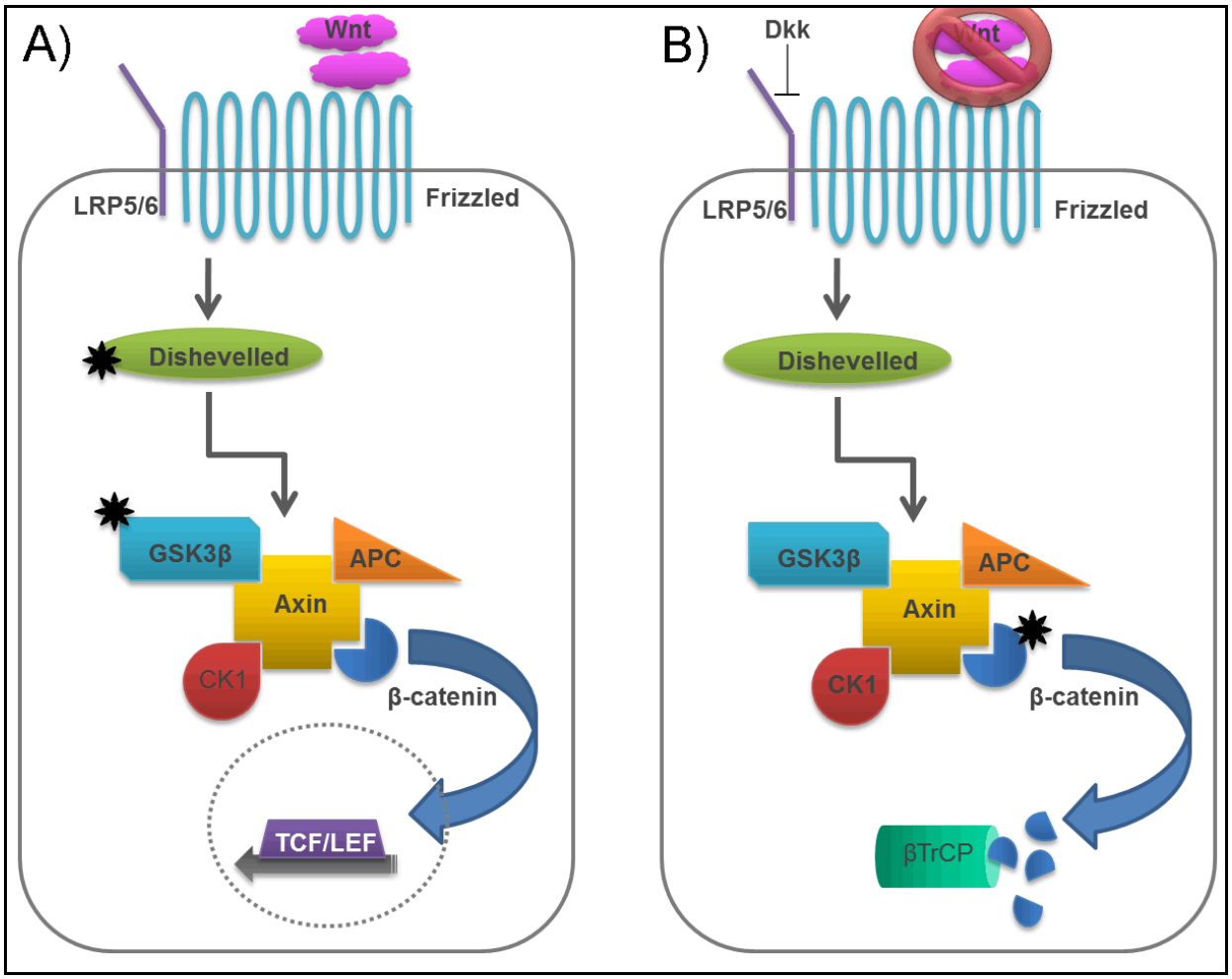

Figure 1. Canonical Wnt/β-Catenin signaling pathway. (A) With and **(B)** without Wnt ligand. Dkk is a Wnt-specific inhibitor; * indicates phosphorylation.

1.1.2 Adherens junction (AJ)

At a steady state and without Wnt ligands, β-catenin can most often be found at the cell membrane in the adherens junction (AJ) complex. In AJs, β-catenin links the classical/type-I transmembrane cadherins (E-, P- N-cadherins and N-cadherin 2) to α-catenin and the actin cytoskeleton via β-catenin arm domain interactions (**Figure 4A**; see also section **1.4.2**). A cadherin/catenin complex with E-/N-cadherin and either β-catenin, p120-catenin or γ-catenin

(plakoglobin) is assembled in the endoplasmic reticulum of the cells and is required for the membrane localization of cadherins [24, 25]. Importantly, the binding of these catenins help stabilize E/N-cadherin by masking the cadherin is proline (P), glutamate (E), serine (S), and threonine (T) ("PEST") domain, which has an ubiquitin/proteasome recognition motif [26]. Therefore, this cadherin/catenin complex initiation is important to overall stability of not only the cadherins but also the AJs.

Currently, it is still unclear whether this membrane-bound pool of β-catenin also has signaling capabilities. It has been historically thought that β-catenin is able to balance its adhering and signaling roles through tyrosine kinase and phosphatase activities in order to mediate both AJ and Wnt signaling tasks [14, 27]. Studies have shown that tyrosine phosphorylation of β-catenin at Tyr142, Tyr654 and also Tyr489, which are near the cadherin-catenin interacting surface, confer AJ integrity. Phosphorylation of Tyr654 via Src kinase or Epidermal Growth Factor (EGF) receptor leads to β-catenin's uncoupling from the E-cadherin. This also occurs when β-catenin is phosphorylated at Tyr489 via Abl [27]. Additionally, the phosphorylation of Tyr142 via Fyn, Fer kinases, along with c-Met leads to the uncoupling of the β-catenin/α-catenin complex, which also lead to the dissociation of the AJ resulting in increased cytoplasmic β-catenin [14, 28]. But whether an increase in cytoplasmic/nuclear β-catenin correlates with increase β-catenin signaling is still under debate [29].

1.2 β-CATENIN IN LIVER HOMEOSTASIS

β-Catenin is a critical protein in liver development, hepatic zonation, metabolism, regeneration, and overall liver health [10]. β-Catenin has a very precise spatiotemporal expression in the developing embryo, and is especially important for specification of hepatic fate, hepatocyte differentiation, and expansion of the liver bud. An embryonic stem cell deletion of β-catenin results in lethality at 7 days post-coitum as a result of ectodermal defects indicating the importance of β-catenin in development and gastrulation [30]. The expression of β-catenin peaks in the developing liver at mouse embryonic day 10 (E10) through E12 in the bi-potent hepatoblasts where staining is localized in both the nucleus and cytoplasm [31]. At these times, proliferation of the developing liver also peaks, suggesting β-catenin expression is key for hepatic morphogenesis [31]. After the β-catenin peak at E12, there is a sharp decrease in total full-length hepatocyte β-catenin. Interestingly, a truncated form of β-catenin appears at day E12.5 and persists in the absence of full-length β-catenin until just before birth [31, 32]. Recent work by our lab has shown that the truncated β-catenin species appears preferentially in embryonic hepatocytes and increases expression of differentiation-specific genes [32]. In addition to its role in embryonic hepatocyte fate, β-catenin is essential for the specification of hepatoblasts into biliary epithelial cells, as a loss in embryonic β-catenin leads to primitive bile duct paucity [33].

Postnatally, β-catenin is also important in neonatal liver growth. β-catenin is responsible for hepatocyte proliferation in mice, especially from day 0 to 30 after birth [34, 35]. It has also been shown that the distribution of β-catenin and destruction complex protein APC help drive lover zonation. The liver is organized into functional units called lobules, where portal triads

(hepatic portal vein, hepatic artery, and bile duct) surround a central vein in a hexagonal pattern. This organization helps with specific metabolic events, as blood flows through the lobules towards the central vein and bile moves in the opposite direction towards the portal triad into the bile ducts (**Figure 3A**). β-Catenin activation and expression of its target genes, such as *Glutamine Synthetase*, is observed in the pericentral liver lobule (near the central vein) whereas APC, a β-catenin degrading protein, is expressed in the periportal area of the liver (near the portal triad) [36]. Furthermore, β-catenin is able to control xenobiotic metabolism, through its regulation of cytochrome P450s in the liver, such as CYP2E1 and CYP1A2, which also follow a pericentral patterning [34]. As a result, β-catenin-driven glutamine, ammonia, and xenobiotic metabolism are major functions of β-catenin in an adult liver [34, 37, 38].

The adult liver also has a unique capacity to regenerate itself in a highly regulated manner. In mouse and rat models, a 2/3 partial heaptectomy is used to gauge the regenerative response of the liver (see **7.2.1** for surgery details). As β-catenin is so critical for liver growth, it is reasonable to assume that it also has an important role in liver regeneration as well. After surgical resection of the liver in rats, overall β-catenin levels are increased with fifteen minutes with preferential nuclear localization, and the influence of nuclear β-catenin is sustained for up to 48 hours [39]. Studies have shown that this β-catenin increase is not due to transcriptional changes of the β-catenin gene *Ctnnb1*, but is due to a decrease in protein degradation as a result of decreased serine phosphorylation [39]. β-Catenin appears to have a direct influence on proliferation in the regenerating liver, as an increase in β-catenin target genes such as *CyclinD1* are noted at similar times of β-catenin nuclear localization [39].

Additionally, to further assess the impact of β-catenin in the liver, our lab and others have recently created a hepatocyte-specific (post-natal) β-catenin knockout mouse (βKO) using Cre-

Lox recombination system in which β-catenin is preferentially deleted from albumin-secreting hepatocytes of the liver (see **7.1.1** for breeding scheme) [34, 37, 40]. The key observations found by our lab were that the βKO mice had a decreased liver-weight-to-body-weight-ratio (about 25% smaller), a decrease in basal proliferation, and an increase in apoptosis [34]. These mice showed marginal intrahepatic cholestasis and mildly anomalous biliary canalicular architecture, but surprisingly there was no apparent defects in cell polarity or adhesion [34, 41]. Novel gene targets of β-catenin were also discovered, such as vitamin C biosynthetic protein Regucalcin [34]. In the context of regeneration, β-catenin-null livers were able to fully recover after surgical resection, though the regenerative process was delayed; specifically, peak proliferation was delayed by at least 24 hours in the βKO mice [34]. Therefore, our characterization of this model showed that β-catenin is essential for overall liver health, but loss of β-catenin in hepatocytes does not lead to liver failure.

1.3 β-CATENIN IN LIVER PATHOLOGIES

Dysregulation of β-catenin plays a significant role in mediating common hepatic pathologies, including hepatocellular carcinoma (HCC), focal nodular hyperplasia, hepatic adenoma, hepatoblastomas, hepatic fibrosis, and cholangiocarcinomas [10]. However, characterization of βKO mice has shown that alterations in β-catenin alone do not necessarily lead to any one liver-specific disease [34, 42, 43]. In most pathological cases exon deletions or stabilizing point mutations of β-catenin lead to an increase in β-catenin signaling. In the case of cancer, the increase in β-catenin can increase survival and proliferative genes, giving the cancer cells a

growth advantage. Therefore, the possibility of targeting β-catenin therapeutically has been highly considered in clinical settings in recent years, especially for HCC [44-46].

1.3.1 Targeting β-catenin in HCC

Targeting β-catenin for HCC is inevitable [11, 12, 44-47]. Therefore, our lab, among others, has started exploring the efficacy of these clinically relevant anti-β-catenin therapies for HCC *in vitro* and *in vivo*. Our lab began by exploring drugs which were been pre-approved for HCC or other pathologies to see if there were any compounds that already existed in which the mechanism of action involved β-catenin. In this way, we could start to test the viability of anti-β-catenin treatments for HCC.

A number of studies have shown an increase in the enzyme cyclooxygenase (COX)-2 in HCC patient tissue samples [48-51]. COX-2 is an enzyme that is important in synthesis of prostaglandins, which mediate pain and inflammation. Additionally, COX-2 inhibitors are common non-steroidal anti-inflammatory drugs (NSAID) routinely used to control pain and inflammation. Previously, other groups have looked into treating HCC with the COX-2 inhibitor Celecoxib [52]; however, some serious side effects have been associated with Celecoxib (renal failure, heart attack, stroke, thrombosis). Therefore, our lab investigated the role of the NSAID R-Etodolac (1,8-diethyl-1,3,4,9-tetrahydropyrano-[3,4-b] indole-1-acetic acid) to compare its efficiency to Celecoxib. R-Etodolac is an NSAID which lacks COX-inhibitory activity, and therefore reduces the serious side effects associated with COX-inhibition [53]. Our data showed that the mechanism of action of both Celecoxib and R-Etodolac included a reduction of total and mutant β-catenin levels in Hep3B and HepG2 HCC cells, in addition to a decrease in Wnt-

dependent signaling and downstream β-catenin targets [11]. R-Etodolac was more effective at lower doses (400 μM), and also decreased transcription of *CTNNB1*. Interestingly, targeting β-catenin in HCC cells with R-Etodolac also enhanced the interaction of β-catenin with E-cadherin [11]. This study showed that it was the NSAID effects of the R-Etodolac which decreased β-catenin levels. Thus, the effectiveness of the clinically-relevant NSAID R-Etodolac in reducing β-catenin levels presents an attractive anti-β-catenin therapy for HCC.

For another potential anti-β-catenin drug, we looked at a drug already used for liver diseases. In the clinic the antiviral Pegylated-Interferon-α2a (peg-IFN) is one of the most common drugs used to treat HCV. HCV infection is one of the largest risk factors for HCC since it causes chronic liver injury. Additionally, HCV-associated HCCs have been characterized as having a high percentage of β-catenin mutations and also high levels of cytoplasmic and nuclear β-catenin as a result of Wnt pathway activation [54, 55]. Interestingly, when patients were treated for HCV with interferons (IFNs), the onset of HCC was delayed, or even prevented, even if the HCV was not effectively treated [56-58]. Our lab investigated the molecular mechanism of peg-IFN on β-catenin and also HCC. The results showed that peg-IFN was able to decrease Wnt signaling activity in human HCC cell lines with and without β-catenin mutations [12]. Our studies showed that peg-IFN up-regulated Ran Binding Protein-3, a β-catenin nuclear export factor, which led to the decrease in nuclear β-catenin and therefore Wnt signaling [12]. In addition to *in vitro* studies, transgenic mice with an activating β-catenin mutation affecting Ser45 were treated with the hepatocarcinogen N-diethylnitrosamine (DEN) and showed a decrease in tumorigenesis with peg-IFN treatment via decrease in hepatocyte proliferation [12]. As with R-

Etodolac, IFNs are also a set of attractive anti-β-catenin drugs which have already been tested for safety in patients.

Novel treatments for cancers with aberrant β-catenin expression are still being developed. The new form of molecular treatments comes as small molecule inhibitors which target specific molecular interactions within transformed cells. The drug ICG-001 was recently identified to selectively disrupt the interaction of β-catenin and its transcriptional coactivator CBP in colon cancer cells [13]. This drug was effective in reducing proliferation and inducing selective apoptosis of cancer cells via reduced TCF-dependent Wnt signaling [13]. This inhibitor was effective without unintentionally interfering with other β-catenin protein-protein interactions, like β-catenin/p300 [13]. Interestingly, ICG-001 was able to exert its effect without changing the protein expression level of β-catenin [13]. Thus, this molecule exemplifies the ability of novel small molecules to be used to target aberrant β-catenin activity in a highly selective and personalized manner. Currently, our lab is focusing on expanding on these types of β-catenin inhibitors, in order to find a better match for HCC-specific therapies.

The caveat for treating HCC with anti-β-catenin therapies, however, is that β-catenin has dual roles in the cell as a component of the Wnt signaling pathway and also the AJs (see **1.1**). Therefore, the question remains whether targeting β-catenin therapeutically will have negative unintended secondary effects as a result of targeting β-catenin at the cell-cell adhesions (**Figure 2).** As mentioned previously, it is uncertain whether the pool of β-catenin found in Wnt singling is the same as that found at the AJs [14]; therefore, we cannot be certain of the outcome of clinically targeting β-catenin until we specifically address this issue, or determine whether there is a compensatory mechanism in place to keep the cell-cell junctions intact. However, promising data from our lab indicates that decreasing β-catenin *in vitro* leads to decreased β-catenin nuclear

localization and signaling, decreased cell survival, proliferation, and soft-agar colonization of human HCC cells, without overtly affecting the cell adhesions [59]. Additionally, the conditional knockdown of β-catenin in hepatocytes did not lead to dissolution of the liver [34], all of which suggests that there is a presumptive compensation for β-catenin loss does not have oncogenic effects of increasing survival and proliferation of HCC cells.

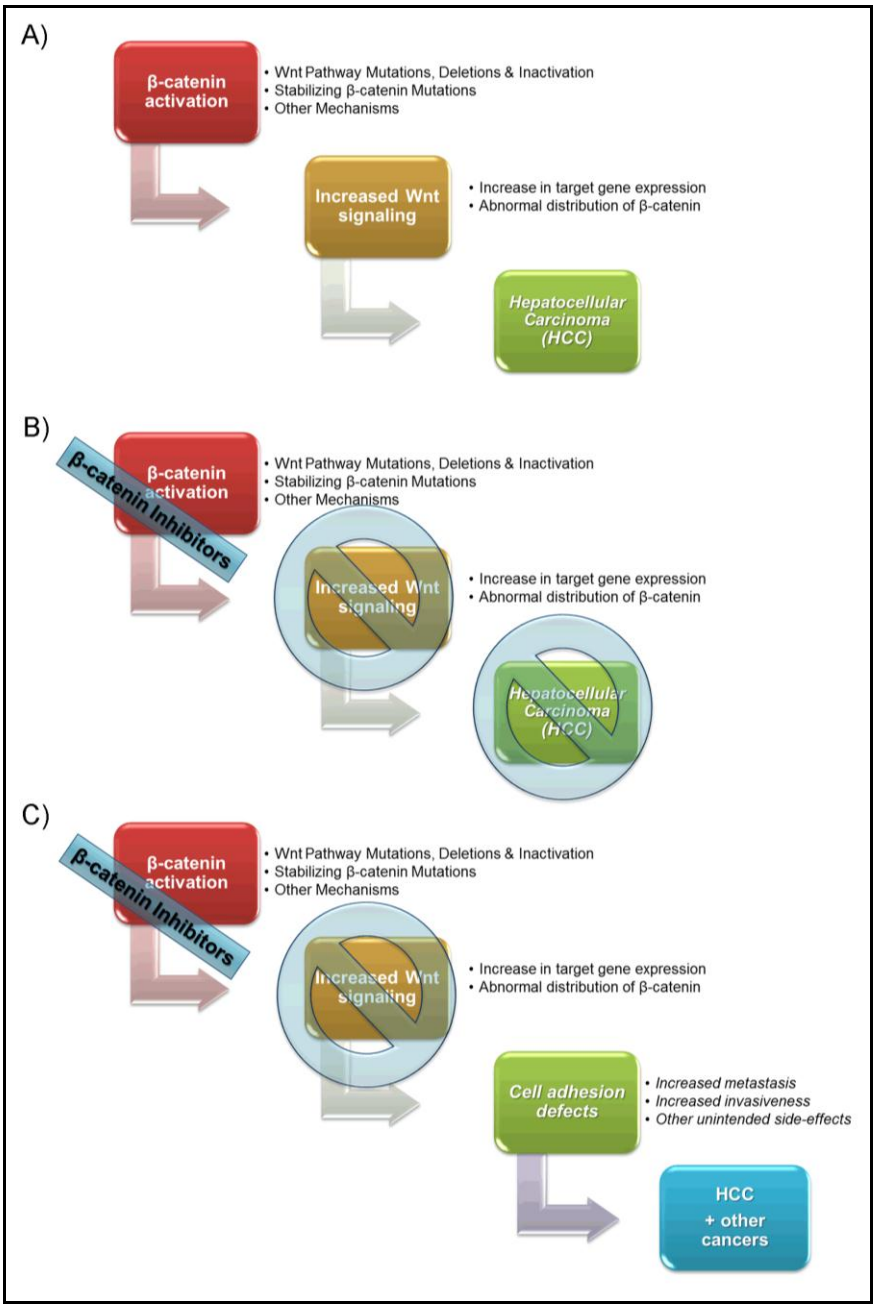

Figure 2. Schematic of targeting β-catenin for hepatocellular carcinoma. (A) The literature has shown that β-catenin activation via Wnt pathway or β-catenin alterations leads to increased Wnt signaling and affects the progression of HCC. **(B)** Proposed anti-β-catenin inhibitors for the treatment of HCC hypothesize a decrease of HCC. **(C)** However, β-catenin's dual role in at the hepatocyte adherens junctions may complicate the proposed anti-β-catenin therapies; if targeting β-catenin for HCC is detrimental to the adherens junctions, the β-catenin inhibitors may increase metastasis or invasion of the cancer cells and/or worsen the prognosis of HCC or other cancers.

1.4 HEPATOCYTE JUNCTIONS

Junctions are critical to overall structure and proper function of the liver. Although cell-cell junctions are rare in non-parenchymal cells of the liver, they are essential for hepatocytes polarity and organization. Hepatocytes are the primary parenchymal cells of the liver and play a role in integrating exocrine and endocrine functions of the liver through discrete membrane domains. Cell-cell junctions not only supply points of adhesion for hepatocytes, but also contribute to intercellular and intracellular communication. For this reason, junctions are frequently affected by in liver injury, toxicity, infection, and cancer [60-63]. The hepatocyte junctions can be divided into three groups: anchoring (AJ, desmosome), occluding (tight junctions), and communicating junctions (gap junction) (reviewed in [64]) (**Figure 3**).

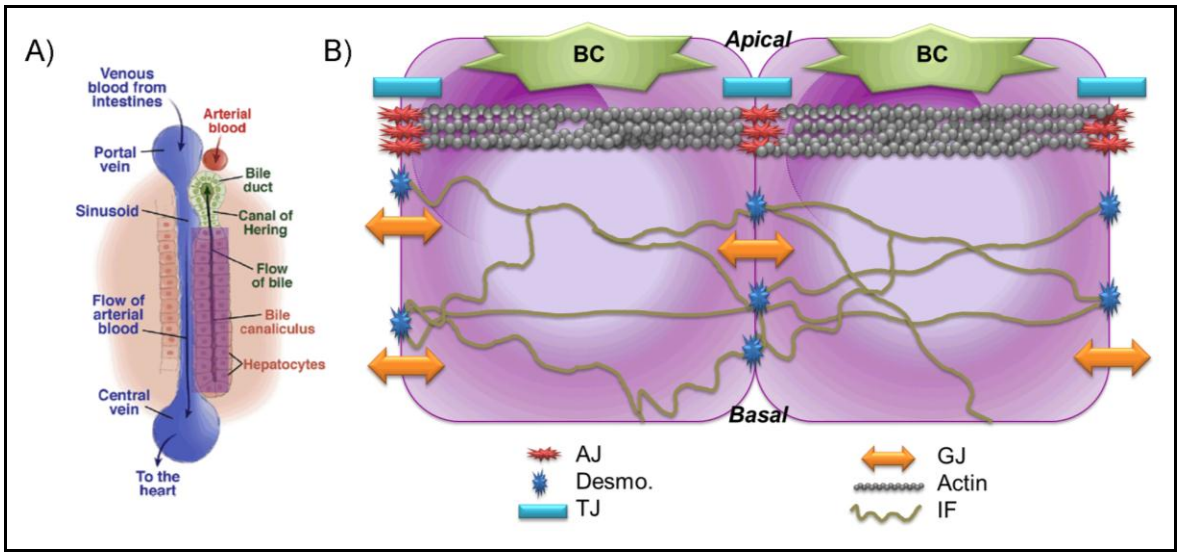

Figure 3. Hepatocyte junctions. (A) Illustration of the segregation of liver cell types and liver-specific functions, which are influenced by cell-cell junctions (image adapted from [65]) **(B)** Schematic of common cell-cell junctions

of the polarized hepatocytes (BC = bile canaliculus; AJ= adherens junction; Desmo. = desmosome; TJ = tight junction; GJ = gap junction; IF = intermediate filament).

1.4.1 The blood-bile barrier

The liver relies on proper organization through organized cell-cell adhesions to facilitate the critical and complex physiological functions of the liver. The apical surface of the hepatocyte generates bile while the basal surface helps exchange metabolites, other nutrients and toxins with the sinusoidal blood. Within the liver, bile flow is directed from the centrizonal to the periportal zone of the liver lobule. Newly synthesized bile is collected in the bile canaliculi, which connects to the bile duct at the portal triad. Bile is then transported to extrahepatic biliary tree and eventually secreted into the intestines. Thus blood and bile are properly segregated in the liver by junctional seals. A breach in the junction can disrupt the blood-bile barriers and cause changes in bile flow and ultimately cholestasis [66]. TJs are the main occluding junction of the liver creating seal which regulates the flow of molecules between hepatocytes in the paracellular space, keeps bile and blood from mixing, and maintains cellular polarity [67, 68]. However, with documented junctional cross-talk between AJs and TJs [69], AJs and desmosomes [70], along with AJs and gap junctions [71] and TJs and gap junctions [68] (see **1.5**), it is unlikely that the TJs are the only junctions responsible for functional segregation.

1.4.2 Anchoring junctions (adherens junctions & desmosomes)

The anchoring junctions help attach cells together via Ca^{2+}-dependent dimerization of extracellular cadherins and internal linkage to the cytoskeleton. As mentioned above in **1.1.2**, the

adherens junctions (AJs) are composed of classical cadherins and catenin armadillo family members that attach cells to one another via extracellular cadherin homodimers and link the plasma membrane to the actin cytoskeleton (**Figure 4A**). From extracellular to the cytoskeleton, the AJs in the hepatocytes normally consist of E- or N-cadherin bound to β-catenin and linked to α-catenin and then F-actin microfilaments. Auxiliary catenins, such as p120-catenin, are attached to the cytoplasmic domain of E-cadherin, but do not participate in the physical linking of the plasma membrane to the cytoskeleton; however, these catenins participate in junctional maintenance by facilitating cadherin turnover and mediating kinase recruitment [14, 27, 72]. In epithelial cells, including hepatocytes, the AJs are located near the apical surface of the cell, just below the tight junctions on an apical-basal axis (**Figure 3B**). The AJs form a belt-like band which is enriched for F-actin, which not only physically anchors cells together but may also bridge the cytoskeletal networks from one cell to another [73]. In addition to adhesion and polarity, AJs and their components are known to help with xenobiotic biotransformation [74] and albumin secretion [75] in the liver.

Desmosomes resemble AJs, but appear later in the evolutionary tree; however, these junctions are not redundant, and both are required for vertebrate existence as indicated by the lethality of global knockouts of their core catenin proteins [30, 76]. In the liver, desmosomes are found mainly along the lateral surface of hepatocytes [77]. Similar to the AJs, desmosomes are composed of cadherins (desmocollins (Dsc) 1-3 and desmogleins (Dsg) 1-4) which also link the cells together in the extracellular space; however, desmosomal cadherins can also interact as heterodimers. The desmosomes connect the plasma membrane proteins to the actin cytoskeleton through cadherin interactions with catenin proteins γ-catenin (plakoglobin) and plakophilin (Pkp; Pkp1-4), and catenin interactions with the adaptor protein desmoplakin (DP; DP1-2) which is

subsequently linked to the cytokeratin intermediate filaments (**Figure 4B**). The desmosomes appear as electron-dense structures via electron microscopy, with distinct outer dense plaques consisting of Dsg, Dsc, γ-catenin, Pkp and DP, and an inner dense plaque where the intermediate filaments loop back on each other. Though the desmosomes are made up of similar components as the AJs, their function is quite unique. Desmosomes are found below the AJs on an apical-basal axis, but are scattered through the lateral membranous space, resembling "spot welds" (**Figure 3B**). The main function of the desmosome is to dissipate mechanical stress, and is therefore critical in highly stressed organs such as the heart and skin [76]. To confer a higher than normal stress tolerance, the desmosomes are involved in specialized hyper-adhesion, where the mature desmosomes are locked into a highly-ordered plaques characterized by their resistance to disruption by calcium-chelators [78]. Though desmosomes are recognizable in liver tissue [77], the role of desmosomes for overall liver function has not been critically addressed in the literature to date.

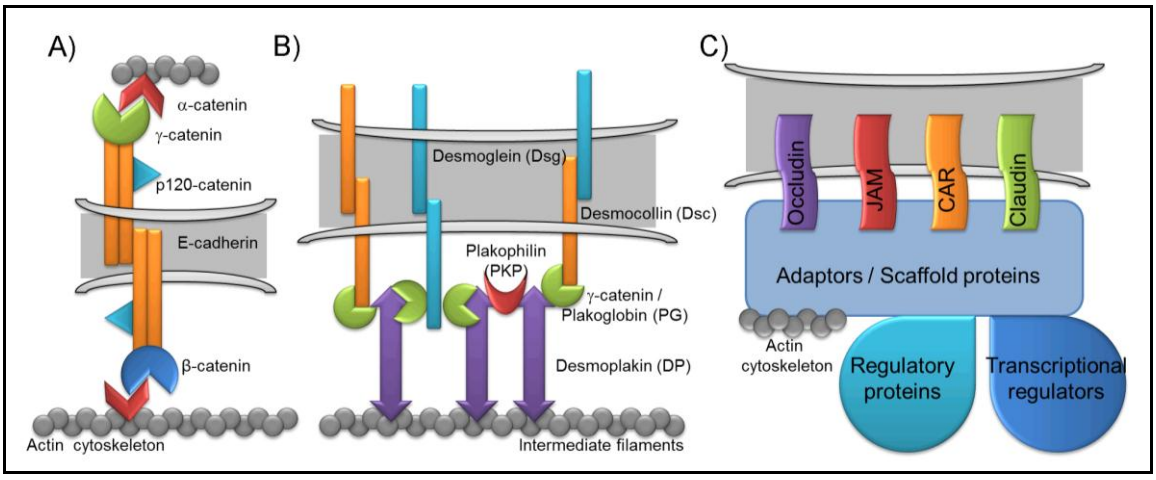

Figure 4. Anchoring and occluding junctions of the liver. (A) Adherens junction; **(B)** desmosome; and, **(C)** tight junction. The tight junction adaptor/scaffold proteins of the hepatocytes are ZO-1/3, MAGI1/3, PAR-3/6, MUPP1

and cingulin; the transcriptional regulators are ZONAB and symplekin; and the regulatory proteins are aPKC, and small GTPases [64].

1.4.3 Occluding junctions (tight junctions)

Occluding junctions encircle the cell at the apical membrane and segregate hepatocyte cellular domains [79]. Tight junctions (TJs) are considered the main occluding junctions of the liver where they surround the bile canaliculi and segregate the flow of bile, which can be caustic to hepatocytes (**Figure 3B**). TJs are composed of a wide variety of proteins which can be classified into two groups: transmembrane components (i.e. occludin, claudins, Junctional Adhesion Molecules (JAMs), and Coxsackieadenovirus Receptor (CAR)) and cytosolic components (i.e. Zonula Occludens proteins (ZO), cingulin, 7H6 antigen, etc.) (**Figure 4C**) [64]. Within the cytosolic TJ components, there are also three sub-groups, which are further classified based on their functional properties: adaptor/scaffolding proteins (core junctional proteins), regulatory proteins (such as small GTPases), or transcriptional regulators [64]. With so many sub-groups and possible combinations of TJ components, the number of TJ proteins discovered at the hepatocyte junction is constantly growing. The core proteins of the TJs are the transmembrane JAMs, claudins and occludin. There are four JAMs and even more related Ig-superfamily members, but only one isoform of occludin. Claudins make up a large family of proteins, and there are currently 20 known claudin family members that have specialized expression and function within each tissue type. The claudins expressed in different liver cell types are: claudins-1, -2, and -3 in the bile canaliculus and hepatocytes; claudin-5 in endothelial cells of the liver; and claudins-7, -8, and -14 liver cell-specific expression has not be specified [64, 68]. Interestingly, some TJ proteins also exhibit lobular expression in the liver. Claudin-3 is

uniformly expressed in the liver, whereas claudin-2 is increased in the pericentral areas of the liver [80], which indicates that they play a role in liver zonation and/or metabolism.

1.4.4 Communicating junctions (gap junctions)

Communicating gap junctions of the liver are composed of connexon proteins organized into channels by the interaction hemichannels of adjacent cells. Each hemichannel is composed of a combination of six connexons (Cx). Currently, there are over 20 connexons described in the literature, each having specialized expression in different cell and tissue types [64]. In the liver the connexons which have been described between hepatocytes are: Cx26, Cx31.9/30.2, Cx32 (the most abundant Cx in the liver at around 90%), Cx37, and Cx39, and Cx 43 [64, 81]. Gap junctional intercellular communication (GJIC) allows molecules to move from one hepatocyte to another through these channels via passive diffusion of small or hydrophilic molecules (**Figure 3B**). In the liver, gap junctions are known to help with xenobiotic biotransformation [74], bile secretion [82, 83], albumin secretion [84], and glycogenolysis [85], both on their own and in concert with hepatocyte AJs.

1.5 JUNCTIONAL CROSS-TALK

As mentioned above in **1.4.1**, there are many known nodes of cross-talk between AJs, desmosomes, TJs, and gap junctions. These are important redundancies built into the physiology of epithelial cells which undoubtedly help in the maintenance of cell-cell adhesions in the event of junctional insults, such as viral infection [60], barrier disruption [66], and therapeutic

treatments. With the inevitability of targeting Wnt/β-catenin and AJ protein β-catenin for HCC (see section **1.3.2**), it is important to understand the potential nodes of cross-talk which may be altered with these treatments.

The cross-talk of anchoring AJs and communicating gap junctions has been historically explored in the heart where gap junctions are most abundant. The formation of gap junctions plaques are known to follow the formation of stable cell-cell contacts, since GJIC requires the junctions to endure large amounts of sheer stress [86]. As a result, disruption of AJ cadherins are known to negatively affect gap junctions [87, 88]. Moreover, other research has shown more direct links between AJ and gap junctions. Experiments in cardiomyocytes show that Cx43 appears to co-localize with β-catenin at AJs in response to Wnt signaling [89]. These interactions not only highlight the cross-talk between AJs and gap junctions, but also β-catenin's potential regulatory roles of other junctions via Wnt signaling. Additionally the interplay of AJs and gap junctions in the liver appear to be important for the induction of cancer. In liver hepatomas, Cx26 levels have been shown to cause induction of AJ protein E-cadherin in order to suppress metastatic phenotypes [90].

Recently in the literature, the notion of cross-talk between the AJs and the occluding TJs has been explored in the liver [41, 69, 91]. Though AJ and TJs are expressed proximally in the hepatocytes, they have completely separate anchoring and occluding roles respectively; therefore, it is interesting to postulate a cross-talk node between these two junctions. It has been shown that a decrease in JAM-A levels leads to an increase in AJ E-cadherin in HCC cell lines [69]. Konopka *et al.* hypothesize that his change in AJ protein with JAM-A disruption may be an attempt of the liver cells to re-establish polarity and adhesiveness [69], but more work needs to be done to investigate whether the changes in the AJ actually help functionally relieve the TJ

disruption caused by a loss of JAM-A. Additionally, the cross-talk between AJs and TJs is also mediated via β-catenin-dependent signal transduction regulation of gene expression. Research has shown that claudin-2 is a downstream target of the Wnt/β-catenin pathway, indicating a direct influence of this signaling pathway on TJ protein expression [8, 92]. Conversely, JAM-A has been shown to influence intestinal cell proliferation by inhibiting Akt/β-catenin signaling [93]. These results highlight the interconnectedness of all epithelial junctions, despite their unique functionalities.

Lastly, though the AJs and desmosomes are known to share a common protein constituent under certain physiological conditions (γ-catenin), there is surprisingly little research on cross-talk between these two anchoring junctions, especially in the liver. As with gap junctions, the assembly of AJs must precede desmosome formation [94-105]. The inhibition of the AJ protein E-cadherin not only decreases AJ formation, but also dampens desmosome formation [100, 106]. Additionally, without γ-catenin AJs can form, but desmosomes cannot [70, 107]. Not surprisingly, two groups have implicated γ-catenin as the cross-talk agent between AJs and desmosomes since it can be localized to both junctions. Both groups have shown that it is the interaction of γ-catenin and AJ E-cadherin which is necessary for the assembly of desmosomes [70, 108]. More recently, it has been shown that the presence of desmosomal plaque protein plakophilin-3 is also essential in this complex to initiate desmosome formation in epithelial cells [108]. It is thought that even though γ-catenin is an unessential AJ protein, the γ-catenin/E-cadherin/plakophilin-3 complex is still needed to recruit desmosomal proteins to the cell membrane [108], blurring the lines of AJ and desmosomal assembly. Together this data suggests that γ-catenin may play a rather dynamic role in regulating multiple junctions.

1.6 β- VERSUS γ-CATENIN

For many years β-catenin and γ-catenin were indistinguishable by investigators of biological systems, especially since the protein "armadillo" sufficed for both proteins in Drosophila. However, β- and γ-catenin are unique entities that developed distinct functions as far back as vertebrate divergence [109, 110]. At times γ-catenin plays a redundant role to β-catenin in the AJ, but is uniquely qualified as a major component in the desmosomes [76, 107], highlighting the non-identical roles of these two proteins (see also **1.4.2**). Though γ-catenin can intervene at the AJ when β-catenin is lost, the reciprocal compensation of β-catenin for γ-catenin loss at the desmosome is not functional [107]. The differences of β- and γ-catenin in cell-cell adhesions and Wnt signaling will be expanded upon in the upcoming sections on γ-catenin (**1.7**).

1.7 γ-CATENIN

γ-Catenin (also known as plakoglobin) is highly homologous to β-catenin with ~80% sequence similarity in the arm domains, and 45-57% and 15-27% N- and C-terminal domain identities respectively [111, 112]. γ-Catenin is essential in embryonic development as well, as a global knockout of this protein is embryonic lethal in mice between day E12 and E16 as a result of heart and skin defects [76], compared to day E7 lethality with an embryonic β-catenin knockout[30]. The primary functional role of γ-catenin is in cell-cell adhesion, where it helps link cadherins to the cytoskeleton in the desmosomes (**1.4.2**; **Figure 4A-B**).

1.7.1 Cell-cell adhesion

It is well accepted that γ-catenin is an essential catenin of the desmosome (see **1.4.2**; **Figure 4B**). As discussed above, recent evidence has also revealed the role of γ-catenin as an important protein in both desmosome and AJ assembly [70, 108]. Additionally, γ-catenin has the capacity to bind E-cadherin at the AJ, especially with a loss or decrease of β-catenin [113-119]. Structural studies indicate that β- and γ-catenin have identical interactions with E-cadherin [111]; however, it is unclear whether this γ-catenin/E-cadherin junction is functionally equitant to the β-catenin/E-cadherin AJ.

As with β-catenin, γ-catenin is able to balance its structural and functional roles through tyrosine kinase and phosphatase activities [114], where it is trafficked between the AJs, desmosomes, and other intercellular locations. Tyrosine phosphorylation of γ-catenin at Tyr549, Tyr693, Tyr724 and Tyr729 by Fer and Fyn kinases or EGFR decreases γ-catenin's binding to DP and increases its binding with α-catenin; conversely, phosphorylation of Tyr643 by Src kinase has the opposite effect and decreases γ-catenin's binding to E-cadherin [114]. Though the residues responsible for γ-catenin's localization to the AJs and desmosomes are well established, the mechanism responsible for γ-catenin's stabilization at specific parts of the membrane or in the cytoplasm/nucleus are less clear. β-Catenin has key serine residues, such as Ser37, whose phosphorylation via GSK3β and other kinases dictate its stabilization and translocation to the nucleus (see **1.1.1**). However, the corresponding mutations on γ-catenin (Ser28Ala) do not infer stability [112]. Nonetheless, studies have shown that serine and/or threonine phosphorylation is

able to stabilize γ-catenin [115]; but, the specific residues, responsible kinases, and physiological cues for serine and/or threonine phosphorylation/stabilization of γ-catenin are still unknown.

1.7.2 Wnt signaling

The role of γ-catenin in Wnt signaling is still controversial. It has been shown that γ-catenin can interact with many Wnt signaling components, including: Axin/APC [112, 120, 121], GSK3β [120], TCF/LEF [122, 123], and βTrCP [124]. Additionally, as with β-catenin, γ-catenin is up-regulated in response to Wnt-1 ligand [125]. However, the question remains whether these associations are functional. The evidence is stacked against a signaling role of γ-catenin in the Wnt pathway, except in cases where there is a complete loss of β-catenin [126, 127]. However, the competition between β- and γ-catenin for similar cytoplasmic and nuclear binding partners makes it difficult to differentiate their roles within the cell, and also complicates the research of both β- and γ-catenins individually and together.

The reason for β-catenin's preferential participation in Wnt signaling might be driven by γ-catenin's solubility and localization within the cell. γ-Catenin has a greater propensity to localize to the cell membrane compartment than β-catenin, as 85% of a cell's γ-catenin is Triton X-100 insoluble (actin cytoskeleton-sequestered), and only 50% of the cell's β-catenin is insoluble [124]. Therefore, γ-catenin's tendency to remain in the membrane may help β-catenin better localize to the cytoplasm and occupy the cytoplasmic Wnt signaling machinery [122, 124]. Overexpression of γ-catenin also has been shown to displace β-catenin from the AJs, leading to an increase in cytoplasmic, and even nuclear β-catenin, which may influence the signaling capacity of β-catenin [122, 128, 129]. Another theory of how γ-catenin can guide Wnt/β-catenin

signaling is that increased γ-catenin in the cytoplasm could overload or preferentially occupy the destruction complex components (Axin or APC) or the βTrCP degradation machinery, decreasing the amount of β-catenin degradation, and consequently increasing in the signaling pool of β-catenin [112, 129, 130]. Therefore, when γ-catenin is increased/over-expressed, it may actually be β-catenin signaling in the nucleus, and not γ-catenin. Neither β- nor γ-catenin is able to bind to DNA directly, but both have been shown to utilize HMG box-containing DNA binding proteins to drive transcription [123]. However, β-catenin co-precipitates with LEF1 and TCF4 with higher affinity than γ-catenin *in vitro* [112, 122, 131]. Therefore, the competition for Wnt signaling may be influenced by the intrinsic affinities of β- and γ-catenin for their shared binding partners. The current tools used to measure Wnt-dependent signaling are unable to differentiate between β- and γ-catenin binding [122, 127].

Moreover, some groups hypothesize that γ-catenin might actively oppose Wnt signaling in the cell. More than one group has reported that in their model system γ-catenin binds to TCF4 but then sequesters it in the cytoplasm, rendering it useless for γ- or β-catenin driven transcription [123, 130-133]. The Alzheimer disease protein presenilin-1 has been shown to mediate this sequestration of γ-catenin/TCF4 to the extranuclear compartment [133]. Detailed mapping of the TCF4 binding regions of β- and γ-catenin show that these proteins have independent interaction sites on TCF4, but that binding of γ-catenin to TCF4 hinders its ability to bind DNA [131]. Additionally, Zhurinsky *et al.* showed that γ-catenin could form an association with LEF1, but that a γ-catenin/LEF1/DNA complex never existed. This group hypothesized that is was the C-terminal of γ-catenin which inhibited the γ-catenin/LEF complex from binding

DNA [123]. This may explain why the C-terminal regions of β- and γ-catenin are the most dissimilar.

Despite all the arguments against γ-catenin in Wnt signaling presented above, this protein still appears to have intrinsic signaling capabilities. Independent of β-catenin, many reports have shown that γ-catenin has its own set of transcriptional targets, including *PTTG1*, *NME1*, and *c-Myc* [121, 134-136]. In addition to Wnt signaling, γ-catenin has been connected with Akt, Erk, and Rho GTPase signaling pathways [135, 137]. Interestingly, unlike β-catenin, it is well accepted that the γ-catenin found at the membrane is the same pool/species involved in signaling [138]. Based on emerging data with respect to Wnt-independent γ-catenin signaling, more attention needs to be given to γ-catenin as an independent signaling effector in the cells. Additionally, more rigorous research efforts should be made to distinguish γ-catenin from the Wnt signaling pathway, instead of assuming it always exerts its influence in the same way as its non-identical sibling β-catenin.

1.8 γ-CATENIN IN HUMAN DISEASES

Alterations in the structure of γ-catenin play a significant role in several human pathologies, most commonly in those tissues which rely on the adhesive strength of the desmosomes. Thus, changes in the membrane localization γ-catenin have been implicated in skin/heart pathologies, but also in cancer. Unlike β-catenin, there are very few cases of point mutations causing aberrant γ-catenin expression; regardless, changes in γ-catenin localization have been noted in the development, progression and metastasis of many types of cancer (see **1.8.2**).

1.8.1 Skin and heart diseases

Classical diseases of the desmosomes, and desmosomal protein components, most often present as skin and heart defects. Therefore, γ-catenin mutations most often lead to the inherited diseases arrhythmogenic right ventricular cardiomyopathy (ARVC) and/or diffuse non-epidermolytic palmoplantar keratoderma with woolly hair and cardiomyopathy ("Naxos" or "Woolly hair" syndrome). These are the only two diseases known to have mutated forms of γ-catenin. The ARVC γ-catenin mutation has been identified as *Ser39_Lys40insSer*, where an extra serine residue is inserted in the N-terminus resulting in preferential ubiquitination and degradation of the mutant γ-catenin protein [139]. In Naxos syndrome, the *2157del2* mutation is a nonsense two base pair deletion which prematurely stops translation, leading to a smaller γ-catenin species (75 kDa) [140]. *In vitro* studies of these mutants have shown increased wound healing and migration with both mutations, and decreased cell-cell adhesion with the Naxos mutation [141], indicating that the change in γ-catenin is affecting the cell junctions. Interestingly, both the diseases have an ARVC component; but very different etiologies. The ARVC mutant γ-catenin still localizes to the membranes, while the Naxos mutant γ-catenin does not, but instead localize to the nucleus [141]. This implies that γ-catenin mutations are detrimental in two ways: 1) affecting cell-cell adhesions; 2) extra-junctional influences.

1.8.2 Cancer

The role of γ-catenin in cancer is highly debated in the literature, especially the assignment its role as a strict tumor suppressor or oncogene. There are many instances of epithelial cancers in

which γ-catenin has unexplained aberrant expression and localization, including: breast [142, 143], prostate [144], cervical [145], pancreatic [146, 147], colorectal [148-150], urothelial [151], gastric [147], bladder [152-154], lung [155, 156], and liver cancers [157-159]. Whether the changes in γ-catenin expression in these cancers are detrimental or helpful to the cancer prognosis is unclear, and discrepancies in the literature are even apparent within cancers of the same tissue (see **1.9.1** for debate in HCC). Additionally, the etiology of γ-catenin changes in these cancers is not very clear. Loss of heterozygosity (LOH) has been documented in some familial breast and prostate cancer cohorts [144, 160, 161], and a few cases of gastric and prostate cancer with Ser28Leu missense mutation of γ-catenin have been described [144, 147]. However, the reason for γ-catenin's mislocalization cannot be explained in most instances by the aforementioned genetic alterations.

The most convincing argument for γ-catenin as a tumor suppressor comes from its LOH in breast and prostate cancers [144, 160, 161]. However, other studies have indicated that unregulated expression of γ-catenin leads to an aberrant increase in effector genes such as *c-Myc* which are clearly oncogenic [135]. However, γ-catenin itself may not be the culprit in these cancers, but merely a manifestation of oncogenic changes in the cell-cell junctions. Alterations in the desmosomes and AJs are known to play a role in cancer [162]. When desmosomes and/or AJs are disrupted, these junctions not only release potential oncogenic proteins, but also affect the invasive and metastatic potential of those cancers, negatively effecting clinical outcomes. If this is the case, then γ-catenin and the intact desmosomes and AJs should be considered as tumor suppressors. Therefore, whether the changes of γ-catenin in these cancers is correlated with a change in the cell-cell junctions should be an important factor to analyze when considering the role of γ-catenin in cancer.

1.9 γ-CATENIN IN LIVER BIOLOGY AND PATHOBIOLOGY

Not many studies have attempted to elucidate the role of γ-catenin in liver biology or pathobiology. This may be because γ-catenin's role in the liver has been over-shadowed by its close relative β-catenin, since β-catenin liver-specific knockout models are available and there are known β-catenin mutations which predispose individuals to HCC and other liver diseases. Currently, there are no known γ-catenin mutations in HCC. It is necessary to identify the roles of γ-catenin that are distinct from β-catenin in the liver homeostasis since it is apparent that the balance of these proteins influences one another. More importantly, γ-catenin may play an important role in the liver, especially when β-catenin insults occur. γ-Catenin also plays a unique role in desmosomal adhesion; however, this aspect of liver biology has also been understudied. The desmosomes in the liver may be smaller and not as numerous as in other tissue types, but they are not absent [163]. Hence γ-catenin may have an important in liver biology that needs to be investigated.

1.9.1 Hepatocellular carcinoma

As expanded upon above (**1.3.1**), HCC is a major health burden in the United States. Successful treatment for this type of cancer is rare, and so the disease management regimen is primarily palliative at this point. Studies have shown that in addition to Wnt/β-catenin signaling pathway mutations/aberrations, the levels of γ-catenin may also influence the initiation and progression of HCC. Unfortunately, the data available for γ-catenin expression in HCC is conflicting. Studies by Endo *et al.* indicate that increased γ-catenin expression correlates positively with HCC grade

(highest in poorly differentiated HCC) [157].Conversely, Zhai *et al.* have shown that HCC patients with under-expression of γ-catenin have a poor survival rate [158].

However, as with other types of cancer with mislocalized γ-catenin levels, it may not be the change in γ-catenin expression which determines HCC tumorgenicity, but how these changes affect the junctions which ultimately influences HCC outcome. The studies by Zhai *et al.* correlate the expression of catenins and E-cadherin with HCC prognosis in a patient. They saw that a decrease in β-catenin/E-cadherin or γ-catenin/E-cadherin was the driving factor for poor clinical prognosis and survival rate in HCC patients [158]. Additionally, Cao *et al.* observed that in well/moderately differentiated HCC, <50% of tumor cells exhibited a decrease in junctional proteins (including γ-catenin). But in poorly differentiated HCC cases, over 50% of tumor cells and nearly 100% of cancer cells no longer expressed desmosomal and AJ components [159]. This data not only reinforces the importance of these anchoring junctions in HCC development/progression, but also highlights the importance of keeping these junctions intact while treating HCC as to not worsen the prognosis. However, the cause and effect relationship is still lacking, thus mandating in depth mechanistic studies.

1.10 GOALS OF THIS WORK

The current body of literature on β-catenin in liver-specific diseases has prompted researchers to begin to investigate targeting β-catenin therapeutically in diseases such as HCC. The following studies will help fill the gap in knowledge regarding the effects of therapeutically targeting β-catenin with respect to its roles in cell-cell adhesions at the AJs. Specifically, we would like to know if decreasing the levels of β-catenin may have any unintended negative consequences

which might affect the efficacy of the proposed therapies. Additionally, in light of only a subtle phenotype in mice lacking β-catenin in hepatocytes, we propose to determine the impact of its loss not only on the AJs and Wnt signaling, but also on other cell-cell junctions.

We identify γ-catenin to compensate for loss of β-catenin at the AJ in the liver [91] (**Chapter 2**). As a result of altered γ-catenin expression in mice lacking β-catenin, we also propose to determine the mechanism of how this up-regulation occurs after β-catenin loss or knockdown in the liver and also in HCC cells in order to highlight the differences or similarities in β- and γ-catenin regulation which may be important for future HCC therapies. Lastly, γ-catenin and desmosomes have been understudied in liver pathobiology, despite clinical observations of their dysregulation in HCC (**1.9.1**) [157-159]. Therefore, we also want to determine whether the changes in γ-catenin with loss or downregulation of β-catenin might affect the desmosomes in the liver and if γ-catenin alone may have any influence on HCC prognosis. In this way we will be able to make recommendations to clinicians regarding potential anti-β-catenin therapies. Therefore, we hypothesize that γ-catenin is unable to fully compensate for β-catenin suppression/loss in the liver in Wnt signaling; however, it is able to help preserve a functionally stable AJ and thus maintain liver homeostasis.

2.0 HEPATOCYTE γ-CATENIN COMPENSATES FOR CONDITIONALLY DELETED β-CATENIN AT ADHERENS JUNCTIONS

The major goal of the studies presented in this chapter was to understand the junctional reorganization of the AJs in the recently reported β-catenin-conditional knockout mice (βKO) generated by interbreeding transgenic mice homozygous for floxed-β-catenin gene (Ex1-6) and transgenic mice expressing Cre-recombinase under the Albumin promoter (see Methods **7.1.1**) [34]. Here we identify an important compensatory role of γ-catenin (plakoglobin) at AJs in the βKO livers.

2.1 HEPATOCYTE-SPECIFIC β-CATENIN KNOCKOUT MOUSE (βKO)

The hepatocyte-specific βKO mice exhibit lower liver-weight-to-body-weight ratio, a marginal intrahepatic cholestasis and anomalous biliary canalicular architecture [164]. No gross defects in cell polarity or adhesion are evident in βKO livers. Therefore, we wanted to explore the βKO hepatic phenotype, or lack thereof, through identification of compensatory mechanisms in AJs, which enable the maintenance of a near normal liver histology in the absence of β-catenin.

To confirm genotype of the βKO livers, β-catenin levels were assessed. β-Catenin protein was significantly decreased in the whole-cell lysates (WCL) and cytoskeletal-associated lysates

(CAL) of βKO livers as compared to age- and sex-matched littermate wild-type controls (WT) ($p<0.0005$) (**Figure 5A-B**) (see section **7.7** for protein extraction details). Some remnant β-catenin expression was evident in βKO livers; however, this was due to existence of β-catenin in non-parenchymal cells unaffected by Albumin-Cre expression. Indeed, immunohistochemistry (IHC) and immunofluorescence (IF) in βKO showed loss of membranous β-catenin in hepatocytes and cytoplasmic β-catenin in cholangiocytes, whereas endothelial cells remained β-catenin-positive (**Figure 5C-D**). IP studies for β-catenin were performed next. When blotted for E-cadherin, an epithelial-specific cadherin, the association between β-catenin and E-cadherin was detected only in WT livers, confirming the loss of β-catenin in epithelial compartment (**Figure 5E**). From this data it is evident that β-catenin is conditionally deleted from the parenchymal cells of the βKO livers.

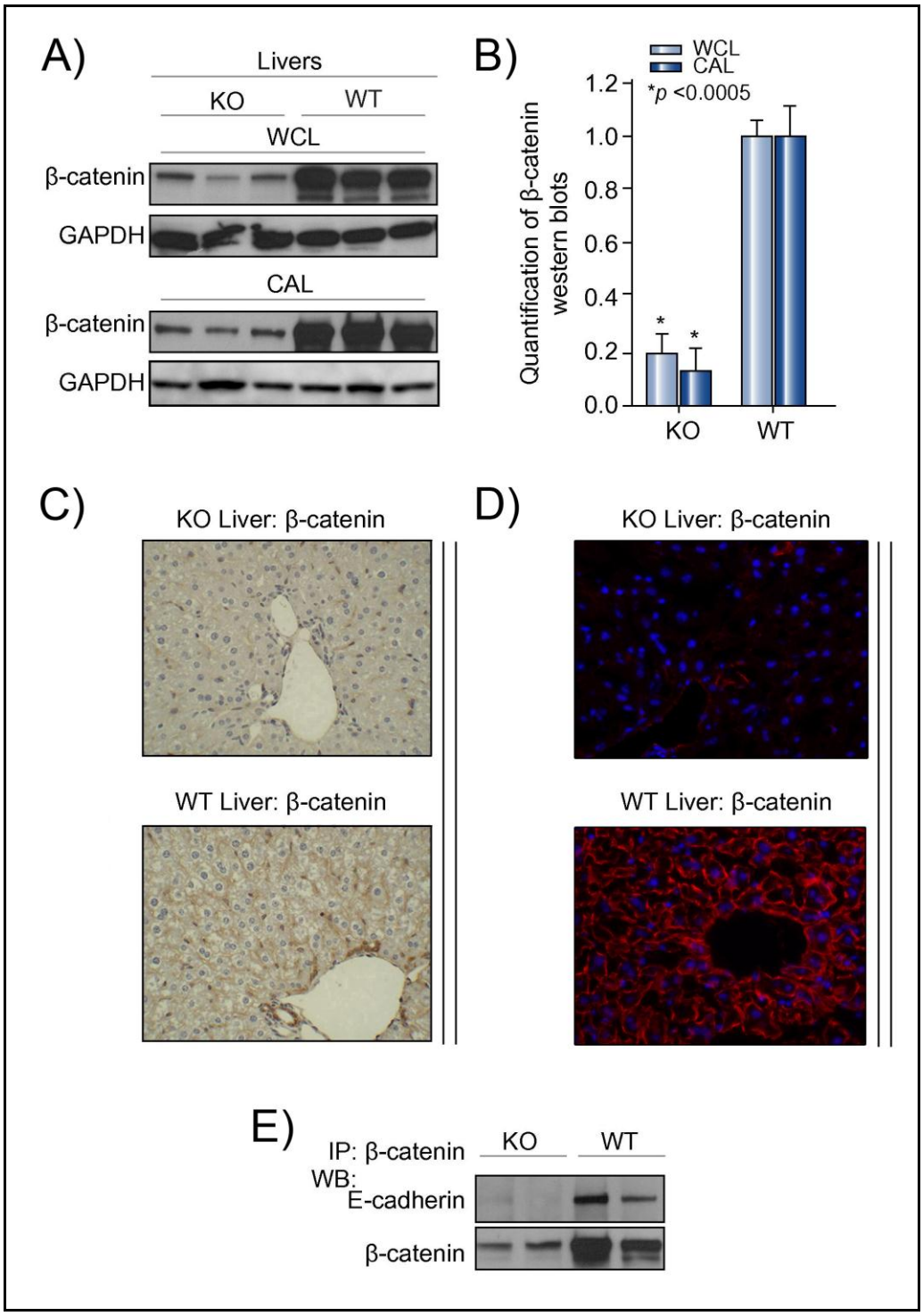

Figure 5. Assessment of β-catenin in βKO and WT livers. (A) β-Catenin (97 kDa) and GAPDH (as loading control; 37 kDa) protein levels in whole-cell lysates (WCL) and cytoskeletal-associated lysates (CAL) of three representative βKO and age-and sex-matched WT livers by WB. **(B)** Normalized (to GAPDH) densitometric analysis reveals significantly lower ($p < 0.05$) average (± SD) β-catenin protein expression in βKO than WT livers

(n > 3). **(C-D)** Liver sections from βKO and WT were analyzed for β-catenin expression by IHC and IF. **(E)** WCLs were immunoprecipitated with anti-β-catenin antibody and probed for E-cadherin (120 kDa) by WB.

2.2 CHANGES AT THE ADHERENS JUNCTIONS

In adult liver, β-catenin is chiefly observed at the hepatocyte membrane regulating cell-cell adhesion as a part of AJs and in the most perivenous hepatocytes displaying additional cytoplasmic and nuclear localization to regulate zonation [36]. Yet, conditional β-catenin loss in hepatocytes resulted in a mild phenotype [34, 37]. To investigate changes in gene expression of AJ components, we analyzed microarray data from βKO and WT livers [34]. Microarray analysis was performed as indicated in section **7.6.3**. βKO livers showed altered expression of α-catenin (+2.4), E-cadherin (+2.6) and γ-catenin (+1.5) relative to WT (**Table 1**). WBs were performed to substantiate the array analysis. Insignificant differences in E-cadherin, α-catenin and F-actin protein levels were observed in CAL and WCL from βKO and WT livers (**Figure 6A**). Interestingly, a significant increase in γ-catenin protein expression was observed in βKO for both WCL and CAL lysates, despite no change in gene expression levels as assed via microarray analysis (**Figure 6A-B**; **Table 1**).

Table 1. Gene expression changes of junctional protein components in βKO livers.

GENE	JUNCTION	Change in βKO vs. WT
α-catenin	AJ	+2.4
E-Cadherin	AJ	+2.6
N-Cadherin	AJ	NC
Desmocollin-2	Desmosome	+2.2
Desmocollin-3*	Desmosome	+3.8
Plakoglobin / γ-catenin	Desmosome / AJ	+1.5
Plakophilin-3	Desmosome	-3.7
Claudin-1	TJ	-2.3
Claudin-2	TJ	-8.0
Claudin-3	TJ	NC
Occludin	TJ	-1.3
JAM-A	TJ	-2.0
ZO-1	TJ	NC
ZO-2	TJ	-1.2

*Not found in liver before; NC = no change

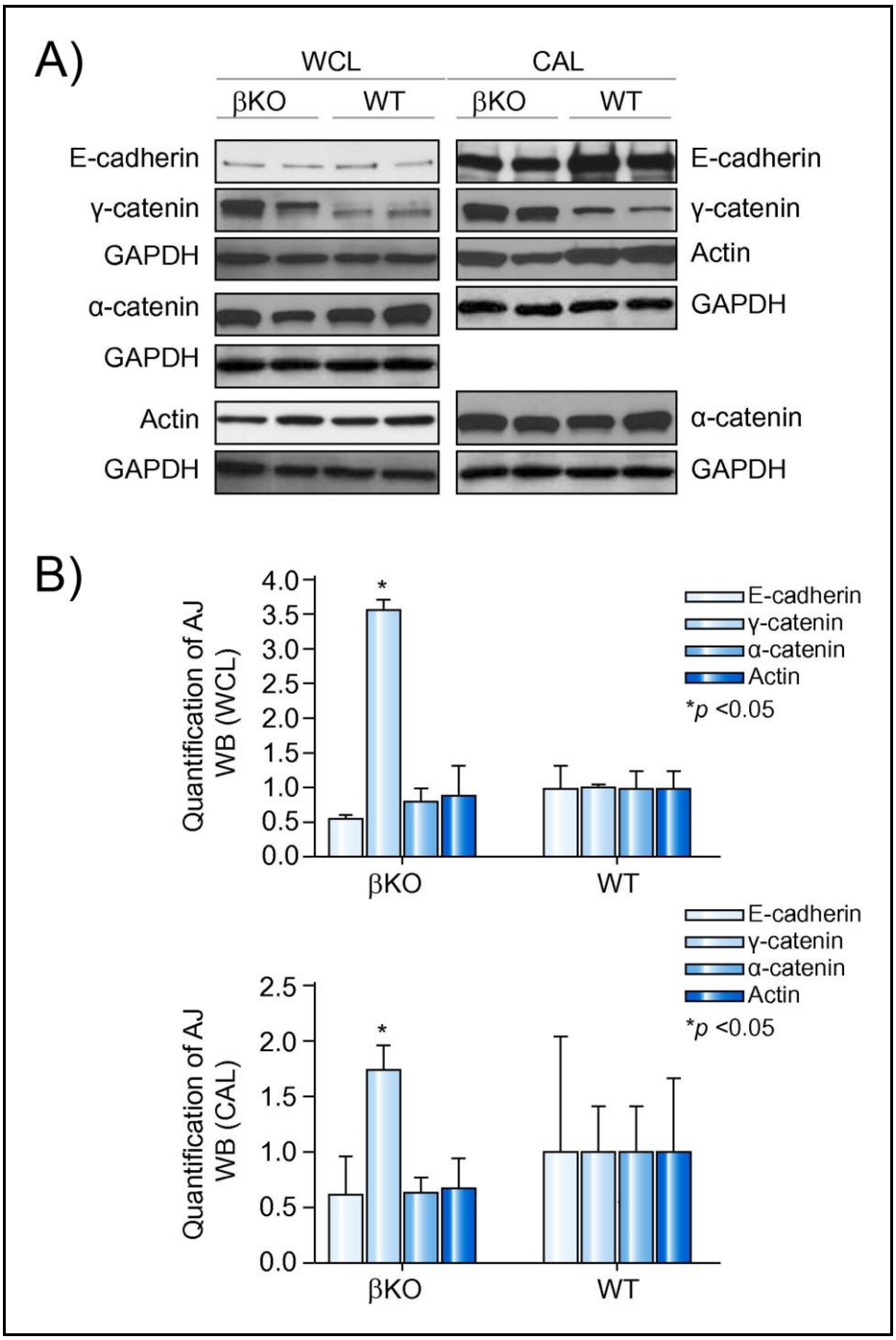

Figure 6. Quantitative analysis of AJ proteins in βKO and WT livers. (A) E-cadherin, γ-catenin (83 kDa), α-catenin (100 kDa), and F-actin (42 kDa) protein levels were detected in WCL and CAL of βKO and WT livers by WB. WB for GAPDH verified comparable loading. **(B)** Normalized (to GAPDH) densitometric analysis reveals significantly higher ($p < 0.05$) average (± SD) protein expression of γ-catenin in βKO than WT livers ($n > 3$), while other proteins showed insignificant differences between the two groups.

IP studies were performed next to see if β-catenin loss impacts association between AJ proteins. When E-cadherin was immunoprecipitated in WT and βKO liver lysates, β-catenin association was detected only in WT (**Figure 7A**), as in **Figure 5A**. We could not detect α-catenin association with E-cadherin in WT or βKO livers (*data not shown*). Interestingly, γ-catenin invariably associated with E-cadherin in βKO while it was only minimally evident in complex with E-cadherin in the WT livers (**Figure 7A**). Conversely, when γ-catenin was immunoprecipitated in WT and βKO livers, an association with E-cadherin was detected in βKO livers only (**Figure 7A**). We also detected F-actin association with E-cadherin by IP in both WT and βKO livers in the same complex. Thus, an intact connection of E-cadherin to actin cytoskeleton is maintained in WT and βKO livers, *albeit* through γ-catenin and not β-catenin in the latter. Lastly, IF studies were utilized to show co-localization of E-cadherin and γ-catenin only at the hepatocyte membrane of βKO and not in WT livers (**Figure 7B**). From this data it is evident that γ-catenin associates with E-cadherin and F-actin *in lieu* of β-catenin when β-catenin is conditionally deleted from the parenchymal cells of the mouse livers.

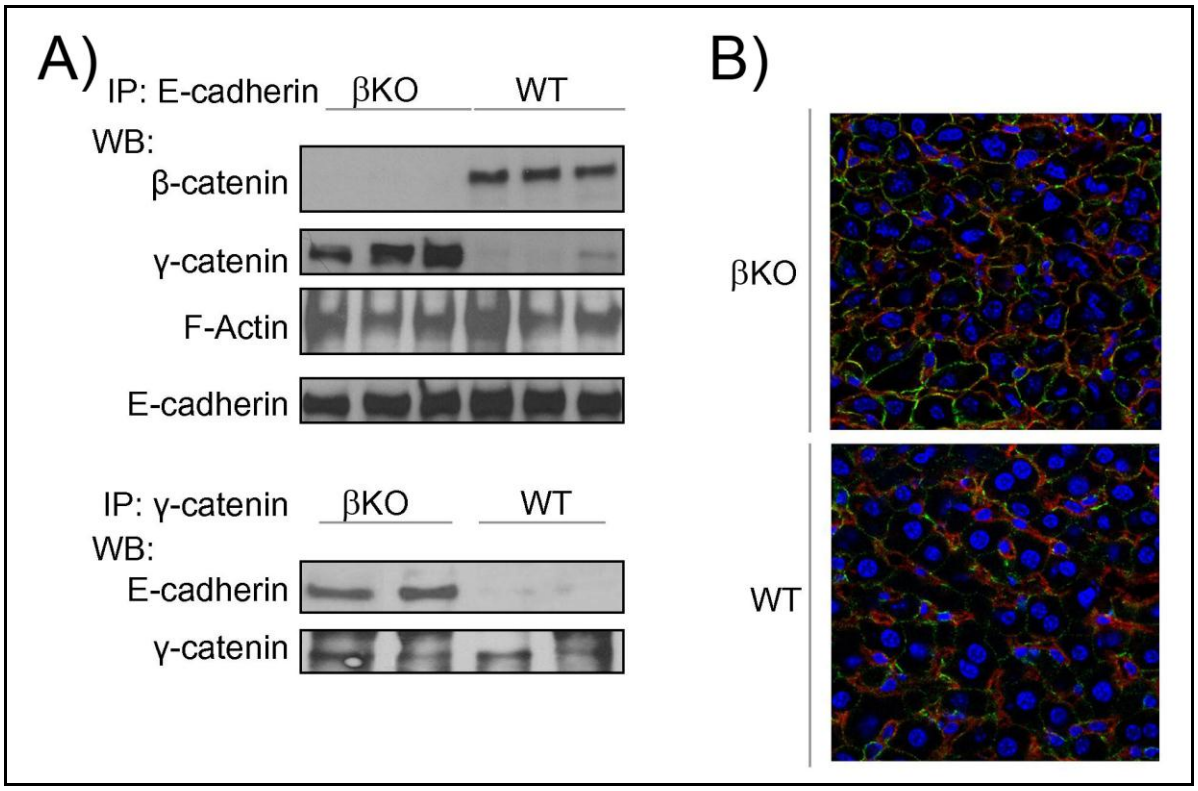

Figure 7. Increase γ-catenin at the AJs of βKO hepatocytes. (A) WCLs were immunoprecipitated with anti-E-cadherin or anti-γ-catenin antibodies. (*Upper panel*) WB performed for β-catenin, γ-catenin, and F-Actin show differential co-precipitation of β-catenin and F-actin with E-cadherin in WT or γ-catenin and F-actin and E-cadherin in βKO livers. (*Lower panel*) The conditions were reversed to show that γ-catenin also co-precipitates with E-cadherin in βKO. **(B)** Increased membranous γ-catenin (green) associates with E-cadherin (red), prominently in the βKO hepatocytes as shown by representative double IF utilizing liver sections from βKO and WT animals.

2.3 LACK OF NUCLEAR γ-CATENIN DURING HEPATOCYTE PROLIFERATION

β-Catenin is a major component of the Wnt signaling pathway, where it translocates to the nucleus to increase target gene expression. However, aberrant activation of β-catenin due to mutations in the *Ctnnb1* gene, or components of its destruction complex such as Axin, results in

stabilization and nuclear translocation of β-catenin that induces target genes critical to tumor growth and survival [10, 59]. Here we address whether γ-catenin can substitute for β-catenin in hepatocyte Wnt signaling.

Previously, others and we have reported a delay in liver regeneration after partial-hepatectomy in βKO mice such that peak hepatocyte proliferation was evident at 72 instead of 40 hours [34, 40]. We hypothesized that if γ-catenin was compensating for nuclear β-catenin function to induce hepatocyte proliferation, βKO livers might show evidence of nuclear γ-catenin at 40 or 72 hours after hepatectomy. Partial hepatectomies were performed as indicated in section **7.2.1**. IF studies detected γ-catenin only at hepatocyte membranes and not nuclei at either 40 or 72 hours after hepatectomy in either group (**Figure 8**). As expected, IHC for Ki-67 on corresponding sections reveal hepatocytes in S-phase of cell cycle in WT at 40 and βKO at 72 hours (**Figure 8**). From this data it is evident that there is a lack of nuclear γ-catenin during hepatocyte proliferation in absence of β-catenin. Thus, γ-catenin is unlikely compensating for nuclear β-catenin function in the liver.

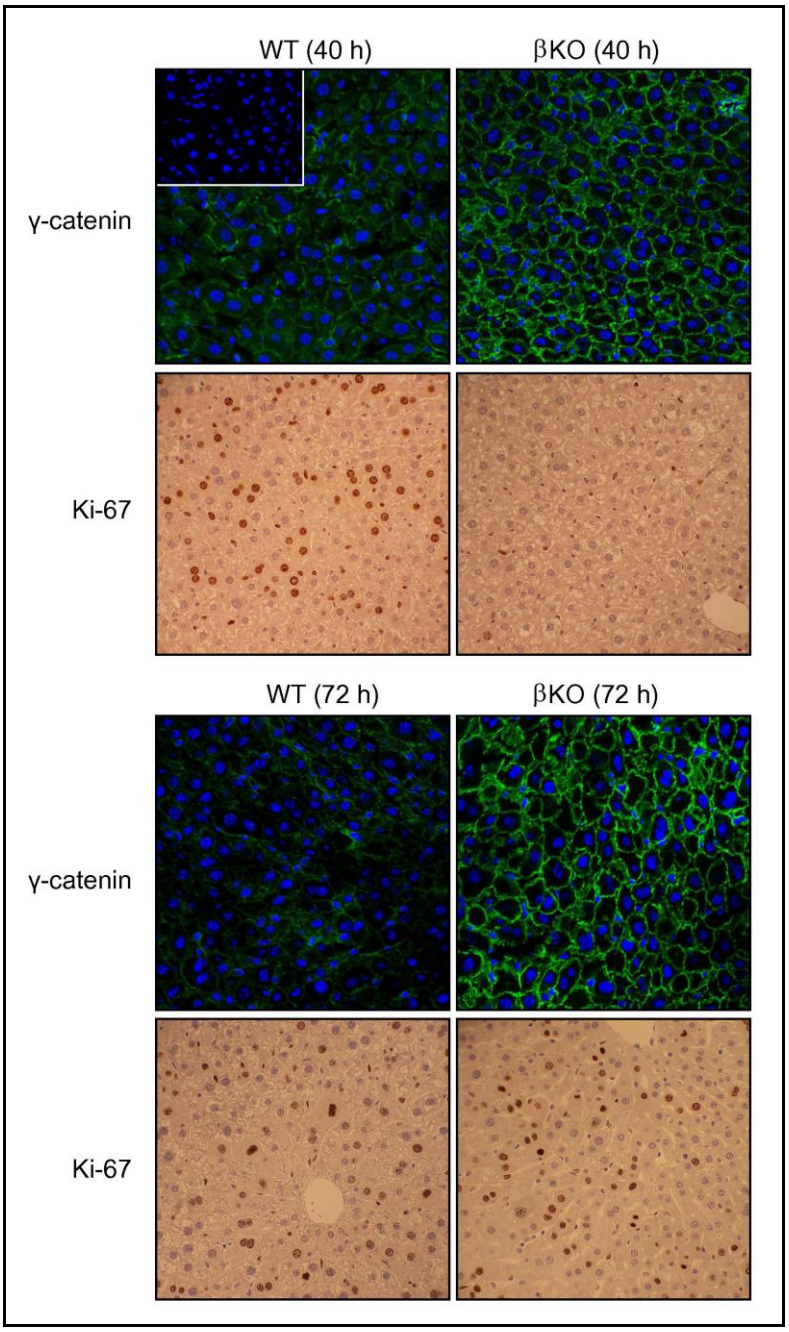

Figure 8. Sustained membranous localization of γ-catenin in WT and βKO livers during liver regeneration.

While an increase in membrane signal of γ-catenin (green) is evident in βKO as compared to WT, it was only localized to hepatocyte membrane at both 40 and 72 h after partial hepatectomy via IF staining, when several Ki-67 positive hepatocytes are observed in WT and βKO livers via IHC, respectively. No signal is detected in negative control when primary antibody is omitted in the reaction (*inset, upper left panel*).

2.4 MECHANISM OF γ-CATENIN STABILIZATION *IN VIVO*

Here we begin to address the mechanism of γ-catenin increase in βKO. For additional validation of microarray analysis, which revealed an insignificant increase in γ-catenin (*Jup*) gene expression (**Table 1**), we performed real-time PCR on βKO and WT livers (see section **7.6.2** and **Table 4** for real-time PCR details and primers respectively). ΔΔCT values confirmed insignificant differences in *Jup* expression between βKO and WT livers in three independent experiments (**Figure 9A**).

We next assessed the phosphorylation status of γ-catenin in each condition. IP studies using γ-catenin antibody, identified greater serine and threonine phosphorylation of γ-catenin in βKO mice when compared to WT (**Figure 9B**). No differences were observed in tyrosine-phosphorylation of γ-catenin. Thus, increase in γ-catenin protein in βKO livers coincide with a greater serine and threonine phosphorylation. From this data it is evident that the increase in γ-catenin appears to be regulated by post-translational modifications in βKO livers.

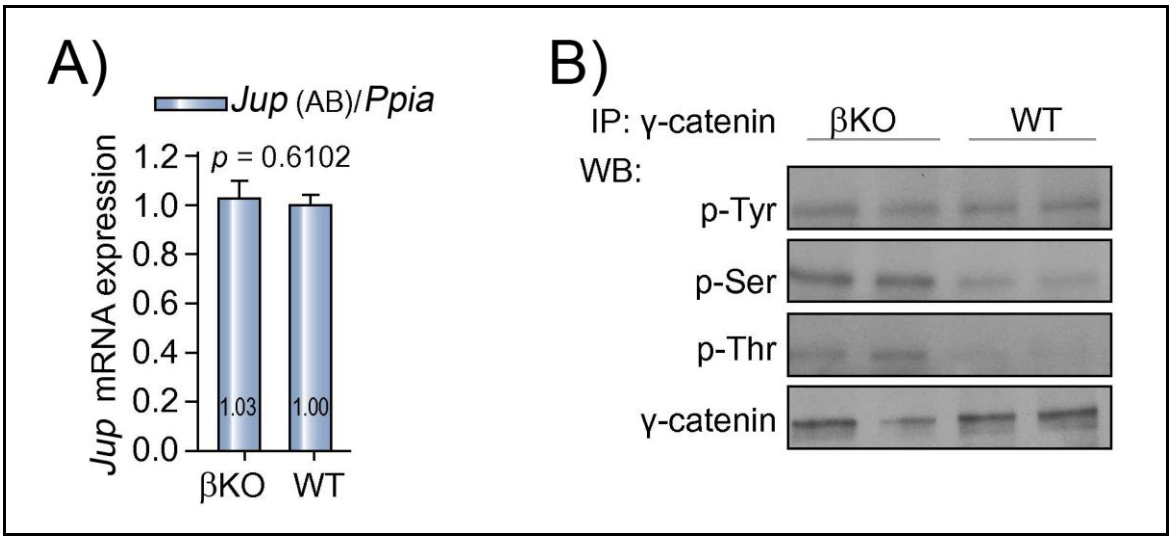

Figure 9. Mechanism of increased γ-catenin in βKO livers. (A) Real-time PCR shown for γ-catenin gene (*Jup*) and cyclophilin-A (*Ppia*) reference gene shows insignificant difference in average mRNA expression (± SD) in βKO versus WT livers (n = 3). Similar results were obtained for all *Jup* and reference gene primer combinations. **(B)** WCLs immunoprecipitated with anti-γ-catenin antibody and western blotted for anti-phosphotyrosine, anti-phosphoserine, or anti-phosphothreonine show greater serine and threonine phosphorylation of γ-catenin in βKO livers as compared to WT, with no change in tyrosine phosphorylation.

2.5 DISCUSSION: γ-CATENIN COMPENSATION FOR β-CATENIN LOSS AT ADHERENS JUNCTIONS

We observed previously that hepatic architecture was well maintained in βKO livers without any histological evidence of adhesion defect despite β-catenin being a critical component of AJ [34]. Here, γ-catenin was identified to compensate for β-catenin loss to maintain AJ in βKO. γ-Catenin is highly homologous to β-catenin [111], and it is a unique component of desmosomes, [165]. Unlike the above paradigm however, in γ-catenin knockout mice, β-catenin, despite its presence at desmosomes, was insufficient to functionally compensate for γ-catenin, causing embryonic

lethality due to faulty desmosomes [107]. In normal liver, we found a significant association of β-catenin and E-cadherin, but not of γ-catenin and E-cadherin, suggesting the former complex to be a major contributor of hepatocyte AJs. Based on their nearly identical interactions with E-cadherin [111], it is conceivable that during steady state, these two catenins may be competing to bind E-cadherin and their relative levels may eventually determine their relative contribution to AJ. Our studies demonstrate that β-catenin is the predominant catenin in hepatocytes and thus the major form associating with E-cadherin. Indeed, increased levels of γ-catenin are known to displace β-catenin from AJ [128]. On the other hand, in the absence of β-catenin, γ-catenin is the clear 'winner'. In fact, an intact E-cadherin/γ-catenin/F-actin ternary complex was evident in βKO livers *in lieu* of E-cadherin/β-catenin/F-actin in the WT (**Figure 7A**). A similar compensation by γ-catenin for β-catenin loss was recently reported to maintain normal cardiomyocyte structure and function as well [119].

The other components of AJ remained only minimally affected. There was an insignificant, *albeit* consistent, decrease in E-cadherin and α-catenin proteins in βKO. For AJ assembly, E-cadherin/β-catenin complex forms in the endoplasmic reticulum and guides E-cadherin to the plasma membrane [166]. This complex prevents exposure of the E-cadherin PEST domain for recognition by the proteasomal degradation machinery [26]. It is conceivable, that loss of β-catenin may contribute to mild E-cadherin destabilization. However, as γ-catenin binds to E-cadherin identically [111], it is likely that excess degradation of E-cadherin is prevented. An increase in E-cadherin and α-catenin gene expression might be compensatory due to decreased proteins. We were unable to detect α-catenin in complex with E-cadherin in WT or βKO livers. Recent studies have provided evidence that β-catenin and α-catenin interactions with F-actin might be mutually exclusive [167]. In our study we detect ternary complexes

composed of E-cadherin/β-catenin/F-actin in the WT or E-cadherin/γ-catenin/F-actin in the βKO livers.

γ-Catenin has also been shown to function as a downstream effector of the canonical Wnt pathway, translocating to the nucleus and regulating target gene expression, as reported in malignant mesothelioma (epithelial) cells *albeit* upon complete β-catenin loss [127]. γ-Catenin contains armadillo repeats, which weakly interact with TCF/LEF, regulating distinct Wnt signaling [122, 123]. At baseline, no nuclear γ-catenin is observed in the βKO livers. Lack of nuclear compensation is also supported by absence in the βKO of β-catenin transcriptional targets such as *Glutamine Synthetase*, *CYP2E1 and CYP1A2*, leading to defects in zonation, ammonia metabolism and xenobiotic metabolism [34, 36, 37, 168]. Similarly, regenerating livers at 40 and 72 hours after hepatectomy (times of peak hepatocyte proliferation in control and βKO livers respectively) [34, 40], do not show any nuclear γ-catenin also arguing against nuclear compensation in absence of β-catenin.

Here we observed that the protein levels of γ-catenin were disproportionately greater than its gene expression suggesting post-translational regulation. We identified increased serine and threonine phosphorylation of γ-catenin in βKO livers along with greater γ-catenin-E-cadherin association. Changes in phosphorylation status of γ-catenin leading to its stabilization and altered subcellular localization have been reported previously [115]. Additional characterization to determine signaling mechanisms regulating γ-catenin phosphorylation and its impact on association with E-cadherin can be found in **Chapter 6**.

β-Catenin activation due to multiple causes occurs in significant subsets of HCC [3, 46]. While β-catenin is no doubt a therapeutic target, it remains unclear what impact β-catenin inhibition might have on the integrity of AJ and ultimately on tumor cell invasion and metastasis.

From our current study, it appears that while β-catenin nuclear functions, which are more critical for tumor cell survival and proliferation, may not be compensated, its functions at the AJ are effectively substituted by γ-catenin. Such pathways that may induce γ-catenin stabilization may need to be spared during anti-β-catenin therapies for cancer treatment to prevent concurrent γ-catenin suppression that may inadvertently increase tumor cell invasion.

3.0 EFFECT OF γ-CATENIN STABILIZATION ON OTHER HEPATOCYTE JUNCTIONS

As previously discussed, the chronic loss of hepatocyte β-catenin in our βKO mice resulted in only a mild liver phenotype of: lower liver-weight-to-body-weight ratio, mild intrahepatic cholestasis, and irregular biliary canalicular architecture [34, 41]. Additionally, there were no gross defects in cell polarity or adhesion which manifested in βKO livers with or without challenges (partial hepatectomy, DDC diet, DEN injection, phenobarbital, or methionine- and choline-deficient (MCD) diet [34, 41, 42, 169, 170]). This was surprising, given that β-catenin is a major component of the AJ in the liver. Initially, we observed that γ-catenin was compensating at the AJs with β-catenin loss (**Chapter 2**) [91]. However, γ-catenin is a major protein component of the desmosomes. Additionally, we observed the loss of β-catenin target genes in our βKO mice, which included known TJ components (claudin-2) [34]. Therefore, to further expand upon the βKO junctional phenotype, or lack thereof, we decided to investigate compensatory changes in other hepatocyte anchoring and occluding junctions in addition to the changes previously noted for the AJs (**Chapter 2**). Here we identify no changes in hepatocyte desmosomes, despite the increase of desmosomal protein γ-catenin at the AJs. Additionally, we see compensatory changes in hepatocyte tight junctions (TJs) with the rearrangement of AJs as a result of junctional cross-talk.

3.1 DESMOSOMES

3.1.1 Hepatocyte desmosomes remain intact with β-catenin loss and γ-catenin increase at adherens junctions

It has been previously shown by our lab and others that γ-catenin is able to compensate for β-catenin loss at the AJ by binding to E-cadherin [91, 113-119] (see also **Chapter 2**). This increase in γ-catenin is not at the transcriptional level, but appears to be a post-translational modification that stabilizes the protein and increases overall expression levels [91, 115, 171] (**Figure 9A**). However, γ-catenin is primarily a desmosomal protein, so is this AJ compensation for β-catenin loss at the cost of desmosomal integrity? Initially we used transmission electron microscopy (TEM) to look for the presence of recognizable desmosomes in βKO and age-and sex-matched WT livers. Here we see that in fact there are identifiable, and visibly identical desmosomes present in both βKO and WT livers (**Figure 10A**). Quantification of the hepatocyte desmosomes revealed no significant difference in intercellular distances at these junctional interfaces, with mean distances of 27.4 nm in the βKOs and 25.1 nm in the WTs ($p>0.10$) (**Figure 10B**).

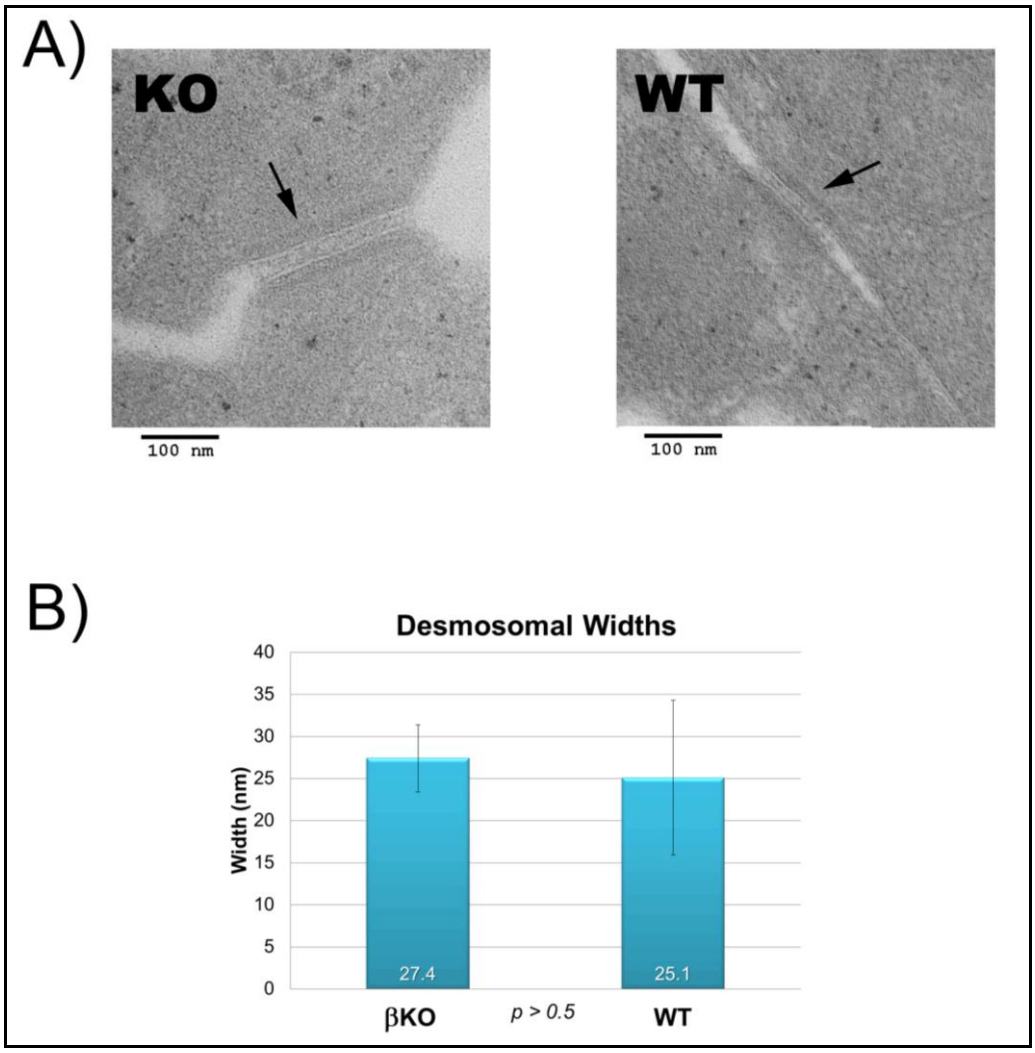

Figure 10. γ-Catenin increase in the βKO mice does not deplete hepatocyte desmosomes. (A) Transmission Electron Microscopy analysis of β-catenin KO and WT livers show the presence of desmosomes (*arrows*). **(B)** The intercellular distance between hepatocytes connected by desmosomes are not significantly different (mean distances βKO=27.4 nm, WT=25.1 nm, $p > 0.05$).

Next, to investigate gene expression changes in desmosomal components, we analyzed microarray data from βKO and WT livers [34]. βKO livers showed altered expression of desmocollin-2 (+2.2 fold), desmocollin-3 (+3.8 fold), and plakophilin-3 (-3.7 fold) (**Table 1**).

Interestingly, desmocollin-3 has never been shown to be present in the desmosomes of the livers, but we see a robust increase in mRNA expression in the βKO livers [159]. Desmocollin-3 is a key desmosomal cadherin, especially in stratified epithelia, and loss of this protein in the mouse results in K-Ras-induced skin tumors [172].

Since changes in mRNA levels do not always correspond to overall protein levels, especially with catenins and cadherins [91], we wanted to further analyze the protein expression of liver-specific desmosomal proteins [64]. We analyzed Triton X-100 soluble (S) and insoluble (I) cytoskeletal-associated (CAL) fractions of β-catenin KO and WT livers for desmosomal protein expression by WB. We confirmed that there was an increase in γ-catenin levels in the βKO livers (**Figure 11B**). This increase in γ-catenin was in both S- and I-fractions, but the most notable change was the appearance of cytoplasmic/S-fraction γ-catenin only with β-catenin loss (**Figure 11B**). Next, we evaluated the levels of desmoplakin I and II (DPI/II), desmoglein-1 (Dsg1), Dsg2, Dsg3, Dsg4, desmocollin-2 (Dsc2), and plakophilin-2 (Pkp2), and there was no change in overall protein levels of these proteins, despite changes in some mRNA levels (**Figure 11A**; **Table 1**). Interestingly, the only desmosomal protein that was changed was Pkp3, which was up-regulated in both the S- and I-fractions (**Figure 12A**), despite a -3.7 fold decrease in mRNA expression (**Table 1**).

Despite the similarities in protein levels of most desmosomal proteins analyzed, we wanted to see if there was a change in desmosomal protein association with γ-catenin. Here we show via immunoprecipitation that there was no change in DPI/II, Dsg1, Dsg2 (*data not shown*), and Dsg4 association with γ-catenin between βKO and WT livers in I-fraction (**Figure 11C**) and S-fraction lysates (*data not shown*). In summary, the lack of ultrastructural changes, along with minimal changes in various desmosomal proteins, and a lack of changes in γ-catenin's

interactions with various desmosomal proteins support the notion that the increase in γ-catenin due to β-catenin loss is not at the expense of the molecular configuration of the desmosomes.

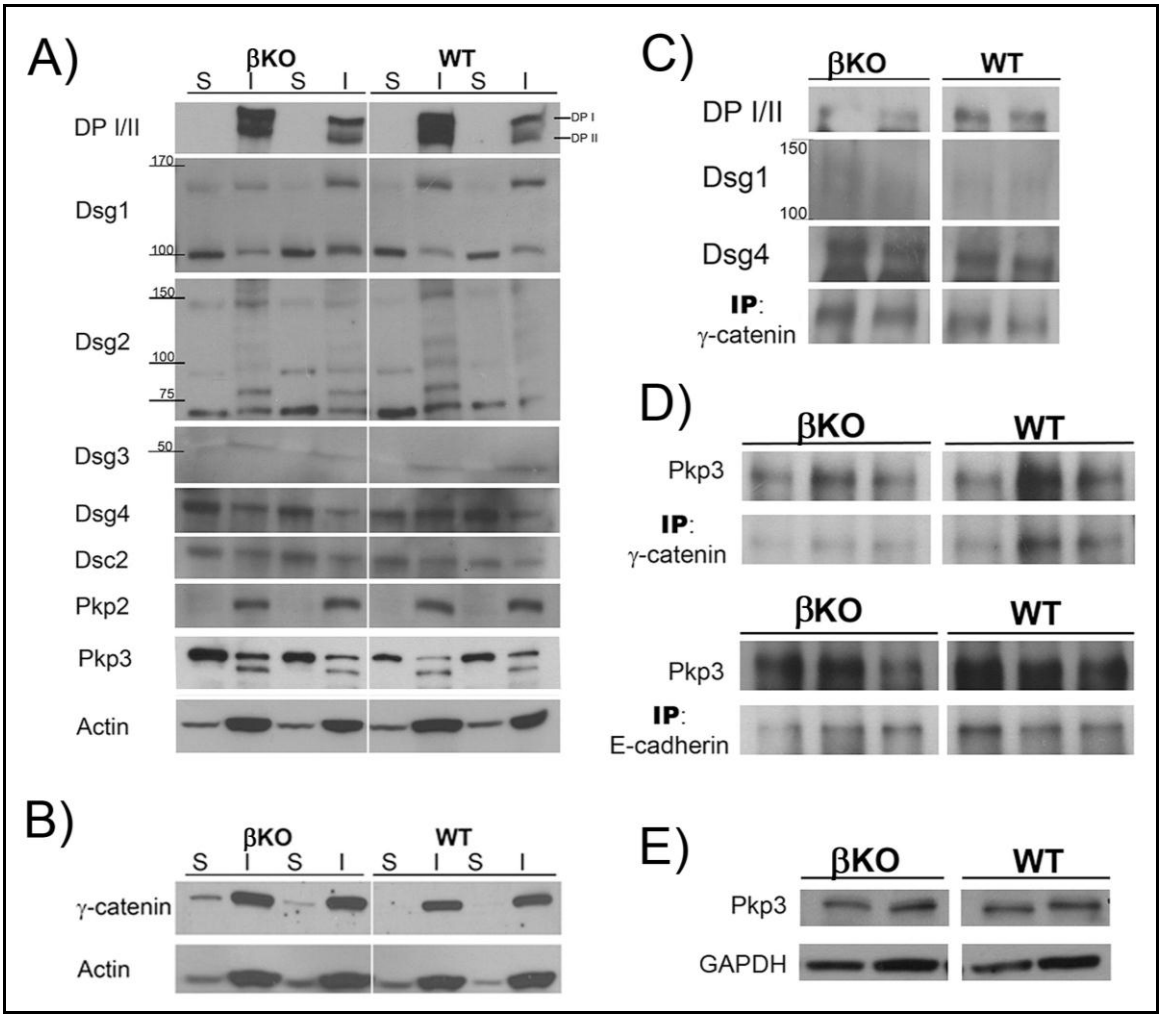

Figure 11. βKO mouse livers show no changes in desmosomal proteins, except plakophilin-3. (A) β-Catenin KO livers have no apparent changes in most desmosomal protein levels in S and I CAL fractions via WB, despite some mRNA changes (**Table 1**): DPI (250 kDa), DPII (210 kDa), Dsg1 (150 kDa), Dsg2 (59–150 kDa), Dsg3 (55–130 kDa), Dsg4 (100–115 kDa), Dsc2 (110 kDa), and Pkp2 (100 kDa). However, Pkp3 (87 kDa) is slightly increased in βKO livers in both in S and I CAL fractions over WT age-and sex-matched livers lysates. **(B)** β-Catenin KO livers show more soluble and insoluble γ-catenin (83 kDa) than WT livers by WBs. WB for Actin (42 kDa)

verifies comparable loading. **(C)** Representative immunoprecipitations of γ-catenin in I-fraction shows no apparent changes in desmosomal protein associations with γ-catenin in βKO and WT livers. **(D)** Pkp3 co-precipitates with γ-catenin (*upper panel*) and E-cadherin (*lower panel*) equally in the S-fractions of βKO and WT livers. **(E)** Nuclear lysates show Pkp3 protein levels are similar between βKO and WT livers.

3.1.2 Changes in plakophilin-3 with β-catenin loss with no apparent consequences on desmosomes

Next, we wanted to address the Pkp3 increase in the βKO livers. Initially, we investigated whether the species of Pkp3 that increased with β-catenin loss had different binding partners which may affect Pkp3 trafficking in the cytoplasm. It was recently published that a complex of γ-catenin, E-cadherin and Pkp3 is required to initiate desmosome formation [108]. Since both γ-catenin and Pkp3 are known binding partners, and both were increased with β-catenin loss, we wanted to see if these proteins associated more readily in the βKO livers, possibly contributing to their mutual stabilization. IP studies showed Pkp3 binding to both E-cadherin and γ-catenin in the S and I (*not shown*) CAL fractions, but there was no change in Pkp3 association with β-catenin loss (**Figure 11D**). This is in agreement with the lack of any changes the in overall desmosomal structure in βKO mice (**Figure 10A**). To further investigate the possible reason for the increase in Pkp3 levels with β-catenin loss, we wanted to see if the increase Pkp3 levels lead to increased nuclear Pkp3, since it has been shown to have potential nuclear functions [173]. Here we show that there was comparable nuclear Pkp3 in the βKO and WT livers (**Figure 11E**). The changes in Pkp3 levels with β-catenin loss cannot be explained by changes in cytoplasmic

binding partners or with nuclear localization. Therefore, additional studies will be necessary to address the role and regulation of Pkp3 after β-catenin loss.

3.2 TIGHT JUNCTIONS

3.2.1 Hepatocyte tight junctions have compensatory changes with β-catenin loss at adherens junctions

In epithelial cells, such as the hepatocytes of the liver, TJs are located between basolateral and apical domains of hepatocytes. These junctions contribute to paracellular adhesion, cell polarity and serve as occluding barriers around the biliary canaliculi [67, 174]. TJs are multiprotein complexes composed chiefly of claudins, junctional adhesion molecules (JAM) and occludin [175] (See **1.4.2**). Anomalies in TJ have been associated with an imperfect blood-bile barrier and also implicated in various hepatic pathologies, namely cholestasis [176, 177]. A cross-talk between AJ and TJ, and among other plasma domains has been reported [79]. Additionally, AJ proteins can influence the expression of TJ proteins [178].

Based on the observed cross-talk between the components of AJ and TJ [69], we analyzed βKO and WT livers for any alterations in gene expression of TJ components by microarray. βKO livers showed altered expression of claudin-1 (–2.3), claudin-2 (–8.0) and JAM-A (–2.0) but insignificant changes in the expression of claudin-3, occludin, ZO-2 or ZO-1 (**Table 1**). By WB, we assessed WCLs from WT and βKO livers for TJ proteins. Despite decreases in gene expression, JAM-A and claudin-1 protein levels were significantly elevated in βKO (**Figure 12A-B**). However, concordant with lower gene expression, a significant decrease

in claudin-2 protein was observed in βKO (**Table 1**; **Figure 12A-B**). Insignificant differences in occludin and claudin-3 levels were evident, which was in accordance with the microarray data (**Figure 12A-B** and [164]; **Table 1**).

3.2.2 Compensatory changes in hepatocyte junctions does not cause leaky tight junctions

Follow-up studies on TJ ultrastructure and function were performed in the lab of Dr. Jai Behari at the University of Pittsburgh, Department of Medicine in collaboration with the Center for Biological Imaging and Dr. Satdarshan P.S. Monga (published in *Hepatology* in 2010 [164]). To determine if the changes in JAM-A, claudin-1 and claudin-2, negatively affected the structure of the TJ, the Behari lab looked for the formation of TJs via TEM. There were no distinguishable changes in the ultrastructure of the TJs surrounding the bile canaliculi in the βKO livers [164]. Next, to test the functionality of the hepatic TJs, the integrity of the blood-bile barrier was tested by measuring the leakage of FITC-conjugated dextran (FC-40) from the blood into the bile (see [164] for experimental details). If the βKO TJs were compromised, and leakier, as a result of the changes in the claudin and JAM expressions, then we would have expected a peak in the FC-40 in the bile of the βKO to occur before that of the WT mice. However, the Behari lab's assessment of the hepatic TJs showed a delay and decrease in the βKO FC-40 peak [164]. Interestingly, it appears that the βKO TJs are "tighter" than the WT junctions.

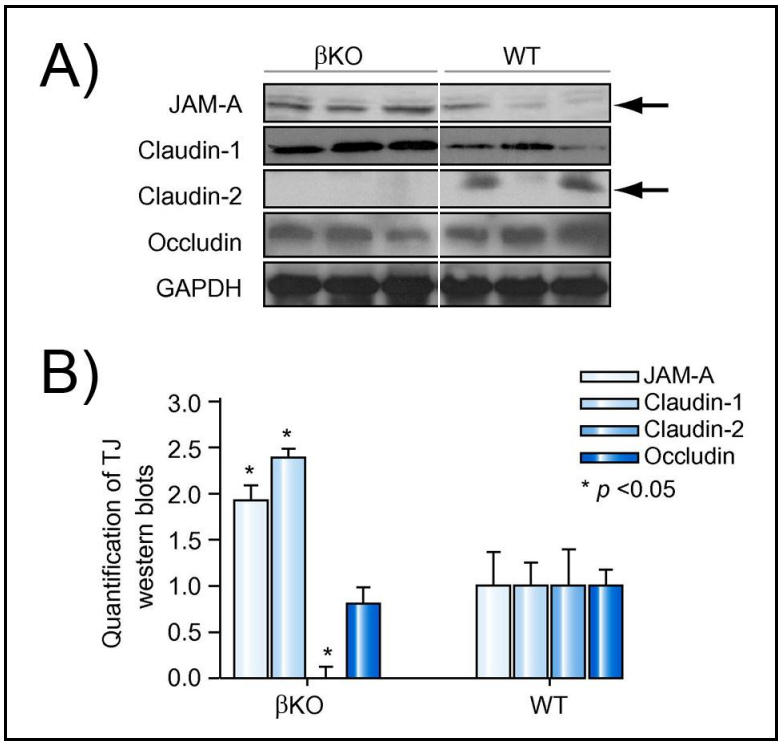

Figure 12. βKO mouse livers show compensatory changes in tight junction. (A) Examination of TJ protein expression by WBs using WCLs shows an increase in JAM-A (35 kDa) and claudin-1 (22 kDa), no change in occludin (60-82 kDa) and absence of claudin-2 (22 kDa) in βKO livers. GAPDH served as the loading control. **(B)** Normalized (to GAPDH) densitometric analysis reveals significantly (*$p < 0.05$) lower average (± SD) protein expression of claudin-2 and higher average (± SD) expression of JAM-A and claudin-1 in βKO than WT livers (n > 3), while occluding remained unchanged.

3.3 DISCUSSION: HEPATOCYTE JUNCTIONS

It was important to initially address the influence of γ-catenin changes with β-catenin loss on the desmosomes, especially since the trafficking of γ-catenin to the AJ is controlled post-transcriptionally [91, 115, 171]. Additionally, disruptions in desmosomes have been shown to play a role in cancer progression [159, 179, 180], and thus compensation by γ-catenin at AJs may

come at the expense of desmosomal integrity, which may have indirect and unintended negative side effects upon β-catenin therapeutic targeting in HCC. We have previously shown using a hepatocyte-specific β-catenin KO mouse that γ-catenin is increased and compensating for β-catenin loss at AJ by complexing with E-cadherin [91]. We now show that such compensation does not have any functional consequences on desmosomal structure in the liver. This was confirmed in multiple ways, including the observation that the γ-catenin/E-cadherin/Pkp3 cytoplasmic complex, which has been shown to be important for desmosomal assembly [108], was unchanged in βKO mice. We also show the structure of desmosomes was not affected *in vivo* via TEM. We did however see an increase in Pkp3, but are unable to explain the significance of this increase at this time. We speculate that Pkp3 increase after β-catenin loss may be more directly related to the changes in Wnt signaling than changes in AJ, in the same way claudin-2 levels are changed in βKO mice since we did not see changes in Pkp3 localization via co-precipitations or nuclear lysates. This observation still remains under scrutiny in our lab.

We also identify changes in protein expression of various TJ components in the absence of β-catenin. TJ are a set of multi-protein complexes that contribute to cell-cell adhesion, polarity, segregating various domains of the hepatocytes, enabling proper hepatocyte secretory function [67, 79]. Claudin-2 was absent in βKO livers. This is not surprising since it is a known Wnt target gene [178] and we also reported its downregulation in βKO livers previously [34]. While gene expression of JAM-A and claudin-1 were decreased in the βKO livers, their protein levels were increased, suggesting a post-transcriptional regulation. Interestingly, despite these changes, our collaborators reported lack of any major functional defect in the integrity of hepatic TJ in βKO mice by assaying for biliary excretion of FITC-conjugated dextran after intravenous injection [164]. It is conceivable that loss of claudin-2 may be compensated by claudin-1 and in

fact result in tighter TJ, since claudin-2 expression is usually contributes to leakiness in epithelia while claudin-1 does not [92, 181, 182]. However, claudin-1 compensation for the absence of claudin-2 may also be imperfect since the former is a barrier forming while the latter is a pore forming claudin. Additionally, changes in JAM-A might also have a compensatory impact on maintaining TJ in absence of claudin-2 and β-catenin. However, both of these hypotheses will need to be confirmed.

While the notion of cross-talk between TJ and AJ exists, a recent study for the first time showed an inverse relationship between E-cadherin and JAM-A in HCC cells [69]. Based on our observations, we suggest that β-catenin/claudin-2 may be another important means of cross-talk between AJ and TJ through which aberrations in one junction may be transmitted to the other through both structural and signaling cues. It is yet untested whether this particular cross-talk node is universally recognized, or whether this may be a function of HCC pathogenesis.

4.0 SPONTANEOUS REPOPULATION OF β-CATENIN-NULL LIVERS WITH β-CATENIN-POSITIVE HEPATOCYTES AFTER CHRONIC LIVER INJURY

Chronic exposure to 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC), a porphyringenic drug, results in hepatobiliary injury, atypical ductular proliferation (ADP), appearance of hepatic progenitor cells (A6-positive oval cells), and limited fibrosis. The etiology of this xenobiotic is aberrant secretion of porphyrin from the biliary tract which leads to the formation of porphyrin crystal plugs in the bile ducts [183]. Obstruction of the bile ducts leads to slowed bile flow, a rise in serum bilirubin, and ultimately interhepatic cholestasis.

Oval cells are the putative adult stem cells of the liver. They are considered facultative stem cells which usually appear in response to hepatobiliary injury, especially when hepatocyte proliferation is simultaneously blocked; specifically, this oval cell response occurs with specific xenobiotics, such as DDC, Dipin, and high-fat diet + ethanol [65]. Oval cell activation induces ADP, which is pathologically manifested as an increase in biliary ductules, leukocyte infiltration, and an increase in extracellular matrix components [184]. Chronic ADP eventually leads to periportal and biliary fibrosis. This ductular reaction is known to be a part of cholestatic disease etiologies. Additionally, the chronic activation/appearance of oval cells has also been linked to the induction of liver cancer [65, 185], which raises the possibility that oval cells could also be cancer stem cells.

Recently, our lab has shown that these oval cells co-stain with β-catenin [186]. Additionally, our lab demonstrated that there was a blunted oval cell response with loss of β-catenin and acute DDC exposure (under 24 days) [186]. Therefore, we were interested in expanding upon these results to identify the role of β-catenin in chronic DDC exposure. Chronic DDC exposure is known to lead to unresolved ADP, and liver injury that eventually resembles primary sclerosing cholangitis (PSC). Here we show that as with the acute DDC exposure, chronic exposure (80-150 days) to DDC lead to ADP and A6-positive oval cell infiltration, but this time it was to a greater extent in βKO livers (data previously published by Thompson *et al.*, 2011 [169]). Additionally, there was a surprising amelioration of hepatocyte injury at later time points in the βKO mice, which coincided with spontaneous β-catenin-positive hepatocyte repopulation of the β-catenin-null livers.

4.1 GREATER BILIARY INJURY BUT ONLY MODEST HEPATOCYTE INJURY IN βKO MICE ON DDC DIET

Gross analysis of the DDC livers showed that the βKO livers were enlarged, with macroscopic non-cancerous nodules after 150 days. Because of the additional weight of the nodules, the liver-weight-to-body-weight ratio of the βKO mice was equal to the WT mice. To try and address the etiology of the hepatic nodules, trichrome staining was performed to assess fibrotic load. The βKO livers had a greater degree of fibrosis, especially between portal triads at 150 days on DDC diet as compared to the WT mice (data previously published by Thompson *et al.*, 2011 [169]).

To further assess the extent of hepatic/biliary injury, serum biochemistry analysis was performed to test the levels of total bilirubin, alkaline phosphatase, AST, and ALTs in the blood of WT and βKO mice on DDC diet for 80 and 150 days. High bilirubin and/or alkaline phosphatase levels indicate cholestasis while ASTs and ALTs are good indications of hepatic damage. The liver function tests for mice on long-term DDC diet showed that the βKO mice had significantly higher serum bilirubin at 80 days and alkaline phosphatase levels at 150 days, indicating increase intrahepatic cholestasis and biliary injury over WT mice [169]. Conversely ALTs were significantly higher in the WT at 80 and 150 days [169], which was unexpected. Interestingly, these results indicate that the βKO mice had less hepatocyte injury than the WT mice on DDC diet, despite increase in ADP (data previously published by Thompson *et al.*, 2011 [169]).

4.2 PROGRESSIVE REPOPULATION OF βKO LIVERS ON DDC DIET WITH β-CATENIN-POSITIVE HEPATOCYTES

We next wanted address why the βKO mice had diminished hepatocyte injury after long term DDC feeding as compared to the WT mice. To our surprise, IHC analysis of the βKO livers showed that these β-catenin-null livers were expressing β-catenin-positive hepatocytes (**Figure 13A**), and had re-expression of β-catenin that was also apparent via WB analysis after 150 days of DDC diet (**Figure 13D**). It was imperative for our analysis to determine the chronology of this repopulation; therefore, we looked at βKO livers on DDC diet at times before 30 and 80 days, when we see small β-catenin-positive clusters, to see if we could find when single β-catenin-

positive hepatocytes arise. At 7 days on DDC diet, we were able to find singular β-catenin-positive hepatocytes in the βKO livers via IF (**Figure 13B**). These β-catenin-positive hepatocytes were located periportally, but did not co-localize with the A6-positive cells (**Figure 13B**). Additionally, these β-catenin-positive cells stained for epithelial- (E-cadherin) and hepatocyte-specific (CCAAT-enhancer-binding protein α - CEBPα) markers (data previously published by Thompson *et al.*, 2011 [169]). Further analysis of βKO livers via IHC showed that β-catenin-positive biliary cells did not begin to appear until at least 80 days of DDC exposure (**Figure 13C**). As we previously reported, βKO mice showed that despite a hepatocyte-targeted/Albumin-Cre driven deletion of β-catenin, there was a lack of normal β-catenin expression in the bile ducts of these mice [34] (**Figure 13C**), possibly because of the shared hepatoblast origin of hepatocytes and cholangiocytes. Taken together, this data indicates that the β-catenin-positive hepatocytes most likely did not originate from the oval cells, since β-catenin-positive hepatocytes precede the appearance of β-catenin-positive bile ducts in the βKO livers.

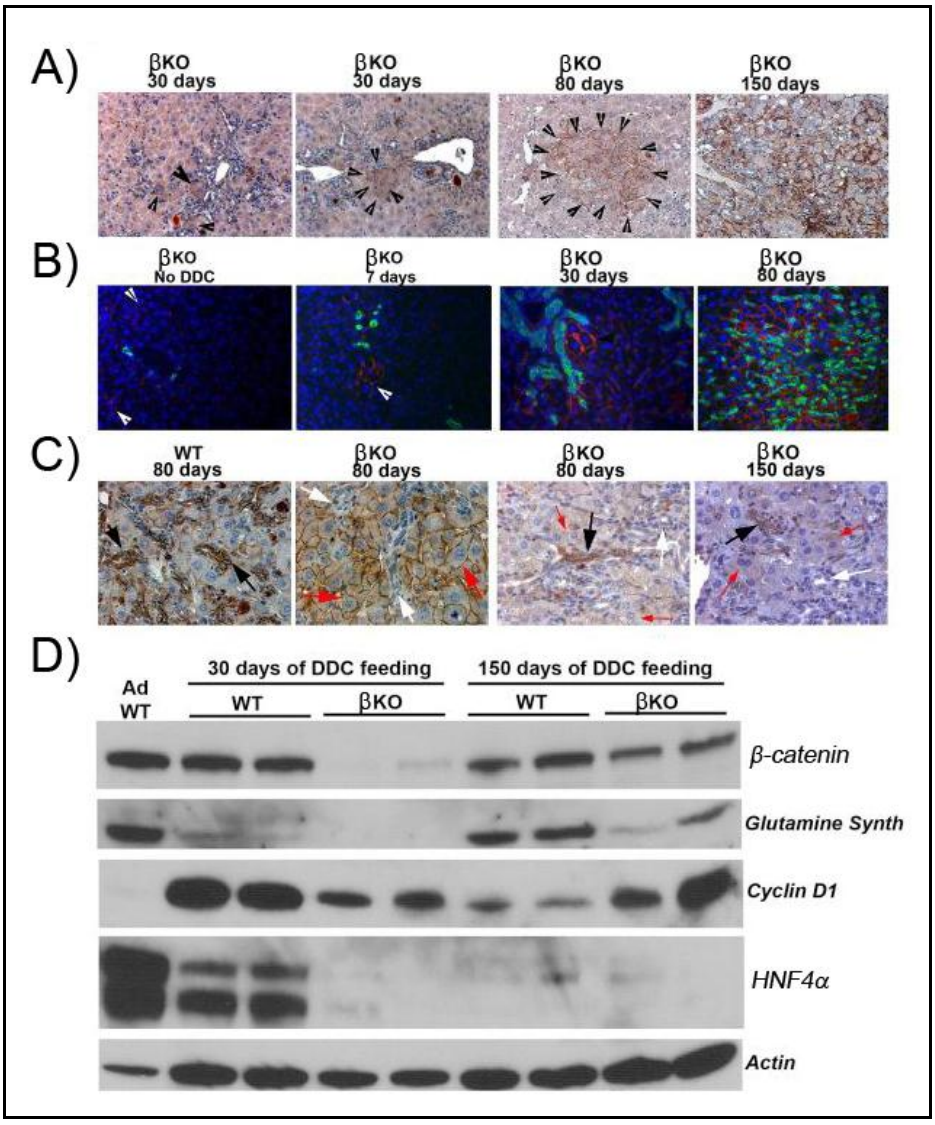

Figure 13. Progressive repopulation of βKO livers on DDC diet with β-catenin-positive hepatocytes. (A) IHC staining of βKO livers after 30, 80 and 150 days on DDC diet show β-catenin-positive hepatocytes (*arrowheads*). Images shown are at 200x magnification. **(B)** Double IF staining for A6 (green) and β-catenin (red; *arrowheads*) shows no co-localization of these markers at baseline and in βKO livers after short and long-term DDC diet. Images shown are at 200x magnification. **(C)** IHC staining for β-catenin after 80 days of DDC diet shows strong expression of β-catenin in atypical ductular cells in WT (*black arrows*) and lack thereof in βKO liver (*white arrows*) even within newly developed clusters of β-catenin-positive hepatocytes (*red arrow*). However, some β-catenin-positive duct cells (*black arrow*) do appear, whereas others remain negative (*white arrow*) amid β-catenin-positive hepatocytes (*red arrow*) at 80 and 150 days on DDC diet. Images shown are at 400x magnification. **(D)** WB analysis

of β-catenin, its downstream targets (GS, cyclinD1), and hepatocyte-specific markers (HNF4α). WB for Actin verifies comparable loading. Data previously published by Thompson *et al.*, 2011 [169].

4.3 PROGRESSIVE REPOPULATION OF βKO LIVERS ON REGULAR CHOW DIET WITH β-CATENIN-POSITIVE HEPATOCYTES

It is possible that there were β-catenin-positive hepatocytes in the livers before DDC exposure. This led us to analyze baseline βKO livers on regular chow at different ages. Surprisingly at three months of age around 1-2 hepatocytes per 200x magnification were β-catenin-positive in the βKO livers as detected by IF (**Figure 13B**). As an additional control, livers from 5 to 8-month-old mice were assessed for degree of spontaneous repopulation with β-catenin-positive hepatocytes without any DDC exposure by IF (n = 6) and IHC (n = 5). By IF, four βKO livers showed less than 1% β-catenin-positive hepatocytes, whereas two βKO livers had around 20%-30% spontaneous repopulation (**Figure 14**). Five additional βKO livers showed less than 1% hepatocytes to be β-catenin-positive in the βKO by IHC as well (*data not shown*). Thus, a majority of the βKO livers did not show many β-catenin-positive hepatocytes at baseline, suggesting only a DDC injury-dependent spontaneous repopulation with β-catenin-positive hepatocytes, which may have initially escaped Albumin-Cre driven β-catenin deletion. However, a very small subset of βKO livers did show the presence of greater numbers of β-catenin-positive cells for unknown reasons, *albeit* nowhere to the extent observed after DDC exposure.

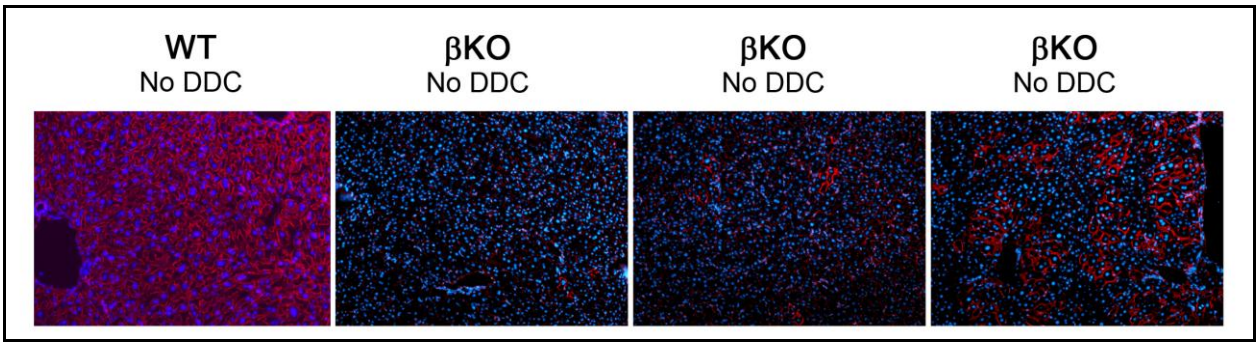

Figure 14. Progressive repopulation of βKO livers on regular chow diet with β-catenin-positive hepatocytes. IF showing β-catenin-positive hepatocytes in WT liver and three βKO livers at baseline. Although two livers represent a common phenotype of a few β-catenin-positive hepatocytes in βKO, one liver was an exception and showed greater repopulation (*right-most panel*). Images are shown at 100x magnification.

4.4 β-CATENIN-POSITIVE HEPATOCYTES DO NOT EXPRESS HEPATIC STEM CELL MARKERS

We also examined the status of the β-catenin-positive cells for some known markers of hepatic progenitors. In βKO livers at baseline, none of the β-catenin-positive cells were positive for commonly used markers of oval cells such as Ep-CAM, Cd133 or LGR5 (**Figure 15**). Interestingly, in the βKO livers occasional β-catenin-positive hepatocytes were α-fetoprotein-positive (AFP) as were a few non-β-catenin-positive hepatocytes (**Figure 15**). Thus, this analysis suggests that β-catenin-positive cells in the βKO livers are mostly mature hepatocytes, which amid chronic insult due to chronic DDC exposure may display growth and survival advantage to gradually repopulate the βKO liver.

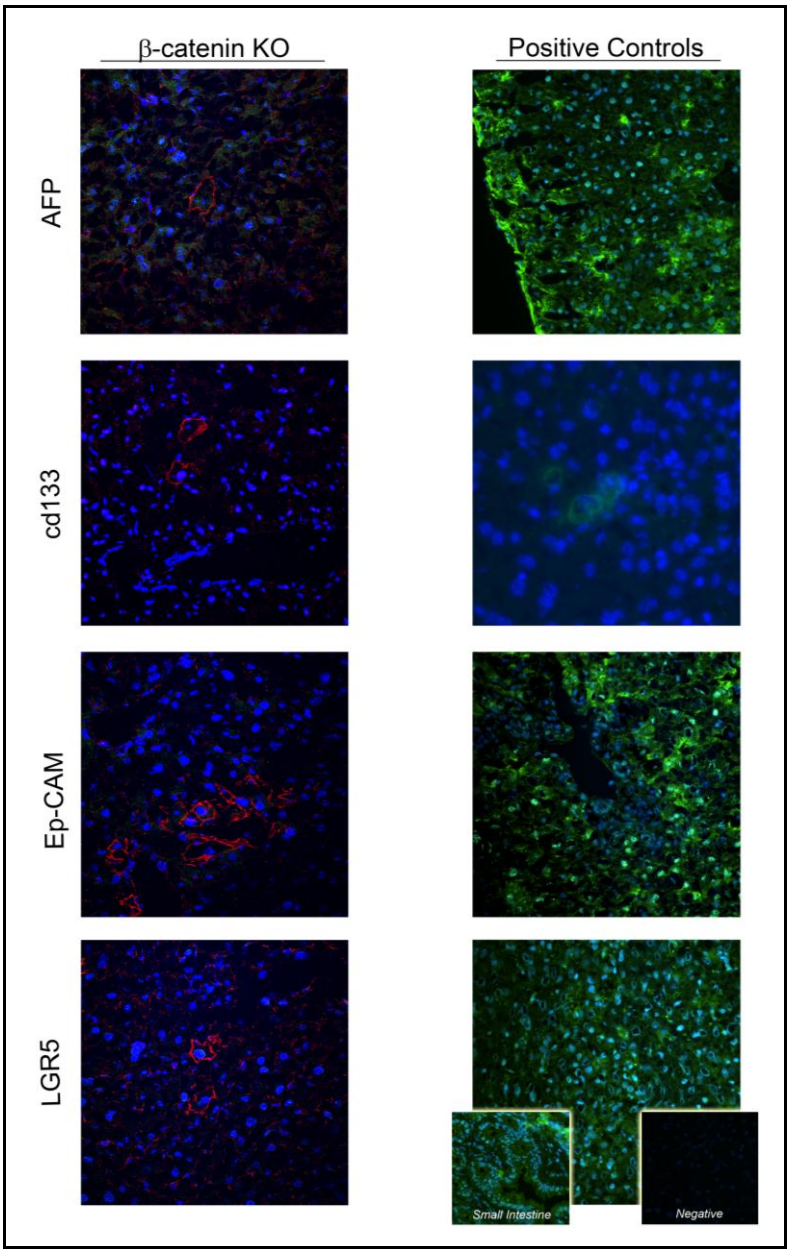

Figure 15. β-Catenin-positive hepatocytes in βKO livers do not express hepatic progenitor markers. There was no co-localization in the βKO livers of β-catenin (red) with Cd133, Ep-CAM, or LGR5 (green), whereas the positive controls showed positive signal in DDC liver or small intestine (*right column*). In addition, some β-catenin-positive cells (red) were positive for α-fetoprotein (AFP; green), whereas some non-β-catenin-positive cells were also β-catenin-positive. Images shown are at 400x magnification.

4.5 PROGRESSIVE LOSS OF CRE-RECOMBINASE IN βKO LIVERS ON DDC DIET

Lastly, we were interested in the mechanism of the β-catenin repopulation of the βKO livers. We hypothesized that the β-catenin positive cells must have somehow escaped the influence of Cre-recombinase. To examine whether the increase in the number of β-catenin-positive hepatocytes correlates with a decrease in expression of the transgene, we performed real-time PCR analysis for Cre-recombinase at 30, 80, and 150 days after DDC feeding. Real-time PCR analysis was performed for Cre-recombinase in three separate mouse livers using three different reference genes. The analysis showed that there was no significant difference in mRNA expression of Cre-recombinase between untreated age- and sex-matched βKO mouse livers and the 30 days DDC-fed βKO livers (*data not shown*). However, between 30 and 80 days after being on the DDC diet, there was a significant decrease in Cre-recombinase expression ($p = 0.00043$) (**Figure 16**). This expression continued to significantly decrease with ongoing DDC exposure (30 to 150 days on DDC, $p = 0.00005$; 80 to 150 days on DDC, $p = 0.091$). Thus, this analysis suggests repopulation of the βKO liver after long-term DDC feeding is by hepatocytes, which have escaped expression of the Cre-recombinase transgene.

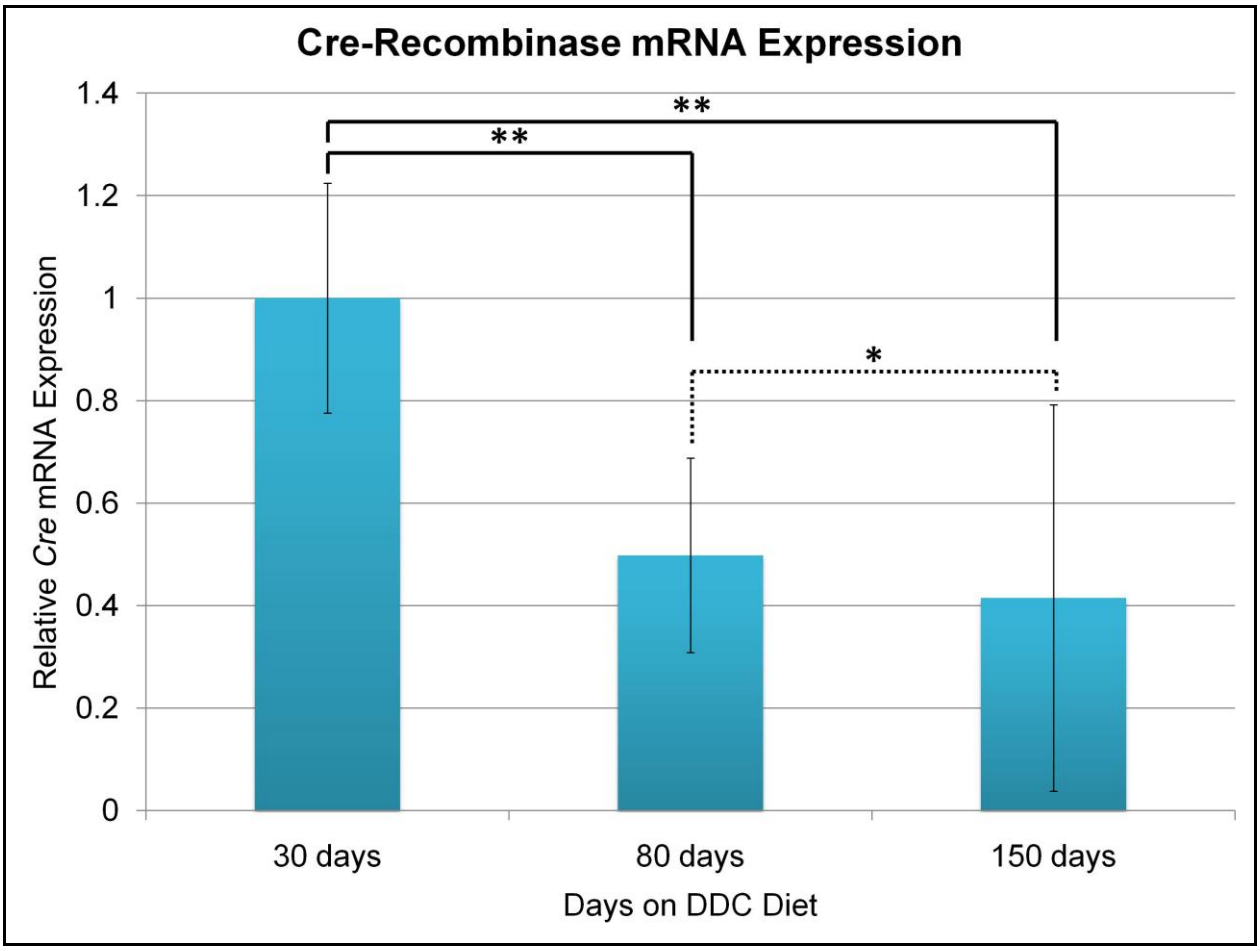

Figure 16. Progressive loss of Cre-recombinase expression in βKO livers on long-term DDC diet. Cre-recombinase mRNA expression in βKO mice at 30, 80, and 150 days after DDC diet shows a progressive and significant decrease (*$p < 0.1$; **$p < 0.001$). mRNA expression values were normalized to cyclophilin-A (*Ppia*) as a reference gene. Bars show +/- SD values of triplicate readings.

4.6 β- AND γ-CATENIN ARE MUTUALLY EXCLUSIVE AT HEPATOCYTE ADHERENS JUNCTIONS

As reported here by our lab, and also recently by other labs, the Albumin-Cre/Lox system of knocking out genes in hepatocytes may sometimes be "leaky," especially after about six months

of age, causing some repopulation of βKO livers with β-catenin positive hepatocytes [43, 169]. We used this anomaly as a proof-of-concept for the work on "Hepatocyte γ-catenin compensates for conditionally deleted β-catenin at AJs" (**Chapter 2**). We performed immunofluorescence staining on liver sections of an 8 month old βKO mice (on regular chow) to assess the localization of β- and γ-catenin at the membrane of hepatocytes with and without β-catenin. We previously showed conditionally deleted βKO mice had an increase in γ-catenin co-localization with E-cadherin at the AJ where β-catenin is normally located (**Figure 7B**). Here we see that in a mosaic β-catenin-positive/β-catenin-negative liver section that β-catenin (*red*) and γ-catenin (*green*) appear to be mutually exclusive at the hepatocyte membranes, with relatively no co-localization (**Figure 17**). Analysis of the γ-catenin localization in the β-catenin positive nodule shows a desmosomal-like staining pattern, while γ-catenin localization in the remainder of the βKO liver section has an AJ-like staining pattern (**Figure 17**: *inset, green channel*).

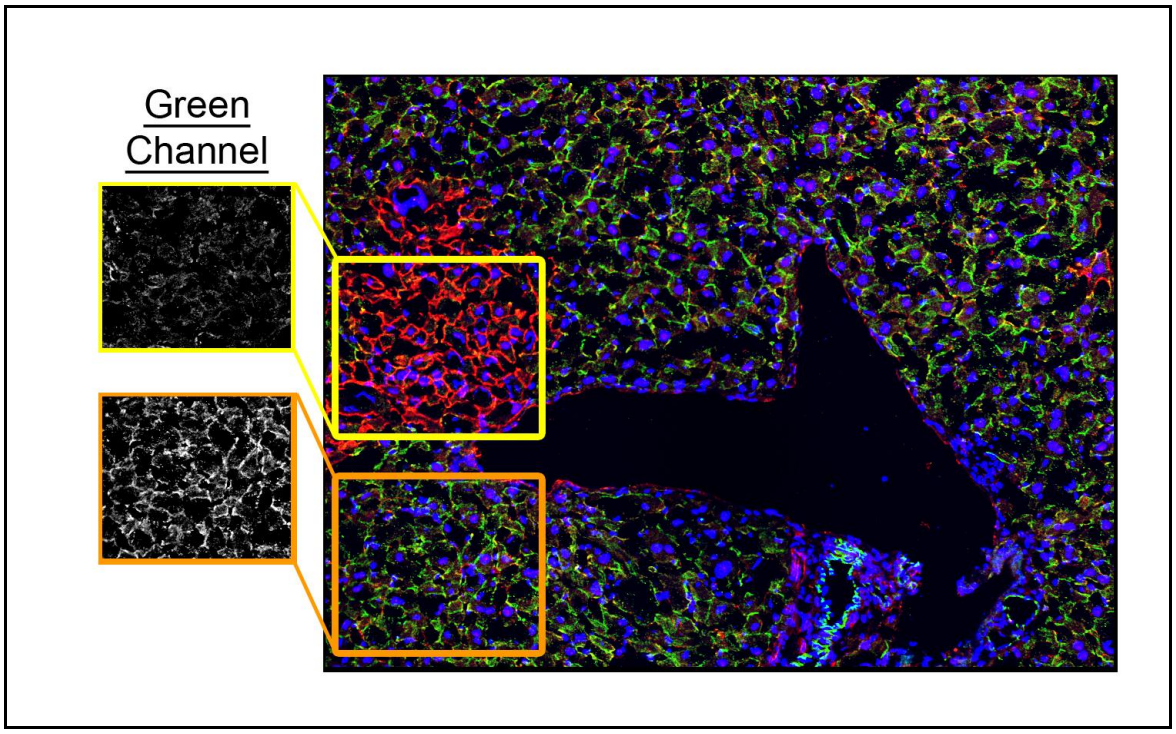

Figure 17. β- and γ-Catenin are mutually exclusive at hepatocyte adherens junctions. Membranous γ-catenin (green channel) and β-catenin (red) are mutually exclusive at the cell membranes / AJ of βKO livers which become repopulated with β-catenin-positive hepatocytes at elderly ages as shown by representative double IF images of fixed liver sections. Images shown are at 400x magnification.

4.7 DISCUSSION: SPONTANEOUS REPOPULATION

These studies were initiated after observations that there was a dampened A6-positive oval cells activation in βKO mice following acute liver injury, indicating an important role of β-catenin in oval cell activation and proliferation [186]. However, chronic DDC exposure actually led to a paradoxical increase in ADP and A6-positive oval cell infiltration in the βKO mice, indicating that there was an initial delay in the βKO mice that was not sustained after chronic hepatobiliary injury. Therefore, we also expected to see an increase in liver injury in the βKO mice as well.

We did see a significant increase in bilirubin at 80 days and alkaline phosphatase at 150 days in the βKO mice indicating increased biliary injury [169]. However, the βKO mice had significantly lower serum AST and ALT levels, indicating only modest hepatocyte injury as compared to the WT mice [169]. Further investigation showed that this phenomenon was due to the unexpected spontaneous repopulation of the βKO livers with β-catenin-positive hepatocytes that had escaped the influence of Cre-recombinase.

These events prompted us to look more critically at our βKO mice. Much to our surprise, variable repopulation of the β-catenin-null liver also occurred spontaneously without chronic injury, as we were able to detect β-catenin-positive cells in a sub-population of older mice (**Figure 14**). This observation was made around the same time by other labs as well, highlighting the leakiness of the Albumin-driven Cre-LoxP system [43]. There is efficient deletion of β-catenin at two months of age in these animals, but as the mice age past four months there is appearance of a small population of β-catenin-positive cells [34, 43]. Further analyses showed that β-catenin-positive hepatocytes are mature hepatocytes, and do not involve the hepatic progenitor cells (**Figure 15**). It appears that the older mice are able to escape the influence of the Cre-recombinase [34] (**Figure 16**). Whether this is an artifact of liver-specific/Albumin-driven knockouts remains to be determined. As of now though, this model is still an effective tool for looking at hepatocyte-specific deletions of β-catenin and other proteins, as long as the appropriately aged mice are used, and the caution of spontaneous repopulation is heeded and recognized in the assessment of data.

Recently, it has been shown that mature hepatocytes have a unique capacity to increase and decrease their ploidy, which may be due to metabolic or xenobiotic insults which allows the hepatocytes to adapt and create a genetically diverse population of cells within the liver [187].

Therefore, it is possible that the liver may have an innate ability to create a population of hepatocytes that are able to adjust better to chronic injury. This may be an explanation for the repopulation of β-catenin-null livers, where the hepatocytes are able to elude the influence of Cre-recombinase in order to reintroduce β-catenin into the liver.

Despite unexpected realizations of the limitations of our *in vivo* conditional-knockout model, the repopulation of the β-catenin-null livers with β-catenin positive-hepatocytes has created a new and useful tool for our lab and others. We now have an *in vivo* model which has a mosaic expression of β-catenin-positive and -null hepatocytes which can be utilized for other experiments. Recently, these mosaic knockout mice were helpful as a proof of concept of the results put forth in the manuscript "Hepatocyte γ-catenin compensates for conditionally deleted β-catenin at adherens junctions" [91], showing that β- and γ-catenin are mutually exclusive at hepatocyte AJs (**Figure 17**). Further characterization of the repopulated β-catenin-null livers should serve as a useful tool in understanding not only for transgenic mouse models, but also general liver and hepatocyte physiology.

5.0 γ-CATENIN AT ADHERENS JUNCTIONS AFTER β-CATENIN LOSS: BIOLOGICAL IMPLICATIONS IN HEPATOCELLULAR CARCINOMA

We have previously demonstrated that γ-catenin is increased at the AJs but not in the nucleus of βKO mice with chronic β-catenin loss (**Chapter 2**) and that this compensation of γ-catenin in βKO mice does not come at the expense of desmosomal structure and integrity (**Chapter 3**). This data is encouraging for the idea of treating HCC with anti-β-catenin therapies. Therefore, we moved to a more easily manipulated *in vitro* model of β-catenin knockdown in HCC cells in order to more comprehensively address the impact of β-catenin loss on HCC cell physiology. Here we show in an *in vitro* model that γ-catenin alleviates β-catenin loss at AJ in HCC cells to maintain cell-cell adhesion function, but is unable to fulfill its role as a component of the canonical Wnt signaling cascade.

5.1 GENERATION OF *IN VITRO* MODEL OF β-CATENIN KNOCKDOWN IN HEP3B CELLS LEADS TO γ-CATENIN INCREASE

Initially we observed that γ-catenin protein levels were significantly increased with the conditional hepatocyte β-catenin knockout *in vivo* in 60 to 120 day-old mice (**Figure 6**), and that this increase in γ-catenin was found to co-precipitate with E-cadherin in the βKO livers

exclusively (**Figure 7**), indicating that γ-catenin was structurally compensating at the AJs for the β-catenin loss. Additionally, we previously showed that in the βKO mice the regulation of γ-catenin increase appears to be post-translational, since there was no change in *Jup* gene expression between βKO and WT mice, but there was an increase in p-Ser/p-Thr γ-catenin protein (**Figure 9**).

5.1.1 β-Catenin knockdown in Hep3B HCC cells

Initially, we wanted to know if changes in γ-catenin due to chronic β-catenin loss i*n vivo* in hepatocytes can also occur acutely *in vitro*. siRNA-mediated acute knockdown (KD) of β-catenin increased γ-catenin protein levels *in vitro* in Hep3B, a human HCC cell line (**Figure 18A**) (see section **7.4.1** and **Table 2** for siRNA transfection details). As in the *in vivo* model, siRNA KD of β-catenin also increased E-cadherin/γ-catenin co-precipitation in the Hep3B cells as compared to the scrambled-siRNA transfected cells (**Figure 18B**). Reciprocally, γ-catenin co-precipitated to a greater extent with E-cadherin upon β-catenin KD in Hep3B cells (**Figure 18B**: *lower panel*). Next we assessed the mechanism responsible for γ-catenin increase following β-catenin downregulation. We sought any change in mRNA expression of the human γ-catenin gene (*JUP*) after β-catenin KD in Hep3B cells. As *in vivo*, we did not observe any change in *JUP* expression despite an increase in γ-catenin protein (**Figure 18C**). Hence, the *in vitro* model using Hep3Bs accurately replicates key observations seen in hepatocyte-specific βKO mice, especially at AJ.

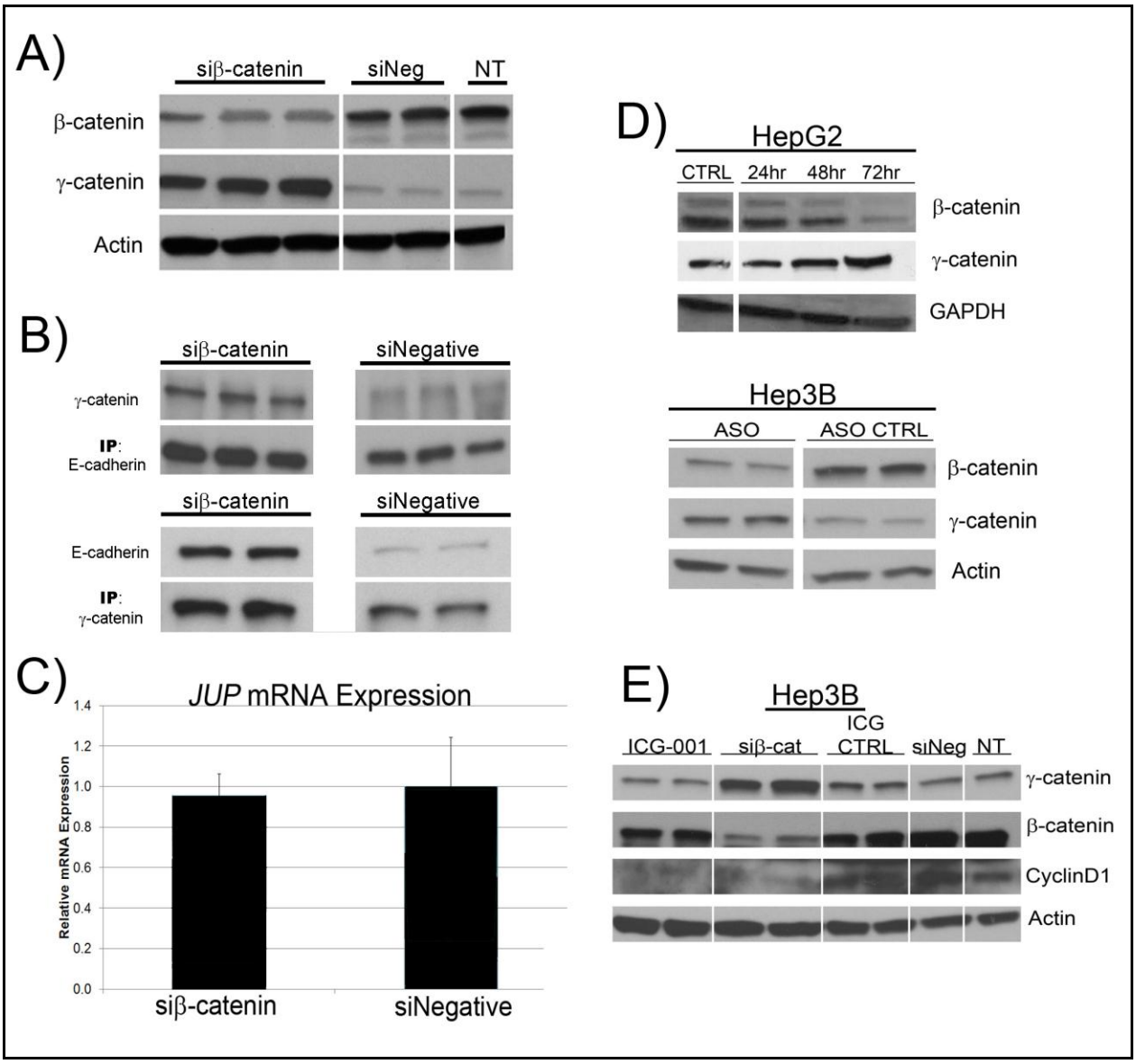

Figure 18. sKD of β-catenin *in vitro* using multiple HCC cell lines and KD methods accurately replicates conditions of *in vivo* βKO, only when protein levels are altered. (A) siRNA (siβ-catenin) induces efficient knockdown of β-catenin (92 kDa) in Hep3B cells and leads to an increase of γ-catenin (83 kDa) protein levels. WB for Actin (42 kDa) verifies comparable loading. **(B)** WCLs from siβ-catenin and siNegative treated Hep3B cells were immunoprecipitated with anti-E-cadherin or anti-γ-catenin antibodies. WB performed for γ-catenin (*upper panel*) and E-cadherin (120 kDa; *lower panel*) show differential co-precipitation with siβ-catenin. **(C)** Real-time PCR for γ-catenin gene (*JUP*) and *GAPDH* reference gene shows insignificant difference in average mRNA expression (+/- SD) in siβ-catenin versus siNegative Hep3B treated cells at 48 hour knockdown (n = 3). **(D)** HepG2

and Hep3B cells treated with ASO to β-catenin shows a decrease in β-catenin protein and subsequent increase in γ-catenin by WB. Both the WT and truncated forms of β-catenin in HepG2s show decrease with ASO. WBs for GAPDH (37 kDa) and Actin verify comparable loading. **(E)** Knockdown of β-catenin activity using small molecule inhibitor ICG-001 does not change the protein levels of β- or γ-catenin, despite the decrease in β-catenin transcriptional activity as indicated by a decrease in cyclinD1 (37 kDa).

5.1.2 Other HCC cell lines and alternative knockdowns

To verify that the observed increase of γ-catenin in Hep3B cells after siRNA-mediated β-catenin KD was not cell-specific or reagent-specific, we next employed antisense oligonucleotide (ASO; see **7.4.2**) to KD β-catenin in not only Hep3B but also in HepG2 cells, which carry a monoallelic deletion of exon-3 in the *CTNNB1* gene. ASO against β-catenin decreased its protein levels and concomitantly increased the levels of γ-catenin in both cell lines (**Figure 18D**). These data demonstrate that an increase in γ-catenin occurs with β-catenin decrease regardless of the method of protein KD, cell line used, or presence of full-length or mutated β-catenin in HCC cells.

5.1.3 Blockade of β-catenin transactivation does not lead to γ-catenin stabilization

We next asked if the increase in γ-catenin occurred secondary to a decrease in β-catenin levels, its activity, or both. We utilized a small molecule inhibitor of β-catenin's nuclear functions, ICG-001 that disrupts the interaction of β-catenin and its coactivator CBP, a known histone acetyltransferase, to down regulate β-catenin/TCF-dependent target genes expression [13] (see also **1.3.2**; see **7.4.2** for ICG-001 treatment details;). Indeed, treatment of Hep3B cells with ICG-001 led to decrease in β-catenin target cyclinD1 (**Figure 18E**) and TOPflash luciferase reporter

activity (*data not shown*). However, unlike siRNA or ASO, ICG-001 did not decrease overall β-catenin protein expression (**Figure 18E**). Intriguingly, ICG-001 treatment did not lead to any increase in γ-catenin in these cells, and if at all a modest decrease was evident in these cells (**Figure 18E**). Thus, this data suggests that cells sense a decrease in total β-catenin protein and not its activity to trigger an increase in γ-catenin protein.

5.2 *IN VITRO* MODEL OF DOUBLE β- AND γ-CATENIN KNOCKDOWN

To try and effectively tease out the individual roles of β- and γ-catenin in HCC cells and to address biological implications of γ-catenin stabilization following β-catenin KD, we investigated the feasibility of double KD (dKD) of β- and/or γ-catenin in addition to single KD (sKD) in Hep3B cells via siRNA (see section **7.4.1** and **Table 2** for siRNA dKD transfection details). Having an *in vitro* model gives us the ability to modulate the two catenins singly or in combination and determine the impact through the simple readouts. Hep3B cells lent themselves well for such a model, although the role and regulation of γ-catenin upon β-catenin sKD was also evident in other cell lines such as HepG2 cells and by other modalities of KD such as ASOs (**Figure 20D-E**). WB analysis of siRNA-treated Hep3Bs at 24, 48, and 72 hours show that the sKD and dKD of these protein is visible by 24 hours post-transfection and persists to at least 72 hours post-transfection (**Figure 19A**). Importantly, the increase in γ-catenin with β-catenin sKD is apparent at all time points (**Figure 19A**). Additionally, the Hep3B cells transfected with single or double siRNAs appear healthy and viable at all time points tested, and lacked any obvious

changes in cell morphology (*data not shown*). From this data it is evident that *in vitro* dKD of β- and γ-catenin is achievable in Hep3B cells.

Since γ-catenin associated with E-cadherin in the absence of β-catenin, and β-catenin/E-cadherin association prevents degradation of E-cadherin by masking the PEST domain in E-cadherin [26], we next asked if γ-catenin/E-cadherin association could have similar function in E-cadherin stability. Hep3B cells subjected to sKD and dKD of catenins were assayed for E-cadherin levels by WB. While comparable levels of E-cadherin protein were observed in control siRNA-transfected Hep3B cells and after sKD of β-catenin or γ-catenin, a notable decrease was observed with dKD (**Figure 19B**). This data suggests that just like β-catenin, γ-catenin can bind effectively to E-cadherin and prevent its degradation in HCC cells.

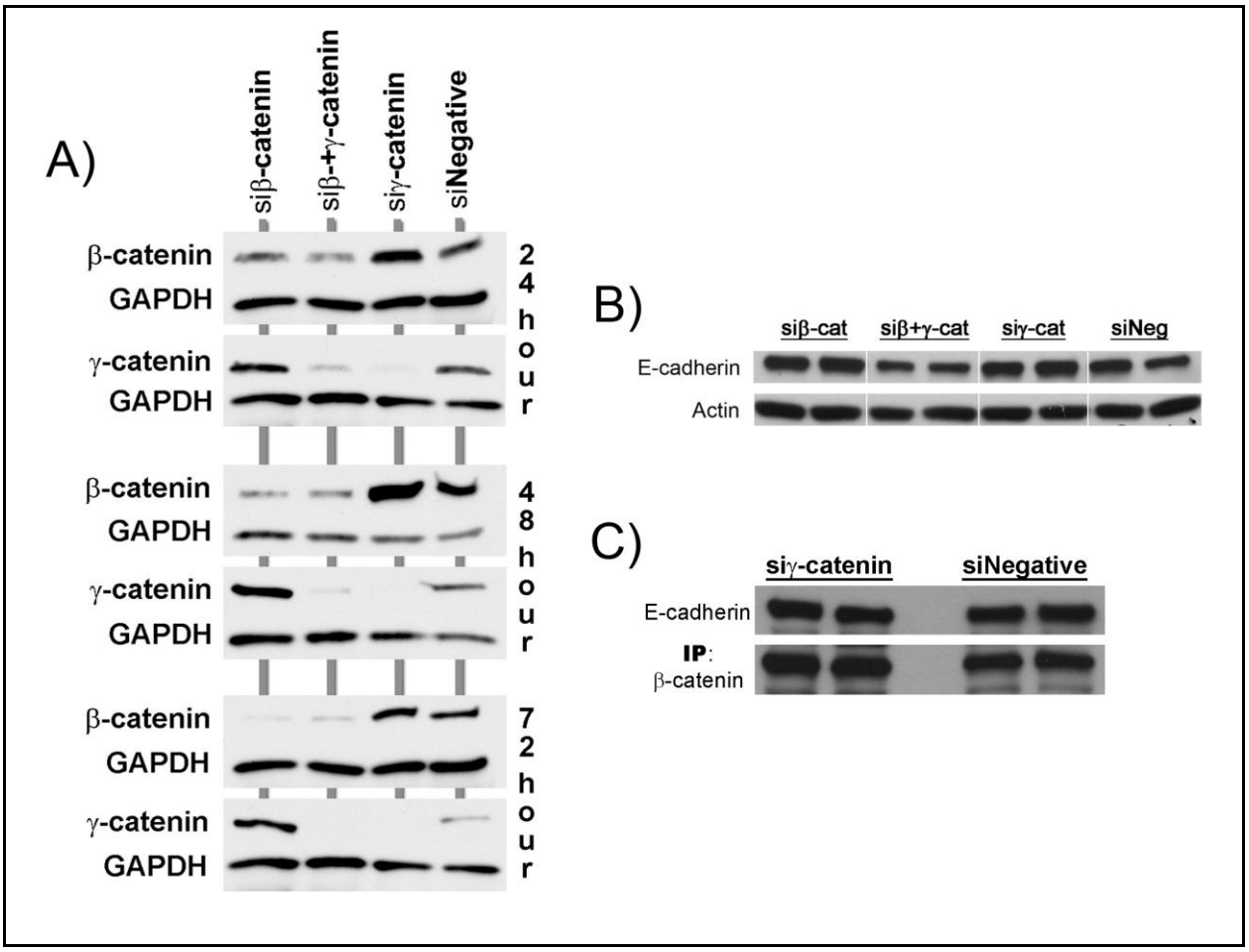

Figure 19. dKD of β- and γ-catenin with siRNA in human Hep3B HCC cells persists from 24 through 72 hours of transfection. (A) Confirmation and time course of sKD and dKD of β- and γ-catenin using siRNA technology in Hep3B cells *in vitro*. KD begins at 24 hours and persists to at least 72 hours after transfection. Representative blots shown here (n > 3). WB for GAPDH verifies comparable loading. **(B)** WB of sKD and dKD Hep3Bs show a decrease in E-cadherin expression with dKD of β- and γ-catenin. WB for Actin verifies comparable loading. **(C)** WCL from siγ-catenin and siNegative treated Hep3B cells were immunoprecipitated with an anti-β-catenin antibody. WB shows no changes in β-catenin's association with AJ protein E-cadherin. WB for β-catenin indicates pull-down efficiency.

5.2.1 β-Catenin increase with γ-catenin loss in HCC cells

Interestingly, γ-catenin KD in Hep3B cells lead to an increase in β-catenin protein expression, especially at 24 and 48 hours of γ-catenin siRNA transfection (**Figure 19A**). This is in agreement with the recent cardiomyocyte-specific γ-catenin knockout mice, which show an increase in β-catenin protein levels; however, the literature is mixed on the role of this β-catenin pool [188, 189]. One study shows that the increase in β-catenin with γ-catenin loss is localized to the AJ [188], while another report suggests this increase to be nuclear since it led to an increase in β-catenin target gene expression [189]. Therefore, we looked at the AJ composition with γ-catenin sKD to see if there are any changes in β-catenin/E-cadherin association. We observe no apparent changes in their association in γ-catenin sKD (**Figure 19C**), indicating that the increase in β-catenin is most likely occurring in the cytoplasmic/nuclear and potentially the signaling pool of β-catenin in HCC cells.

5.3 γ-CATENIN DOES NOT COMPENSATE FOR β-CATENIN-MEDIATED WNT SIGNALING

We have reported previously a lack of nuclear γ-catenin in hepatocyte-specific β-catenin KO mice even at time of β-catenin-dependent hepatocyte proliferation, such as 72 hours after partial-hepatectomy (**Figure 8**), indicating that γ-catenin was not compensating for nuclear β-catenin signaling *in vivo* [91]. To test this *in vitro*, we used a TOPflash luciferase reporter to assess TCF-dependent signaling of β- and γ-catenin (see **Figure 23** for vector map and **7.4.4** for transfection

details). As expected, sKD of β-catenin led to decreased TOPflash activity (**Figure 20A**) (see **7.11.1** for luciferase assay details). A dKD of β- and γ-catenin showed a decrease in Wnt signaling, but there was no additional impact of γ-catenin KD on the TOPflash reporter activity (**Figure 20A**), suggesting that γ-catenin does not contribute to Wnt signaling and therefore cannot compensate for β-catenin loss in this capacity in HCC cells.

Interestingly, there was a significant increase in TOPflash activity with γ-catenin sKD at 48 hours post-transfection (**Figure 20A**). This was in agreement with the increase in β-catenin levels in the non-E-cadherin bound fraction after γ-catenin sKD (**Figure 19A**), and further supports the hypothesis that the increased β-catenin after γ-catenin sKD participates in Wnt signaling [189]. To directly address this biologically, we examined [3H]thymidine-incorporation with dKD and sKD of β- and γ-catenin as a measure of cell proliferation (a β-catenin-dependent event). As expected, and in agreement with TOPflash reporter activity, we observed a decrease in [3H]thymidine incorporation after sKD of β-catenin or dKD of both catenins. However, also in concordance with TOPflash results, there was an increase in proliferation with γ-catenin sKD, *albeit* at 72 hours and not 48 hours after KD (**Figure 20B**), which is most likely due to physiological delay in transactivation of cell cycle related target genes governing this event. From this data it is evident γ-catenin does not compensate for β-catenin-mediated Wnt signaling *in vitro*, but γ-catenin knockdown is able to increase β-catenin-TCF reporter activity in HCC cells.

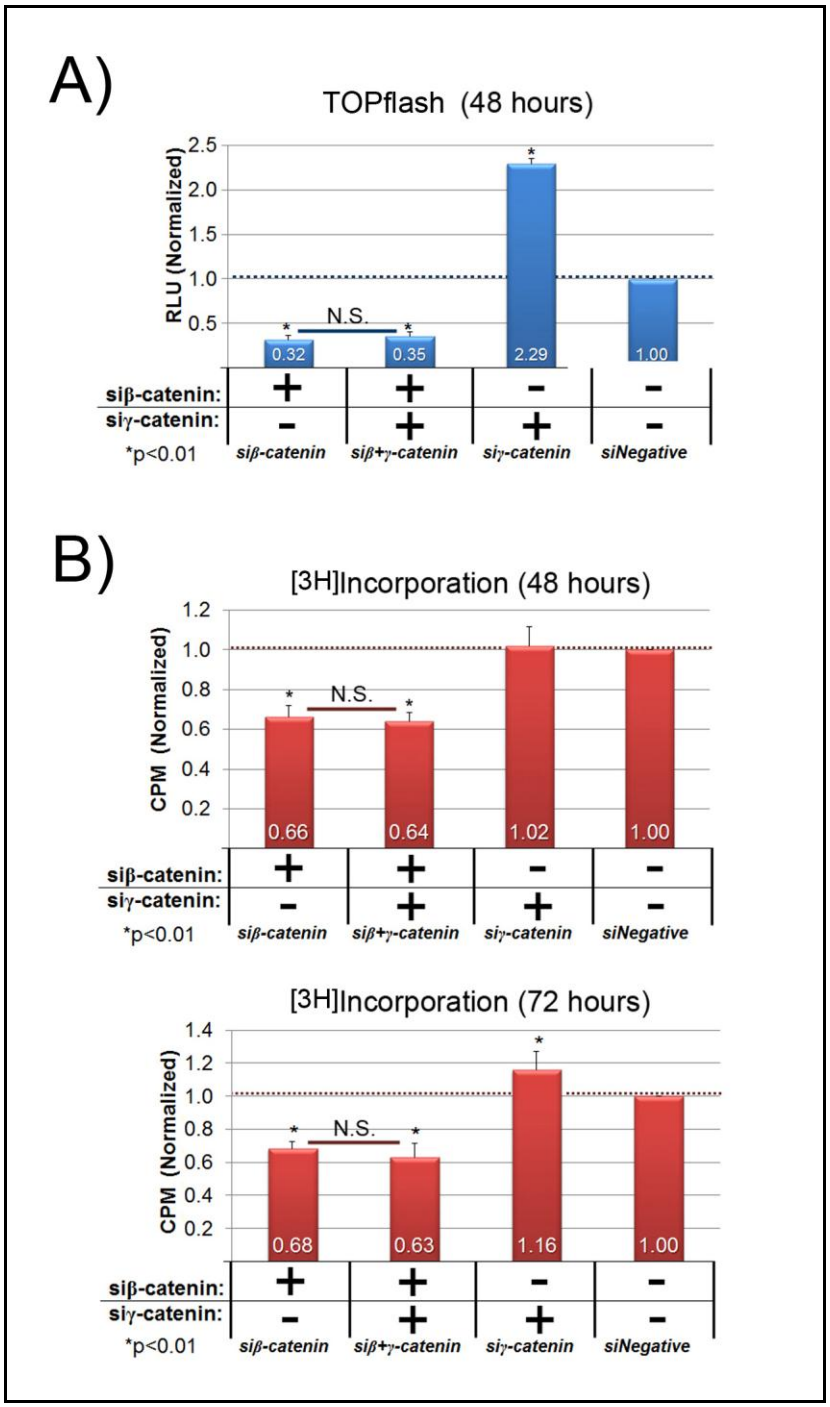

Figure 20. γ-Catenin does not compensate for β-catenin-mediated Wnt signaling *in vitro*. (A) Wnt reporter (TOPflash) activity for sKD and dKD shows a significant decrease in activity with sKD of β-catenin and dKD, but a significant increase with sKD of γ-catenin at 48 hour KD ($p<0.01$; +/- SD). **(B)** (*Upper panel*) Thymidine-incorporation values for sKD and dKD shows a significant decrease in activity with sKD of β-catenin and dKD, but

no difference with sKD of γ-catenin at 48 hour KD. (*Lower panel*) [3H]Thymidine-incorporation values for sKD and dKD at 72 hours shows a significant decrease in activity with sKD of β-catenin and dKD, but a significant increase with sKD of γ-catenin at 48 hour KD ($p<0.01$; +/- SD; *indicates significance as compared to siNegative; N.S. = not significant).

5.4 ONLY DOUBLE KNOCKDOWN OF β- AND γ-CATENIN NEGATIVELY AFFECTS CELL-CELL ADHESIONS

5.4.1 HCC cell migration

We next queried if γ-catenin upregulation after β-catenin KD in HCC cells would conserve cell adhesions comparable to β-catenin. Hep3B cells after sKD or dKD were used for scratch-wound assay to measure cell migration as described in **7.12.1**. We observe that while a sKD of either β-catenin or γ-catenin alone led to no significant change is the percentage of wound closure after 24 hours, dKD of both catenins significantly enhanced wound closure at this time when compared to Hep3Bs treated with a negative control siRNA (**Figure 21A-B**). This data indicates that the dKD of β- and γ-catenin is detrimental to overall cell adhesion thus increasing cell migration, while sKD of β-catenin is adequately compensated by γ-catenin. Additionally, sKD of γ-catenin has no impact on AJs since E-cadherin/β-catenin association remains unaltered as shown earlier (**Figure 18B**).

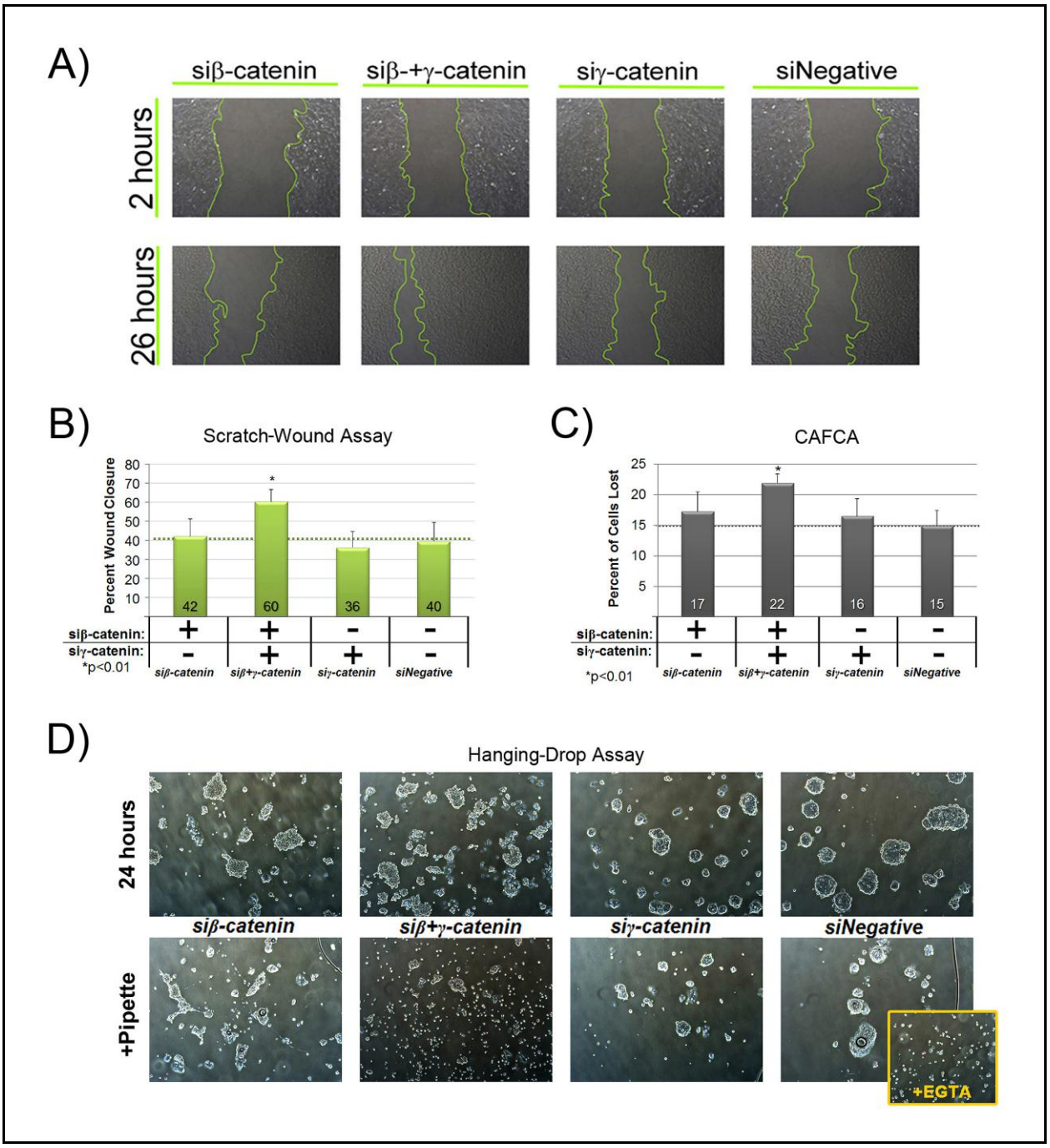

Figure 21. dKD of β- and γ-catenin affects cell migration and cell-cell adhesion in HCC cells. (A) Representative images of wound closure at 24 hours after initiation of the wound and 48 hours after transfection with various siRNAs. **(B)** Scratch-wound assay for sKD and dKD Hep3B cells shows a significant increase in wound closure percentage for dKD of β- and γ-catenin only (*$p < 0.01$; +/- SD). **(C)** Centrifugal Assay for Cell Adhesion (CAFCA) with sKD and dKD Hep3B cells shows a significant decrease heterotypic cell-cell adhesion

with dKD of β- and γ-catenin only as measured by increase in percentage of cells which de-adhere from the monolayer after centrifugation at 375g for 10 minutes (*$p < 0.01$; +/- SD; *indicates significance as compared to siNegative). **(D)** Hanging-drop assay shows that at 24 hour after drop formation (72 hour transfection) the colonies formed appear similar in size, though the dKD colonies appear to be less symmetric than the sKD and siNegative colonies (*upper panel*). After pipetting, the dKD cells dissociate the most, indicating weaker homotypic cell-cell adhesions (*lower panel*). Addition of EGTA, a calcium-chelator, was used as a control to show that colonies formed were calcium-dependent or cadherin-based cell-cell adhesion (*inset*).

5.4.2 Heterotypic cell-cell adhesions

To more directly test the functionality of γ-catenin at cell-cell adhesions after β-catenin KD, we next employed a centrifugal assay for cell adhesion (CAFCA) to compare the overall strength of cell-cell adhesions after sKD and dKD using Hep3B cells as described in **7.12.2**. siRNA-treated Hep3B cells were plated on an untreated monolayer of Hep3Bs and subjected to a centrifugal force of 375g. At this force we observed a significant decrease in cell-cell adhesion as measured by an increase in the percentage of cells lost, only with the dKD of β- and γ-catenin, when compared to control or sKD (**Figure 21C**). Therefore, dKD, but not sKD of β- and γ-catenin, negatively impacts cell-cell adhesions.

5.4.3 Homotypic cell-cell adhesions

To further validate cadherin-mediated cell-cell adhesions specifically (as in [113]) we used a hanging-drop assay, where transfected cells were cultured in suspension (see **7.12.3** for details). After 24 hours in suspension, all the treatments formed colonies of relatively similar size; however, the dKD colonies appeared smaller and more asymmetric than others (**Figure 21D**,

upper panel). To confirm that we were measuring calcium-dependent or cadherin-mediated adhesion, the single-cell suspension of Hep3B cells was also cultured in the presence of EGTA, a calcium-chelator. This led to the absence of any colony formation after 24 hours, indicating that indeed these colonies required calcium to make new cell-cell adhesions (**Figure 21D**; *inset*). When the colonies of hanging-drop cultures were triturated, only the dKD conditions dissociated readily to a single cell suspension, indicating weaker homotypic intercellular adhesions (**Figure 21D**, *lower panel*). From this data it is evident that dKD of β- and γ-catenin in Hep3B cells negatively affects cell-cell adhesion. Taken together with **5.4.2**, the data supports maintenance of AJ by γ-catenin upon β-catenin loss and that concomitant decrease of β- and γ-catenin will be detrimental to intercellular adhesion and effectiveness of AJ.

5.5 DISCUSSION: BIOLOGICAL IMPLICATIONS IN HCC

The first part of our study demonstrated that decreasing β-catenin levels blocks canonical Wnt signaling, which is the major contributor towards HCC cell proliferation and survival. While β-catenin sKD increases γ-catenin, this increase is unable to compensate for β-catenin signaling as a part of the canonical signaling pathway as shown by TOPflash reporter activity. Similarly, despite γ-catenin increase in βKO livers, several liver-specific targets of β-catenin such as *GS*, cytochrome P450s *CYP2E1* and *CYP1A2* continued to be significantly downregulated, again affirming no role of γ-catenin in compensating as a component of Wnt signaling in a normal liver [34].

Next we showed that adhesion of Hep3B cells to an untreated monolayer of Hep3B cells via CAFCA was decreased upon dKD of β- and γ-catenin. This result was not profound, but significant. We can ascribe the modest changes in the CAFCA results to junctional cross-talk and compensation, which we have previously observed in the *in vivo* KO of β-catenin [41, 91], and have also been seen in the *in vivo* models of γ-catenin knockdown/knockout as well [189, 190]. There are known nodes of cross-talk between AJ and desmosomes [70], AJ and tight junctions [69], along with AJ and gap junctions [71] (see section **1.5**). We propose that these important redundancies built into the overall physiology of epithelial cells help enable maintenance of cell-cell adhesions with any therapeutic treatment that would affect a junctional protein such as β-catenin.

In order to tease out the exact roles of β- and γ-catenin on desmosomes and AJ, we used more specific assays for isolating cadherin-mediated adhesions. The hanging-drop assay confirmed that cadherin-mediated adhesion is greatly decreased with the dKD of β- and γ-catenins, but not with either sKD. This indicates that at least one of these catenins is necessary for the proper maintenance of cadherin-mediated junctions, corroborating the data put forth by previously [113]. This data may also be influenced by the fact that we see an overall decrease in E-cadherin levels in our dKD Hep3Bs, which would reduce the pool of E-cadherin available for dimerization and assembly of AJ and would most likely reduce the overall number of AJ as well (**Figure 21B**). The decrease in E-cadherin levels may be a result of the lack of catenins available to traffic E-cadherin to the membrane. β-Catenin is known to interact with E-cadherin in a way that masks the PEST domain of E-cadherin, which is necessary for E-cadherin's recognition by the proteasome [166]. Thus, based on the similarities of β- and γ-catenin's interactions with E-cadherin [111], we assume that γ-catenin may also play a role in E-cadherin stabilization by

masking its PEST domain, at least *in vitro*, and after acute β-catenin KD. Indeed, various studies have shown that the interactions of β-catenin and γ-catenin with similar regions on E-cadherin are indistinguishable [111]. Thus, at least one of the two catenins is necessary for the maintenance of cadherin-mediated junctions and for steady state expression of E-cadherin.

The data presented in this chapter supports the conclusion that γ-catenin increase secondary to β-catenin sKD does not worsen HCC cell phenotype by promoting tumor cell proliferation, migration or Wnt activation. Others have published studies indicating that an increase in γ-catenin leads to enhancement of metastasis through genomic instability and migration [135]. However, we accredit the lack of detrimental phenotypes seen with γ-catenin increase with sKD of β-catenin to a strictly compensatory function of γ-catenin *in lieu* of β-catenin at the AJ. Thus, γ-catenin appears to have a protective effect when β-catenin levels are decreased. However, on its own γ-catenin appears to have rather mixed effects. While on one hand sKD of γ-catenin did not negatively impact cell-cell adhesions or migration, we did observe an increase in proliferation preceded by an increase in β-catenin signaling. Therefore, in our studies we observed that γ-catenin does not explicitly act as an oncogene or tumor suppressor, but that its role in HCC cells may be closely linked to that of β-catenin or the intact AJs, although additional studies like hepatocyte-specific conditional KO of γ-catenin would be necessary. This was also shown previously in a HCC patient samples, where it was not the overall levels of catenins that determined the prognosis of HCC, but instead progression correlated better with AJ expression as measured by combined expression of catenins and E-cadherin [158]. Specifically, the authors observed poor prognosis and survival rate in HCC patients exhibiting decrease in both γ-catenin and E-cadherin and not a decrease in γ-catenin

alone [158]. Our data thus suggests that only combined inhibition of β-catenin and γ-catenin leads to more migration of cells and perhaps may induce local invasion and metastasis. We assume that the AJs are also greatly affected in this cases as indicated by decrease in cell-cell adhesions. Therefore, anti-β-catenin therapies for HCC appear to be feasible, as long as γ-catenin is spared.

6.0 γ-CATENIN AT ADHERENS JUNCTIONS AFTER β-CATENIN LOSS: MECHANISMS IN HEPATOCELLULAR CARCINOMA

We have previously demonstrated in βKO mice that the increase in γ-catenin protein levels is not regulated at the mRNA level, and that γ-catenin stabilization is most likely a result of increased serine/threonine phosphorylation of cytoplasmic γ-catenin (**Chapter 2**). Here further elucidate the mechanism of γ-catenin stabilization via serine/threonine phosphorylation, identifying protein kinases A (PKA) and PKG as the most likely candidates for these phosphorylation events. This allows for β-catenin-lowering agents as a viable option for treatment of HCC, provided γ-catenin stabilization mechanisms are spared.

6.1 NO CHANGE IN *JUP* TRANSCRIPTION WITH β-CATENIN KNOCKDOWN

We showed in **Chapter 5** that there is no change in human *JUP* mRNA levels in an *in vitro* model of acute β-catenin loss in HCC cells, and that the stabilization of γ-catenin in the context of β-catenin loss must be regulated at a post-transcriptional level, as in the *in vivo* mouse model of chronic β-catenin loss (βKO).

6.2 SERINE/THREONINE PHOSPHORYLATION STABILIZES γ-CATENIN AFTER β-CATENIN KNOCKDOWN

Based on *in vivo* observations that γ-catenin increase with β-catenin loss in βKO livers was associated with increased serine and threonine phosphorylation of γ-catenin, we asked whether such posttranslational modification could in fact stabilize γ-catenin *in vitro.* As described in section **7.4.3**, we treated Hep3B cells with Okadaic acid (OA), a well-known inhibitor of serine/threonine phosphatases [191, 192]. Treatment of Hep3B cells for 3 hours with OA led to an increase in total γ-catenin protein levels further supporting the role of serine/threonine phosphorylation in its stabilization (**Figure 22A**).

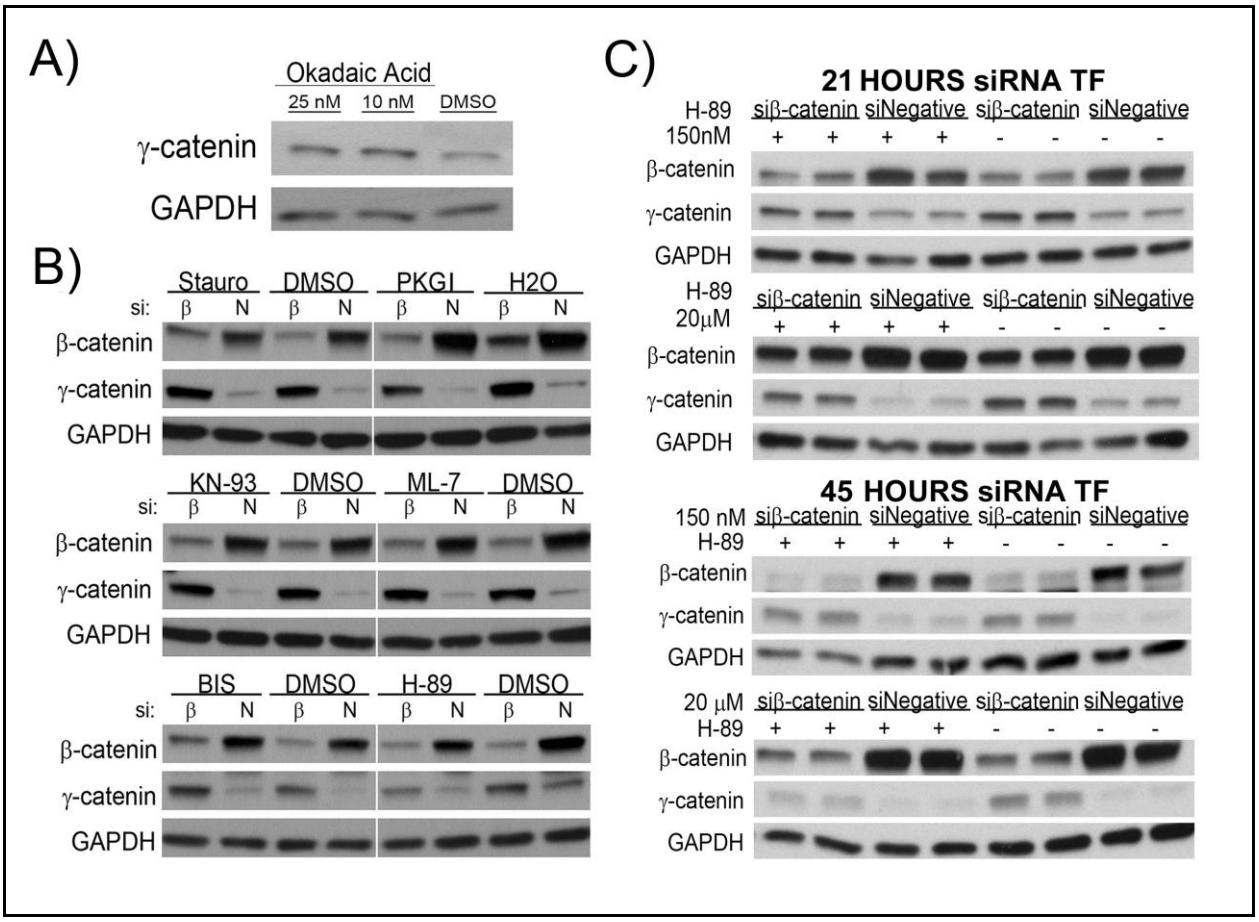

Figure 22. Mechanism of γ-catenin stabilization after sKD of β-catenin. (A) Treatment of Hep3B cells for 3 hours with 10 or 25 nM Okadaic acid (OA), a known serine/threonine phosphatase inhibitor, results in increase in γ-catenin (83 kDa) as shown in a representative WB. WB for GAPDH (37 kDa) verifies comparable loading. **(B)** Treatment of Hep3B cells with various serine/threonine kinase inhibitors for 3 hours after 45 hours of transfection with control or β-catenin siRNA reveals a decrease in stabilization of γ-catenin after PKG inhibitor (PKGI) and H-89 (PKA inhibitor) only in representative WBs. **(C)** As shown in a representative WB, treatment of Hep3B cells with H-89 at 150 nM and 20 μM for 3 hours after 21 or 45 hours of transfection with siβ-catenin or siNegative reveals a dose-and time-dependent decrease in extent of γ-catenin stabilization.

6.3 PKA/PKG REGULATE γ-CATENIN PHOSPHORYLATION AND STABILIZATION

To address which specific serine/threonine kinases may be responsible for γ-catenin stabilization in Hep3B cells after β-catenin sKD, we utilized six different inhibitors with a broad range of activity. As described in section **7.4.3**, at around 45 hours after transfection of Hep3B cells with β-catenin or control siRNA, the cells were treated with the inhibitors: H-89 (inhibits PKA, CaM Kinase II, PKC and Casein Kinase I); PKG Inhibitor (PKGI; inhibits PKG and PKA); Bisindolylmaleimide I (PKC inhibitor); KN-93 (CaM Kinase II inhibitor only); ML-7 (inhibits Myosin Light Chain Kinase) and Staurosporine (broad range). We compared the extent of increase of γ-catenin upon β-catenin sKD following treatment with these inhibitors. A notable decrease in γ-catenin was evident only after Hep3B cells were treated with PKG or PKA inhibitor, while γ-catenin levels continued to increase in the presence of all other inhibitors (**Figure 22B**). The impact of PKA inhibition was more robust than PKG and since the PKGI was utilized at higher dose (200 μM) that also inhibits PKA. Therefore, we concluded an important role of PKA in γ-catenin stabilization.

We further studied response to 3 hours of H-89 at low (150 nM) and high (20 μM) concentration on γ-catenin expression at 21 and 45 hours after β-catenin or control siRNA transfection sKD. Both doses are within the range of specifically inhibiting PKA since concentration of only 30 μM or more impacts other serine/threonine kinases such as CaM Kinase II, Myosin Light Chain Kinase and Casein Kinase I [193]. While a modest decrease in extent of γ-catenin increase upon siRNA-mediated β-catenin was observed in Hep3B cells at 20 hours at

both doses of H-89, maximal inhibition was evident at 48 hours and at 20 μM H-89 concentration thus reinforcing the role of PKA in γ-catenin stabilization process (**Figure 22C**).

6.4 DISCUSSION: MECHANISMS IN HCC

This part of our study addressed the molecular basis of γ-catenin increase following β-catenin loss. In our previous work, γ-catenin increase in β-catenin βKO mice was not due to transcriptional regulation, but we identified enhanced serine/threonine and not tyrosine phosphorylation of γ-catenin [91]. This mechanism was also verified *in vitro* when OA, a known serine/threonine phosphatase inhibitor induced γ-catenin levels in Hep3B cells [191, 192, 194, 195]. γ-Catenin is indeed known to undergo serine/threonine phosphorylation [115]. Since cytoplasmic but not insoluble-membrane associated pool of γ-catenin showed serine and threonine phosphorylation, it suggests that phosphorylation may prevent γ-catenin degradation during cytoplasmic trafficking (**Figure 9B**), although this will need to be further investigated. In our studies, based on the overlap of activities of various kinase inhibitors used and observed dose-dependent response, it appears that PKA is the candidate serine/threonine kinase responsible for phosphorylating γ-catenin to induce its stabilization in the absence of β-catenin. Additional studies to identify the specific effector residues on γ-catenin are ongoing.

Preclinical β-catenin inhibitors being investigated can be broadly categorized into two classes: 1) that block the nuclear β-catenin without impacting the total levels of β-catenin (such as ICG-001 and Pegylated Interferon-α2a) [12, 13], and 2) the inhibitors that suppress β-catenin gene and/or protein expression such as ASO or other indirectly acting agents [11, 59, 196-198].

Results from ASO, siRNA and ICG-001 studies indicate that it is the absolute levels of β-catenin and not a decrease in β-catenin's transactivational activity (**Figure 18D-E**), which provides an impetus for γ-catenin stabilization. Additionally, the compensation by γ-catenin is at the AJ only and the functions of β-catenin as a part of the Wnt pathway remain unfulfilled by γ-catenin (**Figure 20A**). This suggests that there may be a catenin-sensing mechanism in a cell, which monitors the levels of catenins especially at junctions and allows for compensation for these proteins by others via relatively prompt posttranslational modifications. We propose that PKA may be such as sensor, which stabilizes γ-catenin and is also known to phosphorylate β-catenin at Ser552 and Ser675 to induce its activation [20]. Whether PKA normally phosphorylates membrane bound β-catenin is not known, although the two effector serine/threonine sites are in close proximity to Tyr654 and Tyr670, which are the sites phosphorylated by receptor tyrosine kinases known to deregulate E-cadherin/β-catenin complex at AJ [23, 199]. We therefore propose that when β-catenin levels diminish, PKA is unable to activate β-catenin, which now switches substrates to phosphorylate γ-catenin instead and this induces γ-catenin stabilization to at least fulfill the role of β-catenin at the AJ. While this hypothesis needs further experimental proof, it does demonstrates the feasibility of β-catenin-decreasing agents as therapies for HCC, provided γ-catenin stabilization factors such as PKA are spared for adequate compensation at junctions to prevent untoward effects on tumor cell migration, invasion and metastasis.

7.0 MATERIALS AND METHODS

7.1 ANIMALS

7.1.1 βKO mice

Homozygous floxed β-catenin mice (*Ctnnb1*$^{lox/lox}$; C57BL/6 strain) and Albumin-Cre (*Alb-Cre*; C57BL/6 strain) transgenic mice from Jackson Laboratories (Bar Harbor, ME) were strategically bred as previously described [34]. Briefly, mice with homozygous floxed β-catenin gene were bred to Albumin-Cre mice. The offspring with both 1) a floxed β-catenin allele (and wild-type allele) and 2) Albumin-Cre allele were then bred to homozygous floxed β-catenin mice to delete the floxed regions of the β-catenin gene (exons 1-6). Mice with genotypes *Ctnnb1*$^{lox/lox}$*;Alb-Cre*$^{+/-}$ are referred to as knockouts (βKO). Age- and sex-matched littermates with other genotypes were used as wild-type controls (WT): *Ctnnb1*$^{loxp/loxp}$*;Alb-Cre*$^{-/-}$ or *Ctnnb1*$^{loxp/wt}$*;Alb-Cre*$^{+/-}$ or *Ctnnb1*$^{loxp/wt}$*;Alb-Cre*$^{-/-}$. Livers from age- and sex-matched 60–120 day-old βKO and WT mice were used, unless otherwise noted. At least n < 3 βKO and WT animals were used for each analysis to account for animal-to-animal variations. Animals were harvested by CO_2 asphyxiation. All animal studies were approved by University of Pittsburgh IACUC office.

7.1.2 Sample preparation

Mice under isoflurane inhalation anesthesia were killed by cervical dislocation. Animals were harvested by CO_2 asphyxiation. The livers were extracted, washed in PBS, and then the tissue was processed in three ways: 1) embedded in paraffin blocks (after 10% formalin fixation), 2) stored in Tissue-Tek OCT (Sakura Finetek, Dublin OH) at -80^{o}C, and/or 3) flash frozen in liquid nitrogen and stored at -80^{o}C until use. All animal studies were approved by University of Pittsburgh IACUC office.

7.2 SURGERIES AND DIETS

7.2.1 Partial hepatectomy

Surgical removal of 2/3 of the mouse liver was used to induce liver regeneration. Surgeries were performed as previously described [200]. Briefly, three of the five lobes were individually tied and then surgically resected before suturing up the animal. The regenerating livers were harvest at 40 and 72 hours.

7.2.2 DDC diet

Mice were fed a special chow containing 0.1% 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) (Bioserve, Frenchtown, NJ) for 3 to 150 days to induce atypical ductular proliferation as previously described [201].

7.3 CELL LINES

Hep3B and HepG2 human HCC cell lines were obtained from the American Type Culture Collection (Manassas, VA). Hep3B cells express a wild-type β-catenin species, while the HepG2s have an exon-3 deleted/constitutively active form of β-catenin [202]. Cells were cultured in Eagle's minimal essential medium (EMEM) supplemented with 10% vol/vol fetal bovine serum (FBS) at 37^{o}C in a humidified 5% carbon dioxide atmosphere, unless otherwise noted. Cells were passaged using 0.25% Trypsin-EDTA.

7.4 *IN VITRO* TREATMENTS

7.4.1 Small interfering RNAs

Human γ-catenin (*JUP*), pre-validated β-catenin (*CTNNB1*), and scrambled Negative Control 2 Silencer Select small interfering RNAs (siRNA) were purchased from Ambion (Grand Island, NY). For transient transfections using siRNA, the cells were initially plated in 6-well plates and grown to 60% - 80% confluence, followed by serum starvation 4-16 hours in EMEM without FBS. The cells were transfected using Lipofectamine-2000 (Life Technologies, Grand Island, NY) and a total siRNA concentration of 25 to 75 nM (**Table 2**) in OPTI-MEM I Media (Life Technologies, Grand Island, NY) for 24 to 72 hours as per the manufacturer's instructions.

Table 2. siRNA concentrations for single and double knockdown of β- and/or γ-catenin.

TREATMENT	siRNA: *CTNNB1*	siRNA: *JUP*	siRNA (Negative2)
siβ-catenin (sKD)	50 nM	-	-
siγ-catenin (sKD)	-	25 nM	-
siNegative (sKD)	-	-	25-50nM
siβ-catenin (dKD)	50 nM	-	25 nM
siγ-catenin (dKD)	25 nM	-	50 nM
siβ+γ-catenin (dKD)	50 nM	25 nM	-
siNegative (dKD)	-	-	75 nM

7.4.2 β-Catenin/Wnt inhibitors

Small molecule inhibitor of β-catenin's nuclear activity, ICG-001 (MW = 548 g/mol, IC_{50} = 3 μM) [13], was used for cell treatments at 10 μM. ICG-001 selectively disrupts the interaction of β-catenin with its nuclear coactivator CBP which disrupts TCF-driven gene transcription [13]. Additionally, *CTNNB1* (ISIS102708) or control antisense oligonucleotides (ASO; ISIS Pharmaceuticals Inc., Carlsbad, CA) were used at a concentration of 50 nM as previously published [42]. For transient transfection using ASOs, the cells were transfected in a similar way to the siRNAs (**7.4.1**) using Lipofectamine-2000 (Life Technologies, Grand Island, NY) in OPTI-MEM I Media (Life Technologies, Grand Island, NY) for 24 to 72 hours as per the manufacturer's instructions.

7.4.3 Phosphatase/kinase inhibitors

Cells were treated with the phosphatase inhibitor Okadaic Acid (OA) (Santa Cruz Biotechnology, Santa Cruz, CA) at 25 nM and 10 nM as previously published for HCC cells [195] for 3 hours, along with DMSO controls ($[DMSO]_f$ < 2%). Serine/Threonine kinase inhibitors, purchased from Calbiochem (Billerica, MA), were administered for 3 hours, with relevant DMSO controls, at the following concentrations: H-89 at 150 nM and 20 μM [203]; PKG Inhibitor (RKRARKE) at 200 μM [194, 204]; Bisindolylmaleimide I at 1 μM [205]; KN-93 at 5 μM [206]; ML-7 at 30 μM [207]; and Staurosporine at 1 μM [208]. Kinase targets and selectivity for these inhibitors can be found in **Table 3** (adapted from Adapted from Calbiochem "Serine/Threonine Kinase Inhibitor Set" data sheet – Product 539572).

Table 3. Phosphatase inhibitors and serine/threonine kinase inhibitors used *in vitro*.

PHOSPHATASE INHIBITOR	Target Phosphatase	Molecular Weight	Selectivity
Okadaic Acid	PP1 & PP2A	805.0 g/mol	PP2A: K_i = 1 nM; PP1 IC_{50} = 10-15 nM
KINASE INHIBITOR	**Target Kinase**	**Molecular Weight**	**Selectivity**
H-89, Dihydrochloride	PKA	519.2 g/mol	PKA: K_i = 50 nM; MLCK: K_i = 30 µM; CaM Kinase II: K_i = 30 µM; PKC: K_i = 30 µM; CK I: K_i = 40 µM
PKG Inhibitor (RKRARKE)	PKG	943.1 g/mol	PKG: K_i = 86 µM; PKA: K_i = 550 µM
Bisindolylmaleimide I	PKC	412.5 g/mol	PKC_α: IC_{50} = 8.4 nM; PKC_β: IC_{50} = 18 nM; PKC_δ: IC_{50} = 210 nM; PKC_ε: IC_{50} = 132 nM; PKA: K_i = 2 µM
KN-93	CaM Kinase II	501.1 g/mol	CAM Kinase II: K_i = 370 nM
ML-7	MLCK	452.7 g/mol	MLCK: K_i = 300nM; PKA: K_i = 21 µM; PKC: K_i = 42 µM
Staurosporine	Broad Range	466.5 g/mol	PKA: IC_{50} = 15 nM; PKC: IC_{50} = 0.7 – 7 nM; PKG: IC_{50} = 8.5 nM; MLCK: IC_{50} = 1.3 nM; CaM Kinase: IC_{50} = 20 nM

7.4.4 TOPflash reporter plasmid

The reporter construct TOPflash (Upstate, Lake Placid, New York) has three copies of TCF/LEF β-catenin binding sites upstream of a thymidine kinase (TK) promoter and the firefly luciferase (**Figure 23**). For transient transfections using TOPflash, the cells were transfected in a similar way to the siRNAs (**7.4.1**) using Lipofectamine-2000 (Life Technologies, Grand Island, NY) in OPTI-MEM I Media (Life Technologies, Grand Island, NY) at a total concentration of 400 ng for 24 to 48 hours as per the manufacturer's instructions. Cells were also co-transfected with 100 ng Renilla Luciferase plasmid to control for transfection efficiency.

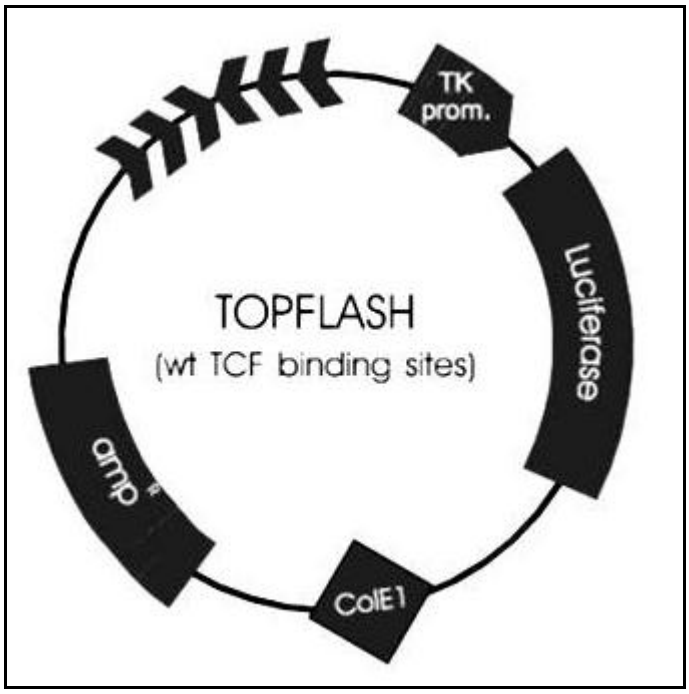

Figure 23. Schematic of TOPflash vector used for Wnt Signaling studies.

7.5 RNA EXTRACTION

RNA was extracted from tissue or cells using TRIZOL (Life Technologies, Grand Island, NY) as per the manufacturer's instructions. Briefly, 1 mL TRIZOL reagent was used per sample/extraction. RNase-free chloroform was added at a volume equal to 20% of the TRIZOL volume and shaken vigorously. Samples were centrifuged at 12000 rpm and 4^{o}C to facilitate a phase separation of the RNA. The top, colorless, aqueous, RNA-containing phase was used to continue the extraction. RNase-free isopropyl alcohol was added at 150% the volume of chloroform and incubated at 4^{o}C to precipitate the RNA. The samples were centrifuged at 12000 rpm and 4^{o}C, and the RNA pellet was washed with 75% EtOH (RNase-free) before resuspension in DPEC-treated or RNase-free water.

7.6 mRNA EXPRESSION STUDIES

7.6.1 Sample preparation

RNA was extracted as in **7.5** and subsequently DNAse-treated to remove potential genomic DNA contamination using the TURBO DNAse Kit (Ambion, Grand Island, NY) as per the manufacturer's instructions.

7.6.2 Real-time PCR

DNase-treated RNA was used for real-time PCR analysis with the SYBR-Green Master Mix (Ambion, Grand Island, NY) as described elsewhere [41]. Briefly, the OD of the DNase-treated RNA was taken and equal μg amounts of RNA from each sample were used to make individual cDNA samples using SuperScript III First–Strand Synthesis System for RT-PCR with an RNase H treatment (Life Technologies, Grand Island, NY). A total of 0.1 μg cDNA along with 1x Power SYBR-Green PCR Master Mix (Applied Biosystems, Grand Island, NY) and the appropriate primers were used for each real-time PCR reaction (see **Table 4**; references: Zhu *et al.*, 2008 [209]; Jackson Laboratories [210]; Primer-BLAST [211]; Cicinnati *et al.*, 2008[212]). The Applied Biosystems StepOnePlus Real-Time PCR System was used for the analysis of the transcripts with the StepOne v2.1 software. Comparative ΔΔCT was used for analysis of the data, and calculations were made without the StepOne software (Applied Biosystems, Grand Island, NY). Primer efficiencies were performed for each primer, and only similar efficiencies were used for analysis. Comparative ΔΔCT from triplicate animals/experiments were compared for statistical significance by Student's t-test.

Table 4. Real-time PCR primers.

TARGET GENE	Species	Forward Primer	Reverse Primer	Source
CTNNB1	Human	5'- GAA ACG GCT TTC AGT TGA GC -3'	5'- CTG GCC ATA TCC ACC AGA GT -3'	Zhu *et al*., 2008
Cre-recombinase	-	5'- GCG GTC TGG CAG TAA AAA CTA TC - 3'	5'- GTG AAA CAG CAT TGT TGT CAC TT-3'	Jackson Laboratories (Bar Harbor, ME)
Ppia	Mouse	5'- CCC CAC CGT GTT CTT CGA CA -3'	5'- TCC AGT GCT CAG AGC TCG AAA -3'	Primer-BLAST (NIH)
GAPDH	Human	5'-TGC ACC ACC AAC TGC TTA GC -3'	5'- GGC ATG GAC TGT GGT CAT GAG -3'	Cicinnati *et al*., 2008
Gapdh	Mouse	5'- ACC CAG AAG ACT GTG GAT GG -3'	5'- CAC ATT GGG GGT AGG AAC AC -3'	Primer-BLAST (NIH)
JUP	Human	5'-AAG GTG CTA TCC GTG TGT CC -3'	5'- GAC GTT GAC GTC ATC CAC AC -3'	Zhu *et al*., 2008
Jup	Mouse	5'- ACG CCA TTG ATG CGG AGG GC -3'	5'- CCC AGG CAG CTG GGT CAT GC -3'	Primer-BLAST (NIH)
Alad	Mouse	5'- ATG TCC GGT AAC GGC GGC -3'	5'- CAA GGC TTT CAG CAT CGC CAC CA -3'	Primer-BLAST (NIH)

7.6.3 Microarray

Livers from three βKO and WT mice were used for Affymetrix (Santa Clara, CA) gene array analysis as previously published [34]. The signals from βKO and WT livers were compared and presented as fold-change.

7.7 PROTEIN EXTRACTION

7.7.1 Whole-cell lysates

Whole-cell lysates were obtained using RIPA buffer (1% Igepal CA-630, 0.5% Sodium Deoxycholate, 0.1% SDS in PBS) containing fresh protease and phosphatase inhibitor cocktail

(Sigma Aldrich, St. Louis, MO) as described previously [34]. BCA protein assays (Pierce, Rockford, IL) were performed to ensure equal protein concentrations for all subsequent experiments.

7.7.2 Cytoskeletal-associated lysates

Cytoskeletal-associated proteins were extracted from both WT and βKO livers as previously described by Hinck *et al*. [166], with additional modifications from Hugh *et al*. [213], and also by our lab [91]. The samples were homogenized in 200 μl *Lysis Buffer A* (1% Triton X-100, 50 mM Tris-HCl pH 7.5, 1 mM EGTA, 5 mM EDTA, 140 mM NaCl, 10% glycerol, 1 mM $MgCl_2$) and fresh protease and phosphatase inhibitor cocktail (Sigma Aldrich, St. Louis, MO) and incubated on ice for fifteen minutes. The samples were homogenized once more and incubated for another fifteen minutes on ice. Next, the samples were centrifuged for twenty minutes at 3000 rpm at 4°C. The supernatant (Triton-soluble or unbound cytosolic fraction; "S"-fraction) was separated from the pellet. The residual pellet was extracted in 200 μl *Lysis Buffer B* (1% SDS, 50mM Tris-HCl pH 7.5, 1 mM EGTA, 5 mM EDTA) and fresh protease and phosphatase inhibitor cocktail (Sigma Aldrich, St. Louis, MO) by homogenizing pellet and then incubating samples at 100^{o}C for ten minutes. The samples were diluted in 500 μl *Lysis Buffer A* and centrifuged for twenty minutes at 3000 rpm at 4°C. This last supernatant represents the Triton-insoluble or cytoskeletal-associated fraction ("I"-fraction). BCA protein assays (Pierce, Rockford, IL) were performed to ensure equal protein concentrations for all subsequent experiments.

7.7.3 Nuclear lysates

Nuclear extracts were performed from fresh liver tissue using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Waltham, MA) as per the manufacturer's instructions. Briefly, 50 – 100 mg of tissue was homogenized in CER I reagent, vortexed, and incubated on ice for ten minutes before ice cold CERII, at a volume equal to 5.5% of the CER I reagent, was added to the samples, vortexed, and centrifuged for five minutes at 13000 rpm. The supernatant was saved as a "cytoplasmic extract," and the residual pellet was extracted with the NER reagent by incubating on ice for forty minutes and vortexing occasionally. The NER was spun down at 13000 rpm for ten minutes, and the supernatant was used as the "nuclear extract." BCA protein assays (Pierce, Rockford, IL) were performed to ensure equal protein concentrations for all subsequent experiments.

7.8 PROTEIN EXPRESSION STUDIES

7.8.1 Western blot

A total of 10–50 μg of protein were resolved on Tris–HCl precast gels (Bio-Rad Laboratories, Hercules, CA) by SDS-PAGE analysis using the mini-PROTEIN 3-electrophoresis module assembly (Bio-Rad Laboratories, Hercules, CA). The resolved proteins were transferred to polyvinylidene difluoride (PVDF) membranes and signal detected by Super-Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). Horseradish-peroxidase-conjugated secondary antibodies were purchased from Millipore (Billerica, MA). See **Appendix A** for

complete list of antibodies and dilutions used for western blots (WB). Image J software (National Institutes of Health, Bethesda, MD) was used for densitometry analysis of relative protein levels and levels were normalized to GAPDH or β-actin reference proteins. Arbitrary units from triplicate animals/experiments were compared for statistical significance by Student's t-test.

7.8.2 Immunoprecipitation

Immunoprecipitation (IP) studies were performed with 500 μg of cell lysates. All sample lysates were pre-cleared by adding 0.25 μg control IgG (same as primary antibody species) and A/G-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA). A total of 0.2–2.0 μg of primary antibody was added to pre-cleared cell lysates for 1 hour followed by addition of 20 μl of A/G agarose beads. Alternatively, 10 μl β-catenin-pre-conjugated A/G agarose beads (sc-1496 AC; Santa Cruz Biotechnology, Santa Cruz, CA) were added to the lysate after pre-clearing. Samples were rotated at 4^{o}C overnight. The proteins were eluted from the beads by adding 2x laemmli buffer and boiling samples. Equal volumes were run on SDS-PAGE gels and immunoblotted as described in **7.8.1**. The comparable input between various groups was verified by WBs against the protein used for pull downs. See **Appendix A** for complete list of antibodies and dilutions used for IPs.

7.8.3 Immunohistochemistry

Immunohistochemical stains were performed as previously described [34]. Briefly, tissue samples were embedded in paraffin and cut into 4μ sections. The slides were deparaffinized with xylene and graded alcohol washes and rinsed in PBS. The tissue sections were microwaved twice

in citrate buffer for twelve minutes total, and then endogenous peroxidases were quenched with 3% hydrogen peroxide. Slides were blocked with Super Block (UltraTek, West Logan, UT) for ten minutes before adding primary antibody diluted in PBS for 1 hour at room temperature. After PBS washes, horseradish-peroxidase-conjugated secondary antibodies (Millipore, Billerica, MA) were added to the slides for 1 hour at room temperature. The secondary antibody signal was detected with DAB (Vector Labs, Southfield, MI), and the signal was quenched with dH_2O before counterstaining with Harris hematoxylin solution (Sigma Aldrich, St. Louis, MO). The slides were re-run through the xylene and graded alcohol washes before mounting the cover slips with DPX (Fluka Labs, St. Louis, MO). Negative controls were generated as above, but without the primary antibody incubation. IHC images were taken on an Axioskop 40 (Zeiss, Thornwood, NY) inverted brightfield microscope. See **Appendix A** for complete list of antibodies and dilutions used for immunohistochemistry (IHC).

7.8.4 Immunofluorescence

Analysis was performed on OCT embedded tissue which was sectioned at 4 – 6μ on a cryostat set at -20^{o}C. The samples were fixed by various methods (See **Appendix A** for antibody-specific fixation methods). The OCT was removes from the tissue sections with three PBS at room temperature. The tissue was then permeablized by various methods (See **Appendix A** for antibody-specific permeablization methods) for ten minutes at room temperature, and washed again three times with PBS. The tissue was then blocked with 2% BSA/PBS for forty-five minutes, and washed five times with PBB (PBS + 0.5% BSA). Primary antibodies diluted in PBB were applied either for 1 hour at room temperature, or overnight at 4^{o}C. After the primary antibody, the sections were washed five times in PBB. The secondary antibody was applied in a

covered chamber for forty minutes at room temperature. For these studies, the following secondary antibodies were used: donkey anti-goat Alexa-488; donkey anti-rabbit Alexa-555; and Cy5 Phalloidin counterstain for actin (Life Technologies, Grand Island, NY). After the secondary antibody incubation, the sections were washed five times with PBB and five times with PBS before applying Hoechst 33258 (Sigma Aldrich, St. Louis, MO) for two minutes at room temperature. The sections were washed three more times with PBS and coverslips were mounted with gelvatol (21 g PVA, 42 mL glycerol, 52 mL dH_20, 106 mL Tris [0.2M, pH=8.5], and a few crystals of sodium azide). Negative controls were generated as above, but without the primary antibody incubation. All images were taken on the Olympus Fluoview 500 or 1000 at the University of Pittsburgh Center for Biological Imaging (CBI). All images shown were taken using a UPLAPO 20x O (NA = 0.80) on the Olympus 500 and a PLAPON 20x O (NA = 0.80) on the Olympus 1000. See **Appendix A** for complete list of antibodies and dilutions used for immunofluorescence (IF).

7.9 ULTRASTRUCTURE STUDIES

7.9.1 Transmission electron microscopy

Liver tissue was fixed with 2.5% glutaraldehyde in PBS by perfusion via the inferior vena cava. The tissue was then cut into 1 mm^3 blocks and washed three times for fifteen minutes in PBS at room temperature. The tissue sample was post fixed in 1% osmium tetroxide (OsO_4) containing 1% potassium ferricyanide ($K_3Fe(CN)_6$) for 1 hour at room temperature. The tissue was washed three times for fifteen minutes in PBS at room temperature and then dehydrated in a series of

alcohol/PBS washes for fifteen minutes each: 30% EtOH, 50% EtOH, 70% EtOH, 90% EtOH, and three times in 100% EtOH. The samples were further dehydrated with two total ten minute incubations in propylene oxide, and then infiltrated for one hour in a 1:1 propylene oxide:Polybed 812 epoxy resin (Polysciences, Warrington, PA). Next, the tissue was infiltrated with pure epon four times for 1 hour each. The tissue was then embedded in pure epon at 37^{o}C for 24 hours and subsequently cured at 60^{o}C for 48 hours. The tissue was sectioned into ultrathin (60 nm) sections and collected on 200 mesh copper grids. Grids were hand stained with 2% uranyl acetate in 50% methanol for ten minutes and 1% lead citrate for seven minutes. Digital transmission electron microscopy (TEM) images were taken with JEOL JEM-1011 Transmission Electron Microscope (Peabody, MA) at 80.0 kV. Quantitative analysis of junctions was done with MetaMorph Software (Molecular Devices, Sunnyvale, CA) at the CBI. Average distances from n > 3 measurements at the same magnification were compared for statistical significance by Student's t-test.

7.10 PROLIFERATION STUDIES

7.10.1 Ki-67 staining

Ki-67 staining was performed similarly to the methods listed in the immunohistochemistry section (**7.8.3**) using a Ki-67 antibody (Dako, Carpinteria, CA).

7.10.2 [3H]Thymidine incorporation

DNA synthesis was measured to assess proliferation of cells as previously described [170]. Briefly, 24 hours after siRNA transfection in Hep3B cells [3H]thymidine (2.5 mCi/ml) was added to culture media. The cells were cultured for [3H]thymidine incorporation for an additional 24 to 48 hours. The cells were then washed with 5% ice-cold TCA and lysed with 0.33 M NaOH. Aliquots diluted in scintillation fluid were used to determine [3H]thymidine incorporation on a Beckman Scintillation counter (Brea, CA). Average counts per minute (CPM) from triplicate experiments were compared for statistical significance by Student's t-test.

7.11 WNT SIGNALING STUDIES

7.11.1 TOPflash luciferase assay

This reporter system has been successfully used to detect both β- and γ-catenin TCF-dependent Wnt signaling [122, 127]. Luciferase assays were performed using Dual Luciferase Reporter Assay System (Promega, Madison, WI). Average relative light units (RLU) from triplicate experiments were compared for statistical significance by Student's t-test. Constructs used for the study are described in **7.4.4**.

7.12 CELL-CELL ADHESION STUDIES

7.12.1 Scratch-wound assay

Wound-healing assays were performed as previously described [214]. Briefly, cells were grown as a confluent monolayer, transfected for 24 hours, and then a wound was made using a p10 pipette tip. During the assay cells were cultured in 0.5% EMEM to decrease the influence of cell proliferation. Cells were imaged periodically for 24 additional hours until the wounds fronts began to merge. The wounds were imaged at four different locations for each time point using phase-contrast filters at 5x magnification on an Axioskop 40 (Zeiss, Thornwood, NY) inverted brightfield microscope and the cell-free area of the scratches was quantified at each time point using ImageJ software (National Institutes of Health, Bethesda, MD). Percentage of wound closure from triplicate experiments was compared for statistical significance by Student's t-test.

7.12.2 Centrifugal assay for cell adhesion

Centrifugal Assay for cell adhesion (CAFCA) was performed as previously described [214] (See **Figure 24**). Briefly, transfected cells were pre-labeled with CellTracker Green CMFDA (Life Technologies, Grand Island, NY) for fifteen minutes at 37^{o}C, then non-enzymatically dissociated from the monolayers using a 1:200 dilution of 500 mM EGTA and 500 mM EDTA in calcium and magnesium-free HBSS. The cells were resuspended in EMEM supplemented with 10% FBS and replated on an unlabeled confluent monolayer of untransfected cells cultured on collagen-coated plates. After thirty minutes the wells were washed once with PBS to removed cells which did not attach, and fluorescence intensity of labeled cells was quantified using a plate

fluorometer at 495/515 nm. The inverted plate was centrifuged at 375g in air-tight chamber filled with warm culture media for ten minutes. The cells were washed with PBS and the fluorescence intensity of the remaining labeled cells was measured. A value of 100% attachment was assigned to the fluorescent measurements recorded for each well prior to centrifuging the plate, and the experimental data was expressed as a percentage of cells lost versus the initial 495/515 nm reading. Percentage of cells lost from triplicate experiments were compared for statistical significance by Student's t-test.

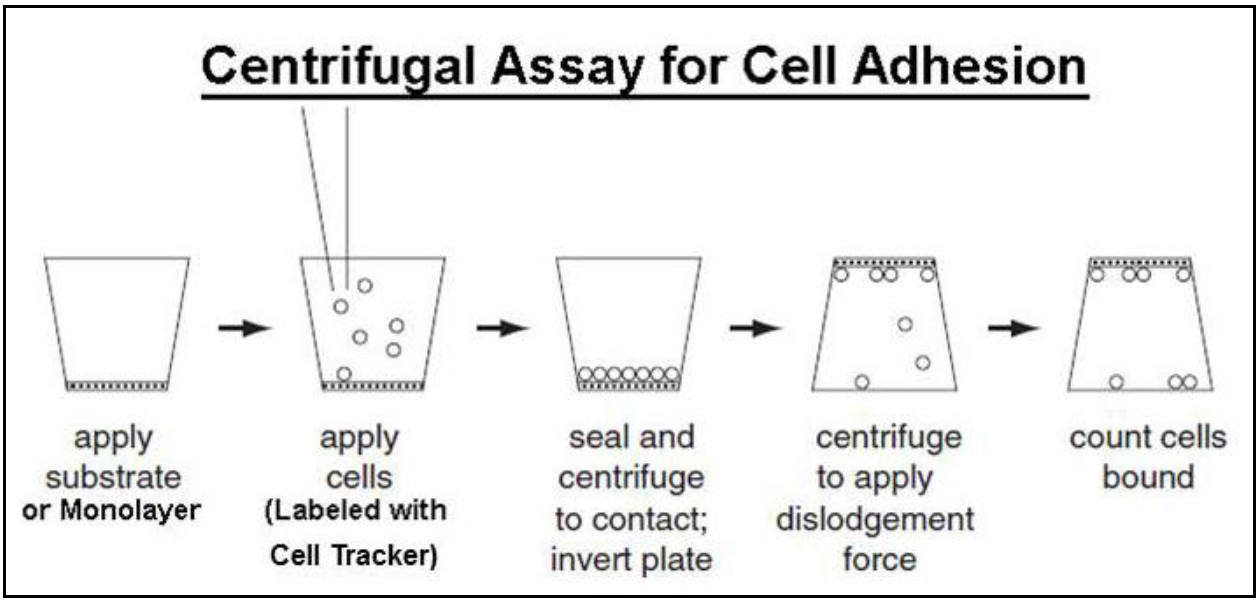

Figure 24. Schematic of Centrifugal Assay for Cell Adhesion (CAFCA). Adapted from McClay & Hertzler, 2001 [215].

7.12.3 Hanging-drop assay

Hanging-drop assay was performed in a similar way as described previously [113]. For this assay treated cells were removed from their monolayer using 0.25% Trypsin-EDTA and counted. The

cells were resuspended in 2% EMEM (to decrease the influence of cell proliferation) at a concentration of 2.5 x 10^5 cells/ml, and 20 μl drops containing 5000 cells/drop were pipetted onto the inner surface of a 10cm^2 dish lid. The bottom of the petri dish was filled with 8 ml PBS to prevent evaporation of the drops. The lid was placed back on the dish so that the drops of cells were hanging from the lid, and the cells were incubated for 20 hours at 37^{o}C in a humidified 5% carbon dioxide atmosphere. Some drops were pipetted up and down twenty times to assess dissociation of cell colonies as a measurement of cadherin-mediated cell adhesion activity. Coverslips were placed over the drops, and images were taken using phase-contrast filters at 5x magnification on an Axioskop 40 (Zeiss, Thornwood, NY) inverted brightfield microscope. Experiments were performed in triplicate.

7.13 QUANTIFICATION AND STATISTICAL ANALYSIS

Student's t-test was performed using Microsoft Excel 2010 (Microsoft, Redmond, WA) to assess statistical significance for a minimum of three different data points per experiment. Unless otherwise noted, significance was measured relative to WT or siNegative treatments. A *p* value of less than 0.01 was considered to be significant, unless otherwise noted. Bar graphs were made for each data set, and representative data are presented with standard deviations (SD) of the mean indicated with error bars.

8.0 CONCLUSIONS

8.1 TARGETING β-CATENIN FOR HCC

We know that targeting of β-catenin for hepatocellular carcinoma (HCC) and for other pathologies with aberrant Wnt/β-catenin signaling is inevitable. *But is β-catenin a viable therapeutic target?* Often overlooked are the dual roles of β-catenin in not only Wnt signaling but also in cell-cell adhesions at the adherens junctions (AJs). *Will anti-β-catenin therapies negatively impact the cell adhesions as well, possibly affecting the outcome of the treatment by inadvertently increasing invasion and metastasis of the tumor cells?* We performed the studies presented here in order to address these questions. Here we identified γ-catenin increase which compensates for β-catenin suppression/loss at the AJs in the liver. Additional studies allowed us to make the following important conclusions: **1)** γ-catenin is unable to compensate for β-catenin in Wnt signaling; **2)** γ-catenin is able to compensate fully for β-catenin at the adherens junctions; **3)** the impact of β-catenin loss and resulting γ-catenin increase on the other cell-cell adhesions is minimal; and **4)** simultaneous loss of β- and γ-catenin is detrimental to cell-cell adhesions. Thus, we conclude that β-catenin appears to be a druggable target for the treatment of HCC.

8.1.1 γ-Catenin is unable to compensate for β-catenin in Wnt signaling

Changes in β-catenin expression in HCC samples are most often linked to aberrant Wnt signaling. Reducing β-catenin within a cell would in turn decrease the amount of β-catenin available to participate in Wnt signaling, affecting cancer cell proliferation and growth. Thus, anti-β-catenin therapies have been developed. The efficacy of these drugs is most often exhibited as a decrease in TCF-dependent Wnt signaling and/or a decrease in β-catenin target gene levels. However, the impact on AJs is rarely addressed. Here we show using conditional βKO mice and also *in vitro* siRNA and ASO treatments that when β-catenin protein levels are decreased in hepatocytes or HCC cells, there is an increase in γ-catenin levels. The increase in γ-catenin levels is concentrated at the AJs, where it takes the place of β-catenin in bridging the connection of E-cadherin to the actin cytoskeleton. Additionally, the increase in γ-catenin does not appear to be oncogenic in the context of β-catenin loss. Yet this increase in γ-catenin is unable to rescue the function of β-catenin as a component of Wnt signaling both *in vivo* and *in vitro*. Thus, in the liver it appears that γ-catenin can take on the responsibility at the AJ, but not in the Wnt signaling. This data helps support the hypothesis that γ-catenin is unable to compensate for every β-catenin-dependent role in the cell. However, the differential signaling capacities of β- and γ-catenin in liver and HCC cells could be exploited anti-β-catenin therapies.

8.1.2 γ-Catenin is able to compensate fully for β-catenin at the adherens junctions

The loss of β-catenin in the *in vivo* and *in vitro* systems presented as a very mild phenotype with no obvious adhesive defects. As mentioned in **8.1.1**, γ-catenin increase with β-catenin loss was

focused to the AJs, where it was able to take the place of β-catenin in linking E-cadherin to the actin cytoskeleton. When we tested the strength of the AJs, formed by β-catenin/E-cadherin (steady state junctions) or γ-catenin/E-cadherin (after β-catenin loss), we were unable to detect any differences between these junctions. We did not detect a decrease in overall AJ numbers with β-catenin loss either. Additionally, there were no changes in migration, heterotypic cell-cell adhesion strength, or homotypic cell-cell adhesion strength of the altered AJs. Taken together, the data presented here indicates that β-catenin is dispensable for cell-cell junctions – as long as γ-catenin is available.

8.1.3 Simultaneous loss of β- and γ-catenin is detrimental to HCC cell-cell adhesion

The cell-cell junctions of the HCC cells were only negatively impacted when both β- and γ-catenins were decreased at the same. It is likely that the effects of the loss of both catenins affect not only the AJs, but other junctions. We did see a decrease in E-cadherin with dKD of these proteins, suggesting a decrease in overall AJ number. Additionally, decreases in E-cadherin levels might also indicate an increase in oncogenic potential and epithelial to mesenchymal transition of these cells [90, 216]. In the instance of double catenin loss, we observed a significant increase in the migratory potential of the HCC cells and a decrease in both heterotypic and homotypic cell-cell adhesion strength. This data indicated that HCC cells without both catenins might have a poor prognosis via increase in poorly differentiated HCCs and an increase in the migration of the cancer to other organs. Thus, when anti-β-catenin therapies are employed to treat HCC in patients, the redundancies of β- and γ-catenin should be recognized in order to make sure the levels of these two proteins are not affected simultaneously.

8.1.4 The impact of β-catenin loss and concomitant γ-catenin increase on other cell-cell adhesions is minimal

The loss of β-catenin prompted changes in other cell-cell adhesions as well; however, these changes also presented as only mild phenotypes. TJ changes with the loss of β-catenin appeared to still support a functional blood-bile barrier. Desmosomal changes were expected with β-catenin loss since γ-catenin is an important desmosomal protein. However, there were no noticeable changes in the desmosomes. Increases in γ-catenin protein levels were not due to changes in *JUP* gene expression, but likely due to an increase in serine- and threonine phosphorylation-dependent stabilization. This observation indicates that the levels of γ-catenin are independently regulated at AJs and desmosomes. Thus, β-catenin is not only dispensable for cell-cell adhesions, but the change in β-catenin levels are well tolerated by other junctions in the hepatocytes, successfully preserving the integrity of the liver.

8.1.5 β-Catenin is a druggable target for the treatment HCC

Taken together, the data presented confirms that β-catenin is a viable target for the treatment of HCC. Targeting β-catenin at a protein level induces compensatory changes in cell-cell adhesions to preserve the integrity of the liver while simultaneously reducing the influence of β-catenin-driven Wnt singling. A lack of β-catenin is endured by the liver and does not appear to lead to specific pathologies; therefore, we anticipate that acute β-catenin targeting in HCC will also be successful in reducing aberrant Wnt signaling without unintended consequences on liver

homeostasis. As a result, it appears that anti-β-catenin treatments will be well tolerated because of the catenin redundancies built into the physiology of epithelial cells.

Currently, there are anti-β-catenin therapies are in preclinical trials. There are two broad classes of β-catenin inhibitors: 1) ones which block the nuclear β-catenin without impacting the total levels of β-catenin (such as ICG-001 and Pegylated-Interferon-α2a) [12, 13] (**Figure 18E**); and 2) inhibitors that suppress β-catenin gene and/or protein expression such as siRNA, ASO or other indirectly acting agents [11, 59, 196-198] (**Figure 18D**). The data presented here indicates that the inhibitors that suppress β-catenin gene and/or protein expression will in fact be successful in the clinic; however, the anti-β-catenin drugs which block the nuclear β-catenin without impacting the total levels of β-catenin maybe closer to use in HCC patients, as a number of these drugs are already used for the treatment of other human diseases. Based on previous studies done with ICG-001, there appears to be no detrimental side-effects of this treatment [13], leading us to believe that the treatment with ICG-001 most likely does not mimic a dKD, especially since the levels of γ-catenin and β-catenin are relatively unchanged as compared to their respective controls (**Figure 18E**). It is possible that a combination of targeted transcriptional and translational anti-β-catenin treatments would be a viable therapeutic option for HCC in the future since both appear to have distinct anti-β-catenin effects. Though the druggability of β-catenin in HCC and other diseases appears to be ideal, realistically, the introduction of siRNA or ASO may prove difficult and not as specific as ICG-001 because of the relative stabilities and molecular properties of these two different types of drugs. Therefore refining a physiologically stable ASO or siRNA to β-catenin which could be delivered in a targeted/liver-specific way would be an important step in the successful fruition of anti-β-catenin drugs for treating HCC.

8.2 CATENIN SENSING MECHANISM

In addition to the work presented on β-catenin as a druggable target, we also gained insight into the regulation of β- and γ-catenin within biological systems which has led us to hypothesize the existence of a *catenin sensing mechanism* within the cell that is regulated in part by PKA. PKA is known to phosphorylate β-catenin at Ser552 and Ser675 which leads to Wnt- and GSK3β-independent, but TCF-dependent, β-catenin signaling [20]. We show here that PKA is involved in the serine and threonine phosphorylation and subsequent stabilization of γ-catenin. We think that PKA has the ability to sense the levels of catenins available in the cytosol and negotiate the functions of β- and γ-catenins in Wnt signaling at the AJs (**Figure 25**). In this way, under basal conditions PKA will help β-catenin maintain normal hepatocyte homeostasis by sustaining appropriate amounts of β-catenin-dependent Wnt signaling. However, in the absence of β-catenin the PKA signals are transduced by γ-catenin, inducing its stabilization and AJ localization. In this way not only are the AJs maintained, but if there is residual β-catenin stored in the cell membrane compartment, it could be displaced by γ-catenin and allowed to participate in Wnt signaling to again maintain homeostatic levels of β-catenin target genes. By conclusively identifying the PKA-dependent mechanism that regulates the interplay between β- and γ-catenin we would gain further insight into just how closely these redundant catenins are regulated, and how this impacts normal cell biology.

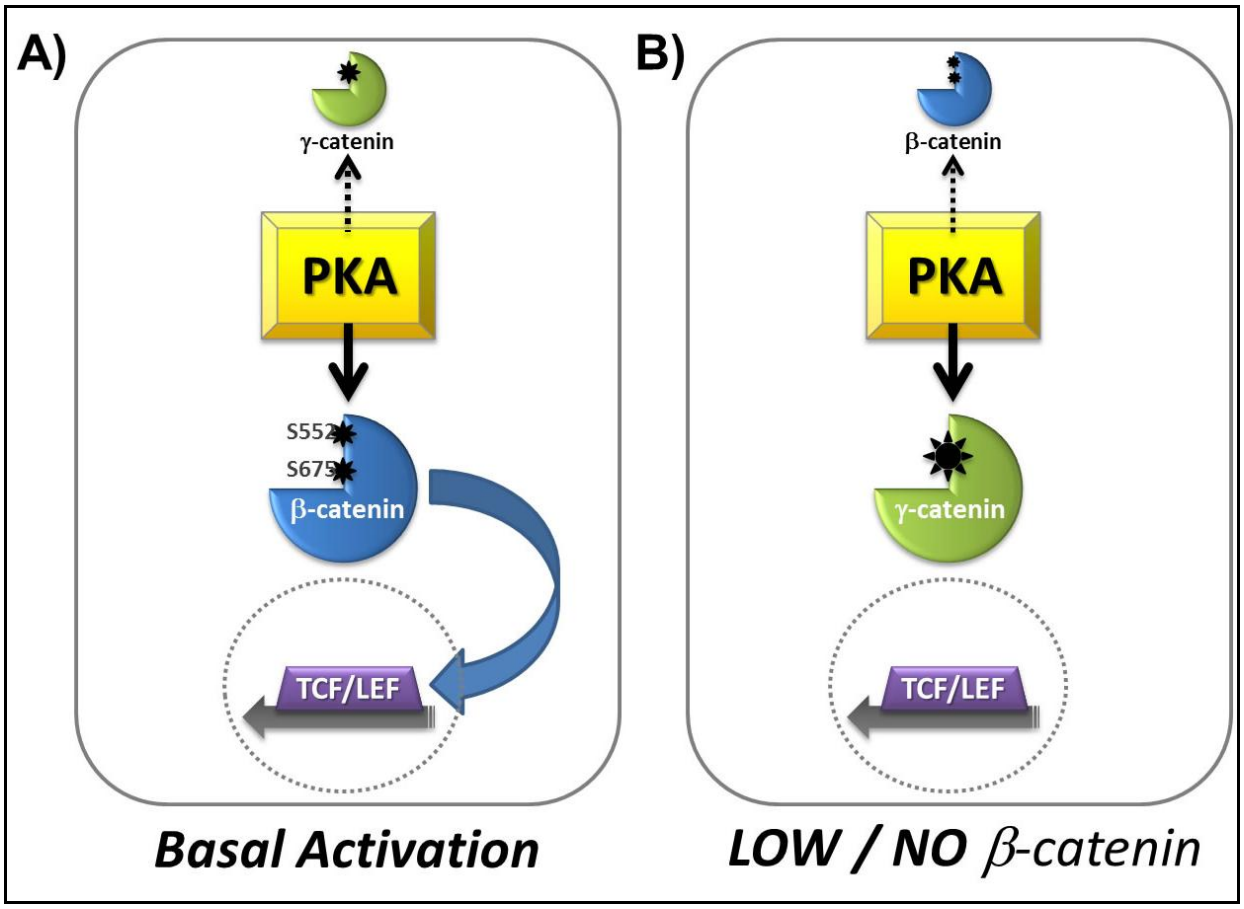

Figure 25. Catenin sensing mechanism via PKA. (A) Under basal conditions in the liver PKA helps β-catenin maintain normal hepatocyte homeostasis by sustaining appropriate amounts of β-catenin-dependent signaling. **(B)** At low levels or in the absence of β-catenin the PKA signals are transduced by γ-catenin, inducing its stabilization (* indicates ser/thr phosphorylation).

9.0 FUTURE DIRECTIONS

There were some observations made within the scope of research presented here that will require further scrutiny.

9.1 DETERMINE ROLE OF PLAKOPHILIN-3 INCREASE WITH β-CATENIN LOSS

In **Chapter 3** we observed a change in the desmosomal protein Pkp3 with β-catenin knockout *in vivo*. We thought that this might be the mechanism by with γ-catenin could be stabilized within the cytoplasm, since recent work by others have shown that Pkp3 binds γ-catenin and E-cadherin within the cytoplasm in order to help with the assembly of desmosomes [108]. Attempts to address this theory *in vivo* using the βKO mice indicated that there were no changes in Pkp3/γ-catenin/E-cadherin complex levels or overall desmosomal structure as compared with WT mice. However, the change may be temporal and a careful *in vitro* time course may be more conclusive. It would be helpful to manipulate Pkp3 levels in conjunction with β-catenin to more directly determine if Pkp3 complex with γ-catenin is responsible is responsible for the reciprocal stabilization of γ-catenin and possibly contributes to the cadherin-based adhesions.

9.2 ADDITIONAL CHARACTERIZATION OF TJ CHANGES WITH β-CATENIN LOSS

Additionally, there were changes in TJ proteins observed in the βKO mice. As the possibility of anti-β-catenin therapies for HCC come closer to fruition, it will be imperative to better understand how β-catenin might impact the paracellular seal created by the TJs in the liver. The leakiness of the junctions is very important to overall liver health, as compromised TJs can lead to cholestasis [66]. Functional data indicated that the βKO hepatocyte TJs were not leakier than the WT TJs [164]; however, the state of the TJs was still not equivalent. We theorized that the switching of JAM or claudin isoforms at the TJs may have contributed to the leakiness of the junctions. However, these hypothesizes should be confirmed with co-precipitation and imaging studies of these TJ components.

9.3 IN DEPTH ANALYSIS OF SERINE/THREONINE PHOSPHORYLATION-DEPENDENT STABILIZATION OF γ-CATENIN

Though significant strides were made in elucidating the mechanisms which controls the upregulation of γ-catenin after β-catenin loss or knockdown, further work needs to clarify the reciprocal relationship between these catenins in order to highlight the differences or similarities in β- and γ-catenin regulation which may be important for future HCC therapies. Initially, it is critical that we identify the specific residues on γ-catenin which are responsible for its stabilization. A few groups, including our, have indicated that it is serine/threonine phosphorylation which is dictating γ-catenin's stabilization. Importantly, we describe here in

Chapter 6 that PKA may be the serine/threonine kinase responsible for the stabilization of γ-catenin. Once key serine/threonine residues are identified on γ-catenin, we can then use site-directed mutagenesis in order to manipulate theses residues and fully understand the impact of γ-catenin stabilization on cell physiology. This knowledge will not only be important for the overall characterization of γ-catenin in biological systems, but will be useful when considering anti-β-catenin therapies. In **Chapter 5** we showed that it is critical that at least one of these catenins is maintained in order to keep the cell-cell adhesions functioning normally. If anti-β-catenin therapies are used for the treatment of cancer, we would want to make sure that those mechanisms which lead to the stabilization of γ-catenin are spared.

9.4 WILL ALL β-CATENIN INHIBITORS HAVE AN IMPACT ON AJS?

We also observed in **Chapter 5** that it was the overall protein levels of β- and γ-catenin which were driving this reciprocal relationship. When we used ICG-001 to block the transactivating potential of β-catenin, we did not see a decrease in β-catenin protein levels or a significant change in γ-catenin levels, though we did see a decrease in β-catenin target gene expression as expected. Whether drugs like ICG-001 impact the functionality of the AJs is still unknown. Therefore it will be critical to perform follow-up studies to determine how ICG-001 will be tolerated by the AJs, since this compound does not appear to trigger the catenin sensing mechanism within the cell.

9.5 STUDY γ-CATENIN'S RESPONSIBILITIES IN LIVER PATHOBIOLOGY

As mentioned throughout, more work needs to be done in order to understand the importance of γ-catenin, and also desmosomes, to liver biology and pathobiology. We have begun to address this topic by creating a liver-specific γ-catenin knockout mouse (γKO) using the Albumin-driven Cre-LoxP system. We plan on characterizing this mouse before and after challenges such as partial hepatectomy, hepatocyte injury, and biliary injury. In this way we will not only be able to address the importance of γ-catenin to overall liver biology, but also the significance of the desmosomes, since research has shown that γ-catenin is critical for the formation of desmosomes [70, 108]. Although acute loss of γ-catenin in HCC cells does not appear to negatively impact cell-cell adhesion (**Figure 21**), we anticipate that the adhesive phenotype associated with the loss of γ-catenin in hepatocytes might be more severe than that observed in the βKO mice since γ-catenin has also been implicated in AJ formation [70, 108]. Additionally, the γKO model will be able to help us address the role of γ-catenin and/or desmosomes in HCC. There is conflicting data on whether the presences of γ-catenin in HCC is correlated with a better or worse patient prognosis [157-159]. It is possible that if γ-catenin is critical in HCC, the absence of γ-catenin from hepatocytes would result in a spontaneous appearance of cancer in these mice. However, if there is no spontaneous cancer formation in these γ-catenin-null livers, we would be able to induce carcinogenesis with DEN or DDC. In this way we could also assess how γ-catenin levels impact the outcome of the HCC.

9.6 HOW DOES γ-CATENIN DECREASE CAUSE β-CATENIN ACTIVATION?

We observed in **Chapter 5** that when γ-catenin was knocked down in HCC cells there was an increase in β-catenin protein levels and also Wnt reporter activity. More studies on the mechanisms regulating this reciprocal β-catenin increase will be necessary. It is unclear at this point whether this increase is initially governed by changes in gene expression or β-catenin stabilization. We can begin to address the regulation of this event *in vitro* by manipulating γ-catenin levels and looking at *CTNNB1* gene expression and also at the phosphorylation status of known β-catenin stabilizing residues. Additionally, it is possible that this observation of β-catenin increase with γ-catenin decrease may have been specific to HCC. Therefore, it would be critical to also expand upon these studies *in vivo* using the γKO mice. Initially we would want to look at overall β-catenin levels to determine whether they are increased with a chronic loss of γ-catenin. If so, we could investigate whether β-catenin has increased localization at the AJs. Additionally, we could stain these livers for β-catenin targets cyclinD1 and GS in order to determine if there is an increase in Wnt/β-catenin signaling as a result of the presumed β-catenin increase *in vivo*. Thus, we would be able to determine whether the increase in activated β-catenin with siRNA to γ-catenin was only a result of acute loss of γ-catenin in HCC cells, or whether this is a universal observation in liver biology.

9.7 CONCLUSIVELY ADDRESS β- AND γ-CATENIN REDUNDANCIES IN THE LIVER

Lastly, to fully understand the physiological impacts of the interplay of β- and γ-catenin within the liver, along with the importance of a catenin sensing mechanism, we have also started generating a liver-specific β- and γ-catenin double knockout mouse (βγKO) using the Albumin-driven Cre-LoxP system. If these mice are viable, we do anticipate a very severe phenotype, especially after challenges as a result of compromised AJs and desmosomes. To our surprise, a viable double knockout of β- and γ-catenin was recently generated in α-myosin heavy chain-expressing ventricular cardiomyocytes [190]. However, there are a number of differences between the cardiomyocyte model and the βγKO we are proposing to use. Firstly, the cardiomyocyte double knockout mouse used tamoxifen-inducible knockout. This system provides only an acute change in β- and γ-catenin levels; however, the inducible knockout could be helpful if the β- and γ-catenin double knockout is embryonic lethal. Additionally, we propose to knock out γ-catenin from a different tissue type. It is important to note that cardiomyocytes only make up 56% of the murine heart [217], while hepatocytes make up 65% of liver cells (but 90% of the liver mass) [218]; therefore, the double knockout in hepatocytes will most likely have a greater impact on the affected organ. Taken together, the results presented in this work along with additional experiments proposed in this chapter will help to paint a clearer picture of not only the role of γ-catenin in liver biology and pathobiology, but will also help us better understand interplay of these redundant catenins.

APPENDIX A

ANTIBODIES

Table 5. Antibodies used for WB, IP, IF, and IHC.

Protein	Antibody Species	Size (kDa)	WB Dilute	IP Dilute	IF/ IHC Dilute	IF Fixation; Permeablize	Company	Product Number
A6	Rat	-	-	-	1:100	Acetone	Gift from Valentina Factor (NIH)	-
α-Catenin	Rabbit	100	1:500	-	-	-	BD	610193
α-Catenin	Rabbit	-	-	1:50	-	-	Abcam	ab51032
α-Fetoprotein	Mouse	-	-	-	1:200	Acetone	Santa Cruz	sc-51506
β-Actin	Mouse	42	1:2500	-	-	-	Millipore	MAB1501
β-Catenin	Mouse	92	1:1000	-	-	-	BD	610154
β-Catenin	Rabbit	-	-	-	1:150	2% PFA; Triton X-100	Santa Cruz	sc-7199
β-Catenin	Goat	-	-	1:50	-	-	Santa Cruz	sc-1496 AC
Cd133	Rabbit	-	-	-	1:200	2% PFA	Abcam	ab16578
Claudin-1	Mouse	22	1:200	-	-	-	Life Technologies	374900
Claudin-2	Mouse	22-23	1:250	-	-	-	Life Technologies	325600
Claudin-3	Rabbit	22	1:250	-	-	-	Life Technologies	341700

Claudin-4	Mouse	22	1:250	-	-	-	Life Technologies	329400
CyclinD1	Mouse	37	1:200	-	-	-	Santa Cruz	sc-20044
Dsc2	Goat	110	1:200	-	-	-	Santa Cruz	sc-34311
Dsg1	Rabbit	150/ 160	1:200	-	-	-	Santa Cruz	sc-20114
Dsg2	Rabbit	59-150	1:200	-	-	-	Santa Cruz	sc-20115
Dsg3	Goat	108/130/ 100/ 80/75/55	1:200	-	-	-	Santa Cruz	sc-14867
Dsg4	Goat	99/115	1:200	-	-	-	Santa Cruz	sc-28069
DP I/II	Rabbit	(I) 210; (II) 250	1:200	-	-	-	Santa Cruz	sc-33555
E-cadherin	Rabbit	120	1:200	1:50	1:200	2% PFA; Triton X-100	Santa Cruz	sc-7870
E-cadherin	Mouse	120	1:1000	-	1:200	Acetone	BD	610182
E-cadherin	Rabbit	135/ 120	1:1000	1:50	1:50	2% PFA; Triton X-100	Cell Signaling	4065
Ep-CAM	Goat	-	-	-	1:50	2% PFA; Triton X-100	Santa Cruz	sc-23788
γ-Catenin	Rabbit	80-87	1:1000	1:100	1:50	2% PFA; Triton X-100	Cell Signaling	2309
γ-Catenin	Goat	80-87	1:200	-	-	-	Santa Cruz	sc-30997
γ-Catenin	Goat	80-87	1:200	-	-	-	Santa Cruz	sc-30996
Glutamine Synthetase	Rabbit	49	1:200	-	-	-	Santa Cruz	sc-9067
GAPDH	Rabbit	37	1:1000	-	-	-	Santa Cruz	sc-25778
HNF4α	Rabbit	54	1:200	-	-	-	Santa Cruz	sc-8987
HNF4α	Goat	40/54	1:200	-	-	-	Santa Cruz	sc-6556
JAM-A	Rabbit	35	1:200	-	-	-	Santa Cruz	sc-25629
Ki-67	Rabbit	-	-	-	1:200	-	Dako	NM-SP6
LGR5	Goat	-	-	-	1:200	Acetone	Santa Cruz	sc-68580

Occludin	Rabbit	65-82	1:200	-	-	-	Santa Cruz	sc-5562
p-Serine	Mouse	-	1:150	-	-	-	Santa Cruz	sc-81514
p-Threonine	Mouse	-	1:150	-	-	-	Santa Cruz	sc-5267
p-Tyrosine	Rabbit	-	1:150	-	-	-	Santa Cruz	sc-18182
Pkp2	Goat	100	1:200	-	-	-	Santa Cruz	sc-18977
Pkp3	Mouse	87	1:200	-	-	-	Santa Cruz	sc-166655
Pkp3	Rabbit	87	1:200	-	-	-	Abcam	ab109441

APPENDIX B

ABBREVIATIONS

ADP: Atypical ductular proliferation

AFP: α-Fetoprotein

AJ: Adherens junctions

Alb: Albumin

ALT: Alanine aminotransferase

APC: Adenomatous polyposis coli

Arm: Armadillo domains/family

ARVC: Arrhythmogenic right ventricular cardiomyopathy

ASO: Antisense oligonucleotides

AST: Aspartate aminotransferase

βKO: Liver-specific β-catenin conditional knockout

βγKO: Liver-specific β- and γ-catenin conditional knockout

βTrCP: β-Transducin repeat-containing protein

CAFCA: Centrifugal assay for cell adhesion

CAL: Cytoskeletal-associated lysates

CBP: Cyclic AMP response element-binding protein

CK1: Casein kinase Iα

CaMKII: Calcium/Calmodulin-dependent kinase II

Cox: Cyclooxygenase

CPM: Counts per minute

Cre: Cre-recombinase

CTNNB1: Human β-catenin gene

Ctnnb1: Mouse β-catenin gene

Cx: Connexon

DDC: 3,5-diethoxycarbonyl-1,4-dihydrocollidine

DEN: N-diethylnitrosamine

dKD: Double knockdown

Dkk: Dickkopf

DP: Desmoplakin

Dsc: Desmocollin

Dsg: Desmoglein

EGF: Epidermal growth factor

EMEM: Eagle's minimal essential medium

FBS: Fetal bovine serum

FC-40: FITC-conjugated dextran

γKO: Liver-specific γ-catenin conditional knockout

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GJ: Gap junction

GJIC: Gap junctional intercellular communication

GS: Glutamine synthetase

GSK3β: Glycogen synthase kinase 3β

HCV: Hepatitis C virus

HCC: Hepatocellular carcinoma

HGF: Hepatocyte growth factor/scatter factor

HMG: High mobility group

HNF4α: Hepatocyte nuclear factor 4 α

I-fraction: Triton X-100 insoluble CAL fraction

IF: Immunofluorescence; Intermediate filament

IHC: Immunohistochemistry

IP: Immunoprecipitation

IFN: Interferon

JAM: Junctional adhesion molecule

JUP: Human γ-catenin/plakoglobin gene

Jup: Mouse γ-catenin/plakoglobin gene

KD: Knockdown

kDa: Kilodalton

KO: Liver-specific β-catenin conditional knockout; knockout

LEF: Lymphocyte enhancer factor

LOH: Loss of heterozygosity

LRP: Lipoprotein receptor related protein

MCD: Methionine- and choline-deficient

NC: No change

NSAID: Nonsteroidal anti-inflammatory drugs

NT: No treatment

OA: Okadaic acid

PCP: Planar cell polarity

Peg-IFN: Pegylated-Interferon-α2a

PBS: phosphate-buffered saline

PCR: Polymerase chain reaction

PEST: Proline (P), glutamate (E), serine (S), and threonine (T) domain

PKA: Protein kinase A

PKC: Protein kinase C

PKG: Protein kinase G

Pkp: Plakophilin

PVDF: Polyvinylidene difluoride

R-Etodolac: 1,8-diethyl-1,3,4,9-tetrahydropyrano-[3,4-b] indole-1-acetic acid

RIPA: Radioimmunoprecipitation assay

RLU: Relative light units

S-fraction: Triton X-100 soluble CAL fraction

SD: Standard deviation

SEM: Standard error of the mean

Ser: Serine

siβ-catenin: sKD of β-catenin with siRNA

siβ+γ-catenin: dKD of β-catenin & γ-catenin with siRNA

siβ-catenin: sKD of γ-catenin with siRNA

sKD: Single knockdown

siNegative: Negative control siRNA

siRNA: Small interfering RNA

TCF: T-cell factor

TEM: Transmission electron microscopy

Thr: Threonine

TJ: Tight junction

TK: Thymidine kinase

Tyr: Tyrosine

WB: Western blot

WCL: Whole-cell lysates

WT: Wild-type

ZO: Zona occuldens

APPENDIX C

OUTLINE OF PUBLISHED WORK

1) **Wickline ED**, Awuah PK, Behari J, Ross M, Stolz DB, Monga SPS (2011), Hepatocyte γ-catenin compensates for conditionally deleted β-catenin at adherens junctions. Journal of Hepatology, 55(6): 1256-1262. (Reference: [91])
    a. **Figure 5A – C & E**
    b. **Figure 6A & B**
    c. **Figure 7A & B**
    d. **Figure 8**
    e. **Figure 9A & B**
    f. **Figure 12A & B**
    g. **Table 1**
2) Thompson MD, **Wickline ED**, Bowen WB, Lu A, Singh S, Misse A, Monga SP (2011), Spontaneous repopulation of β-catenin null livers with β-catenin-positive hepatocytes after chronic murine liver injury. Hepatology, 54: 1333–1343. (Reference: [169])
    a. **Figure 13A - D**
    b. **Figure 14**

c. **Figure 15**

d. **Figure 16**

3) **<u>Wickline ED</u>**, Du Y, Stolz DB, Kahn M, Monga SPS (2013), γ-Catenin at adherens junctions: Mechanism and biological implications in hepatocellular cancer after β-catenin knockdown. Neoplasia, *In Communication*.

a. **Figure 10A & B**

b. **Figure 11A – E**

c. **Figure 17**

d. **Figure 18A – E**

e. **Figure 19A – C**

f. **Figure 20A & B**

g. **Figure 21A – D**

h. **Figure 22A – C**

4) Unpublished Data

a. **Figure 5D**

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