β-catenin in liver: A matter of life and death

by

Kari Nichole Nejak-Bowen

BA in Microbiology, University of Pittsburgh, 1999

MBA, University of Pittsburgh, 2002

Submitted to the Graduate Faculty of

The School of Medicine in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2010

UNIVERSITY OF PITTSBURGH

School of Medicine

This dissertation was presented

by

Kari Nichole Nejak-Bowen

It was defended on

October 6, 2010

and approved by

Dr. Stephen Strom, PhD, Department of Pathology

Dr. George Michalopoulos, MD, PhD, Chair, Department of Pathology

Dr. Wendy Mars, PhD, Department of Pathology

Dr. Xiao-Ming Yin, MD, PhD, Department of Pathology and Laboratory Medicine, Indiana University

Dr. Nirmala SundarRaj, PhD, Cell Biology and Molecular Physiology

Thesis Advisor: Dr. Satdarshan P.S. Monga, MD, Director, Division of Experimental Pathology, Department of Pathology

β-catenin in liver: A matter of life and death

Kari Nichole Nejak-Bowen, MBA

University of Pittsburgh, 2010

β-catenin plays multiple roles in liver health and disease through regulation of proliferation, differentiation and metabolism. Elucidating the molecular basis of how β-catenin regulates these diverse functions and others is the subject of this dissertation.

While β-catenin signaling undergoes temporal activation and its loss dampens liver regeneration (LR), the impact of stimulating this pathway remains unknown. We utilized transgenic (TG) mice expressing Ser45 mutated β-catenin in hepatocytes to show a growth advantage both *in vitro* and during LR through cyclin-D1 regulation. Additionally, hydrodynamic delivery of Wnt-1 gene delivery induced β-catenin activation and hepatocyte proliferation during LR.

Regucalcin or senescence marker protein-30 (SMP30) was identified as a β-catenin target in the liver through the use of hepatocyte-specific β-catenin conditional knockout (KO) mice. SMP30 is a critical enzyme for the synthesis of ascorbic acid in murine hepatocytes, and its loss led to lower serum ascorbate levels in KO. KO hepatocytes displayed massive apoptosis in culture, which was blocked by addition of ascorbate to culture media. Additionally, apoptosis in HepG2 cells due to regucalcin knockdown was rescued by anti-oxidants. Thus, one mechanism of how β-catenin regulates hepatocyte redox state and survival is through the control of regucalcin expression.

KO livers displayed a basal increase in number of apoptotic hepatocytes. We explored the susceptibility of KO and wildtype (WT) controls to activation of the TNF-α mediated apoptotic pathway. Paradoxically, KO mice are refractory to D-galactosamine (GalN)/LPS, Actinomycin D (ActD)/LPS and GalN/TNF-α treatments showing lower morbidity than WT. NF-κB, a major pro-survival factor and its transcriptional targets were increased in KO basally

and after injury due to lack of β-catenin-p65 association, presence of increased basal inflammation and oxidative stress and increased TLR4 expression in KO livers. Additionally, p65 activation occurred earlier in KO than WT after LPS stimulation. Thus, paradoxical protection from TNF-α-mediated apoptosis in KOs occurs owing to pre-existing NF-κB activation that 'primes' the liver for protection against exogenous insult.

Thus, we have identified β-catenin as a pleiotropic factor regulating cell proliferation, cellular redox state and cell survival through specific genetic targets and protein-protein interactions. These findings have broad implications in acute and chronic hepatic diseases.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

There are so many people in my life that have made this thesis possible, and I would like to take the opportunity to thank them here. First and foremost, I would like to thank my husband, Bill, whose unwavering support and love through all phases of my career has been unparalleled. He has been there to celebrate with me on the good days and offered practical and insightful advice on the bad ones, watched the kids when I had to stay late at work, and always had an encouraging word, even when I was at my most disheartened. In addition to providing emotional support, Bill is also a gifted animal surgeon, and has taught me every one of the complicated surgical techniques that I utilized in my graduate work. Thus it is no exaggeration to say that I could never have done this work without him at my side, and I am very grateful and blessed to have him in my life.

There are also many other people who have shaped my graduate career that I would like to thank. First, my kids, for continuously motivating and inspiring me: Will, who is pursuing his own dream of become a doctor as a freshman in college; and my two little ones, Ian and Elli, whose wonder, curiosity, and enthusiasm bring me joy and remind me of what is important in life. Second, my parents, who have both supported all of my academic endeavors, telling me from an early age that I could be anything I wanted to be. Third, my sister, Kristin, and my friends, both at work and in my professional life, who were always there for me to talk to, and

stuck with me through the day-to-day ups and downs. A special shout-out goes to the fellow members of Club 433, who made the lab an entertaining and exciting place to be, and who always had my back. Fourth, my thesis committee, whose helpful comments and ideas over the years have contributed significantly to my research.

Finally, I would like to thank my mentor, Dr. Paul Monga, for allowing me the privilege of working in his lab. When I started graduate school, it was with the perspective of a technician who was used to being told what to do. He taught me to think like a scientist, challenging me to take the next step and design experiments around questions I wanted to answer. He pushed me to accomplish more than I thought I could, and gave me a multitude of opportunities to present my work at the national level, for which I have received recognition and awards. Most importantly, when life got tough, when experiments failed and the balance between work and life became difficult, he never let me give up. His belief in me, as well as his compassion and understanding, carried me through many difficult times. It is with deep gratitude that I say I could not have asked for a better mentor, and that I am fortunate to have had such excellent support and guidance throughout my graduate career. I owe all my current success, as well as any future successes, to him.

1.0 INTRODUCTION

1.1 THE WNT/BETA-CATENIN SIGNALING PATHWAY

1.1.1 The canonical Wnt pathway

Because of its essential role in regulating developmental decisions as well as adult tissue homeostasis, the Wnt/β-catenin signaling pathway has been the subject of extensive research for the past two decades [1]. Characterization of this evolutionarily well-conserved pathway has shown that Wnt signaling is indispensible in processes as diverse as cell fate, development, differentiation, growth, survival, regeneration, and self-renewal [2-6]. Mutations in Wnt pathway components have also been implicated in the initiation and development of cancer and disease progression [7, 8].

Wnt genes encode a large family of secreted glycoproteins that act as extracellular signaling molecules. Binding of Wnt proteins initiates a signaling cascade, which results in activation of β-catenin, the central player in the canonical Wnt pathway. However, in most normal unstimulated adult cells, where the Wnt/β-catenin pathway is inactive, this steady-state condition is ensured by the absence of Wnt protein and the degradation of β-catenin. Free cytoplasmic β-catenin is bound in a complex with Axin, adenomatous polyposis coli (APC), casein kinase 1 (CK1) and glycogen synthase kinase 3β (GSK3β), which collectively comprise

the destruction complex. β-catenin is phosphorylated by casein kinase 1 (CK1) and GSK-3β at specific serine/threonine residues located at the N-terminal region of the protein, which is facilitated by the scaffolding proteins Axin and APC [9]. This sequential phosphorylation targets β-catenin for ubiquitination and ultimate degradation by the proteasome.

Binding of Wnt proteins to the seven-pass transmembrane Frizzled (Fz) receptor on the surface of cells induces association with the low-density lipoprotein receptor related protein (LRP) 5/6. This coreceptor complex triggers activation of the canonical Wnt pathway. Dishevelled (Dvl) is recruited to the Frizzled receptor [10], and the Fz/Dvl complex in turn relocates Axin to LRP5/6 [11]. Axin-bound GSK-3β and CK1 then phosphorylate LRP5/6 [12, 13], which leads to inactivation of GSK-3β [14]. The absence of β-catenin phosphorylation releases it from the Axin/APC/GSK3 complex, resulting in accumulation of cytoplasmic and active β-catenin**.** Although β-catenin lacks a nuclear localization sequence, it then translocates to the nucleus through an unknown mechanism which may involve interaction with either components of the nuclear pore complex [15] or LEF/TCF [16], its nuclear binding partner. Once in the nucleus, β-catenin binds to lymphoid enhancer-binding factor/T cell-specific transcription factor (LEF/TCF), displacing the transcriptional inhibitor Groucho, and in complex with TCF initiates transcription of target genes [4].

The number and variety of target genes activated by Wnt/β-catenin signaling are diverse and include target genes such as Met, Jagged, gastrin, MMP7, survivin, and various FGFs [17-24]. β-catenin also controls expression of cell-cycle regulators important in proliferation such as cyclin-D1 [25, 26], as well as oncogenes such as c-myc [27]. Interestingly, some of the target genes of the pathway are components of the Wnt signaling pathway itself. For example, β-catenin can activate expression of repressors such as Axin2, Tcf1, and Dkk1 [23, 28, 29], or

suppress positive pathway components such as Frizzled and LPR6 [30, 31], indicating the presence of a negative feedback loop to dampen or suppress Wnt signaling. Intriguingly, Wnt/β-catenin signaling can induce expression of genes such as LEF1, which enhances and prolongs the signal, indicating the presence of a feed-forward mechanism which can be exploited by carcinoma cells [32, 33]. The majority of Wnt target genes, however, appear to be cell-type specific and are thus regulated both temporally and contextually. In the liver, these genes include glutamine synthetase, several cytochrome P450s, glutathione-S-tramsferases (GSTs), and leukocyte cell-derived chemotaxin 2 (LECT2) [34-37], as well as our recently discovered target regucalcin [38].

1.1.2 Interaction of β-catenin with other pathways

In addition to the above-mentioned participants involved in the canonical Wnt signaling pathway, several other proteins are known to interact with β-catenin in liver (Figure 1). β-catenin forms a bridge between the actin cytoskeleton and E-cadherin present at the surface of hepatocytes [39-41]. The β-catenin/E-cadherin interaction mediates cell-cell adhesion and is regulated by the phosphorylation of β-catenin at a specific tyrosine residue (Y654) [42, 43]. Specifically in liver, this causes dissociation of the complex and subsequent degradation of E-cadherin, resulting in a loss of adherens junctions and impaired apical trafficking in hepatocytes [44]. Loss of adhesion may also contribute to motility, which is an important component of the cellular response in processes such as development, regeneration, and cancer growth.

TGF-β, a known regulator of self-renewal which has also been implicated in the modulation of hepatocellular carcinoma [45], plays an important role in E-cadherin/β-catenin interactions as well. TGF-β-mediated loss of E-cadherin results in the release of β-catenin from

cell-cell contacts and its subsequent translocation to the cytoplasm. Active β-catenin thus leads to the increased cell motility and invasive phenotype seen in gastrointestinal and liver cancers [46, 47].

Another important interaction involving β-catenin in hepatocytes is the β-catenin/Met complex. Met is the receptor for hepatocyte growth factor (HGF), a known mitogen, motogen, and morphogen for the liver [48]. Our laboratory has previously shown that Met and β-catenin associate on the inner surface of hepatocytes [49, 50]. Binding of HGF to its receptor induces phosphorylated Met to phosphorylate β-catenin at tyrosine residues 654 and 670, which results in its translocation to the nucleus and subsequent upregulation of target genes [51, 52]. Another study by our lab found that injecting the human HGF gene into mice leads to hepatomegaly via dissociation of the Met/β-catenin complex and induction of the β-catenin pathway [53]. β-catenin also regulates HGF-induced cell morphogenesis [54], and interactions between Met and a mutated active form of β-catenin have been found to facilitate hepatocellular carcinoma [55]. The Met/β-catenin pathways thus cooperate to induce hepatocyte proliferation both *in vitro* and *in vivo*.

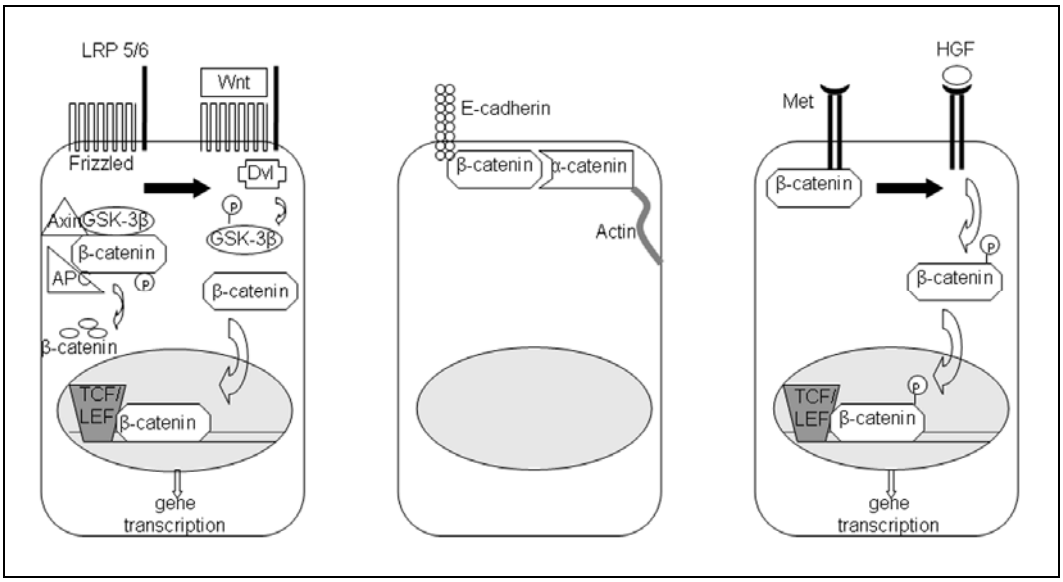

Figure 1: The three major roles of β-catenin in liver physiology. Left: in the presence of Wnt, β-catenin is released from its inactivating complex and translocates to the nucleus, where it activates genes essential for proliferation, growth, and regeneration of the liver. Middle: β-catenin mediates cell-cell adhesion through its interaction with e-cadherin on the hepatocyte membrane. Right: in the presence of HGF, β-catenin, which associates with Met at the surface of hepatocytes, is phosphorylated and translocates to the nucleus to turn on genes important in proliferation and morphogenesis.

1.2 BETA-CATENIN IN LIVER DEVELOPMENT

1.2.1 Embryonic Liver Development

The induction of embryonic liver is a complex process that requires a series of tightly regulated localized signals from multiple cell types. Liver in mouse begins to arise from the definitive gut endoderm at E8.5, or the 7-8 somite stage [56-58]. It is at this time that a family of transcription factors, Foxa, specifies the endoderm to express hepatic genes [59, 60]. The

fibroblast growth factors FGF1 and FGF2, which are expressed in the cardiac mesoderm at this time, are responsible for initiating the expression of these liver-specific genes in the endoderm [61]. FGF8, which is important for morphogenetic outgrowth of the liver, is also expressed during this stage [61]. Endothelial cells also interact with the hepatic endoderm shortly after specification to promote morphogenesis and liver bud formation [62]. The resulting bud migrates into the septum transversum mesenchyme upon bone morphogenic protein 4 (BMP4) signaling, which is also required for hepatogenesis [63]. One of the most critical genes at this stage is Hex, which is maintained by FGF and BMP4 signaling in the commitment phase [64]; expression of this transcription factor is essential for hepatoblast differentiation and liver bud formation [65-67].

The second phase of embryonic liver growth is characterized by expansion and proliferation. The cells are now considered hepatoblasts, which means that they are capable of giving rise to both major lineages of the liver, hepatocytes and biliary epithelial cells [68]. Hepatoblasts or hepatic progenitors are the bipotential stem cells that will be undergoing expansion while maintaining their de-differentiated state during this stage. This event is comparable to the expansion of a lineage-restricted progenitor population in stem cell biology. HGF, expressed in the septum transversum mesenchyme which now surrounds the liver bud, is critical for this stage of liver growth [69]. Transcription factors required for liver development at this stage include Hlx [70] and Prox1 [71]. Albumin and α-fetoprotein mRNA is also being produced at this time, indicating commitment to a hepatic fate [72]. There is significant proliferation of hepatoblasts leading to expansion of the hepatic bud. Finally, the general architecture of the liver is beginning to be established, including the formation of sinusoids and the development of hepatic vasculature [59, 73].

The final stage is characterized by the differentiation of hepatoblasts to mature, fully-functional cell types. At the center of the differentiation process are the liver-enriched transcription factors such as hepatocyte nuclear factor (HNF) transcription factors and C/EBPα, which regulate cell fate decisions in the liver [74, 75]. HNF-4α is essential for differentiation toward a hepatocyte phenotype, as well as formation of the parenchyma [76]; GATA6, which regulates HFN-4α, is also required for hepatocyte differentiation [77]. HNF-6, HNF-1β, and the Notch signaling pathway are required for normal development of biliary epithelia and resulting bile duct structures [78-80]. Finally, the expression of transcription factors NF-κB, c-Jun, and XBP-1 is necessary for growth and morphogenesis [68], which continues until birth.

1.2.2 The Role of Wnt/β-catenin in Embryonic Liver Development

In recent years, a plethora of evidence has emerged identifying regulation of Wnt/β-catenin signaling as a requirement for embryonic liver development. In fact, in Xenopus, the impact of Wnt/β-catenin signaling on liver development can be seen as early as the maternal phase, which occurs before gastrulation. Maternal Wnt/β-catenin, in conjunction with endodermally-derived TGF-β, can induce anterior endomesoderm (AE), a subset of endoderm cells fated to form the liver [81]. Thereafter, repression of this pathway becomes necessary during the competency and commitment stage of liver development [82]. In Xenopus, β-catenin expression after gastrulation is necessary for intestinal formation in the posterior endoderm, while repression in the anterior endoderm allows for expression of Hex, which is required for liver and pancreas development. Repressing β-catenin in the posterior endoderm causes organ buds expressing liver markers to form [83]. Sfrp5, an antagonist of Wnt, is expressed in the ventral foregut endoderm that gives rise to the liver at mouse E8.5 [84], resembling the

expression pattern of Hex. The expression of this inhibitor functions to modulate Wnt-11 activity by delineating borders between organs in the developing gut [85, 86]. Further, calcineurin, a member of the Wnt/calcium signaling pathway, is involved in dorsal-side signaling that leads to the formation of liver during Xenopus embryogenesis through its interference with canonical Wnt/β-catenin signaling [87]. However, a recent study has shown that as soon as foregut endoderm is formed, hepatic specification requires activation of Wnt signaling brought about by Wnt2bb [88]. Additional studies, perhaps using conditional knockout mice in which β-catenin is deleted around E8.5, will be needed to definitively determine whether Wnt/β-catenin signaling is activated or repressed during liver specification.

Our laboratory was the first to demonstrate a mechanistic role for the Wnt/β-catenin pathway in developing liver. Livers from mouse embryos cultured in the presence of a β-catenin antisense oligonucleotide showed a decrease in proliferation and a simultaneous increase in apoptosis, two processes vital to liver development [89]. This correlated well with a subsequent study that found overexpression of β-catenin in developing chicken livers leads to a three-fold increase in liver size, which is due at least in part to an expanded hepatoblast population. Conversely, blocking β-catenin expression through overexpression of pathway inhibitors resulted in decreased liver size and altered liver shape [90]. The effect on cell proliferation noted in both cases may be due to cell cycle mediators such as cyclin-D1. We and others have shown that β-catenin protein expression peaks at E10-12, during which time it is localized throughout the cell including the nucleus, cytoplasm and membrane. Subsequent decreases in β-catenin gene expression and increased protein degradation coincide with a dramatic decrease in total β-catenin protein levels after E16, at which time it is also localized to the membrane of maturing hepatoblasts and hepatocytes [91, 92]. While the early stages coincide with ongoing hepatoblast

expansion which is mediated via increased proliferation and survival, the later stages represent hepatoblast maturation to hepatocytes, and these maturing cells begin to express genes that are associated with measures of hepatic function such as transferrin, cyochrome P450s, coagulation factors, haptoglobin and many others [93].

As found in our previous studies with *ex vivo* embryonic liver cultures, there was a positive correlation between β-catenin and cell proliferation, which has also been supported by additional studies in chicken, zebrafish and Xenopus [83, 88-90, 94]. Thus, these studies have established an important physiological role for β-catenin during early liver development in expansion of hepatoblasts or the hepatic progenitors. β-catenin also plays an important role in the differentiation of hepatoblasts into the two major liver lineage cell types: biliary epithelial cells and hepatocytes. The addition of Wnt3a to embryonic liver cultures induced a biliary phenotype and duct-like arrangement in the developing liver, while lack of Wnt3a causes loss of architecture, proliferation, and increased apoptosis [95]. We also observed a lack of mature hepatocytes in the absence of β-catenin in the explanted mouse livers. This phenotype was confirmed by the concomitant presence of stem cell markers and mature hepatocyte markers in the organ culture [89]. Using another *in vitro* model that recapitulates hepatocyte differentiation, we found an increase in total β-catenin protein as early as 24 hours after the induction of differentiation [49]. This increase was a result of decreased protein degradation and resulted in membranous localization of β-catenin rather than the nuclear localization that is the hallmark of proliferating undifferentiated cells. Finally, deletion of β-catenin from hepatoblasts *in vivo* using β-catenin transgenic mice under the Foxa3 promoter resulted in the decrease of liver-specific transcription factors C/EBPα and HNF4α and an overall hepatic deficiency [96]. These studies

imply that the presence of β-catenin, as well as its location inside the cell, might be a critical event dictating differentiation.

Although the picture not complete, the data thus far suggests that β-catenin levels vary during prenatal hepatic development, and that the temporal expression of β-catenin regulates liver formation. However, spatiotemporal expression of β-catenin is not unique to the liver; the Wnt/β-catenin pathway also shows distinct stage specific effects during cardiac [97, 98], gut [99], and lens development [100]. For example, activating β-catenin expression during embryogenesis enhances cardiomyocyte differentiation, while activation of this pathway later in development causes inhibition of differentiation [101]. In the gut, Wnt expression is present in developing intestines after the appearance of villi, disappears during villi morphogenesis, and then reappears in differentiated postmitotic villus epithelium [102]. Thus, data from organogenesis studies demonstrates that turning β-catenin expression on and off at various stages of development can cause opposing results depending on the timing of induction.

1.3 BETA-CATENIN IN LIVER GROWTH AND REGENERATION

1.3.1 Liver Regeneration

Adult liver has the unique capacity to regenerate after insult and loss of liver mass, and has thus become a useful model to study organ regeneration and controlled growth. A phenomenon unique to liver regeneration is that rather than utilizing progenitor or stem cells, repopulation of the liver occurs almost exclusively through proliferation of mature cell populations, including hepatocytes, biliary epithelial cells, endothelial cells, and stellate cells

[103]. Remarkably, liver regeneration occurs without loss of function; that is, throughout the regenerative process, the liver still performs all the essential functions needed for organism homeostasis. Another property that makes liver regeneration unique is that it can be triggered without causing inflammation or damage to surrounding tissues. The most common method of inducing regeneration experimentally is surgical removal of three of five lobes from the rodent liver, commonly referred to as a 2/3 partial hepatectomy (PHx) [104]. The remaining two lobes grow in size until the liver mass is completely restored, which usually occurs after approximately 7 days in the rat [105]. Because these lobes can be resected within minutes, the entire regenerative process can be precisely timed.

Partial hepatectomy triggers a series of cell signaling pathways and cascades that are very tightly regulated. Many of these pathways are the same as those activated in wound healing and innate immunity, although liver resection itself does not generate an immune response. For example, within minutes after PHx in the rat, urokinase plasminogen activator (uPA) activity increases [106]. uPA is responsible for several critical functions in liver regeneration, such as cleavage of latent hepatocyte growth factor (HGF) to its active form, activation of plasminogen to plasmin, and induction of matrix remodeling [105]. Matrix remodeling is a very important component of regeneration, and is a complex process involving release of bound growth factors, including HGF, by matrix metalloproteinases. HGF has been shown to be indispensible for liver regeneration because it serves as the main mitogenic stimulus driving hepatocytes toward proliferation [107]. Once cleaved and activated, HGF then activates its receptor, Met, within 30-60 minutes after PHx [108], initiating a signaling cascade that results in activation of STAT3, PI3K, and Akt [109, 110]. Pre-existing stores of HGF are rapidly consumed within the first 3 hours [111], causing a 17-fold increase in circulating levels of HGF as early as 2 hours after PHx

[112]. To replenish the stores of HGF depleted by activation and utilization, stellate cells begin to synthesize new HGF. Coinciding with the peak of HGF consumption, levels of HGF mRNA increases dramatically between 3 and 6 hours after PHx, peaking at 12 hours [113]. Thus, the kinetics of HGF consumption and resynthesis are crucial events for liver regeneration and provide an example of the coordinated regulation of liver regeneration.

Another event that occurs within 30 minutes after PHx is the induction of "immediate early genes", including members of the jun, c-fos, and myc families. Transcription of these genes are the result of rapidly activated transcription factors such as Stat3 and NF-κB, which are in turn activated by cytokines such as TNF-α and IL-6 [114]. Genes important for regulation of cell cycle entry are also transcribed either concurrently with cytokine stimulation or immediately following this period in response to growth factors such as HGF and EGF [115, 116]. Interestingly, fetal markers such as alpha-fetoprotein are also upregulated during this time [117], and suggests that regeneration may recapitulate development to some extent.

The hallmark of liver regeneration is proliferation of adult hepatic cell types. The first peak of DNA synthesis happens in hepatoytes and occurs around 24 hours in the rat and approximately 36 hours in the mice [105]. Hepatocyte DNA synthesis and proliferation proceed in a zonal manner through the hepatic lobules, from periportal to pericentral areas [118]. Since 1/3 of the original hepatocytes remain after PHx, they only need to undergo 1.66 rounds of replication before the original number of hepatocytes is fully restored [119]. In fact, hepatocytes are capable of clonal expansion and have an almost unlimited capacity to proliferate *in vivo*. Rat liver was able to continue regenerating even after 12 sequential hepatectomies [120], which suggests that unlike other mature cells in the body, hepatocytes are not terminally differentiated and can divide continuously when presented with appropriate stimuli [103]. Proliferating

hepatocytes also produce growth factors for other cell types, including stellate cells and endothelial cells, in a paracrine fashion. These cells undergo DNA synthesis 24 hours after hepatocytes, peaking at 48 hours after PHx [121]. A key cell-cycle associated gene critical for initiation of cell proliferation is cyclin-D1, which is expressed as early as 6 hours after PHx [122]. As β-catenin is a key driver of cyclin-D1 expression and cell proliferation, it is logical to assume that β-catenin plays a role in liver regeneration after PHx. Indeed, work by our lab and others have described the function of β-catenin in regeneration and postnatal liver growth, which emphasizes its vital role in liver health and repair.

1.3.2 Wnt/β-catenin signaling in liver growth and regeneration

In adult resting liver, the Wnt/β-catenin pathway is mostly quiescent. This steady-state condition is characterized by the phosphorylation and subsequent degradation that is the hallmark of β-catenin turnover; hence, β-catenin is localized at the cell membrane and is largely absent from the cytoplasm and nucleus [123]. In the centrizonal hepatocytes, however, active β-catenin is evident and regulates expression of genes such as glutamine synthetase, CYP2E1, and CYP1A2 [124]. Therefore, when liver is not being challenged by chemical, metabolic, or dietary stress, β-catenin is not required for normal physiologic function [125]. However, during liver regeneration, levels of β-catenin are dramatically increased. In a rat model, an increase in β-catenin protein expression was seen as early as 1-5 minutes post-PHx [126]; this increase was not due to an increase in mRNA expression, but rather to a decrease in protein degradation, the result of a change in steady-state kinetics. This expression was transient, and β-catenin levels returned to normal at 48 hours. Translocation of β-catenin to the nucleus, starting at 5 minutes

after PHx and continuing until 48 hours post-PHx, contributes to the increase in cyclin-D1 and c-myc and the concomitant increase in cellular proliferation [126].

The importance of β-catenin to liver regeneration is highlighted by three studies in which β-catenin is removed or absent from the liver. When a β-catenin antisense oligonucleotide was administered to rats after 2/3 PHx, total β-catenin decreased significantly at 24 hours [127]. Also of note was the decrease in liver weight/body weight ratio as a function of decreased proliferation in these animals. Further, β-catenin conditional knockout mice showed a sick and lethargic phenotype after PHx as opposed to their wild-type counterparts. Additionally, these mice displayed suboptimal regeneration, with delayed regenerative onset and a biphasic trend in proliferation that peaked at day 3 and increased slightly again at day 14 [94]. These results were confirmed by another laboratory that demonstrated a lack of cyclin-D1 induction and a resultant delay in DNA synthesis in liver-specific β-catenin knockout mice [128].

The Wnt/β-catenin pathway also plays a key role in controlling postnatal liver growth. Our laboratory found an increase in β-catenin levels in wild-type mice shortly after birth [91], which serves to promote hepatic growth during postnatal development. Indeed, increased β-catenin translocation to the nucleus correlates with an increase in cell proliferation between 5-20 postnatal days [129]. Further, mice expressing a conditional deletion of β-catenin generated by our laboratory and others showed a significant decrease in the liver weight/body weight ratio (15-25%) in mice older than two months [94, 125]. This decrease is correlated with a basal decrease in cellular proliferation, which is due to a deficient cyclin-D1 response.

Several studies have addressed the role of constitutively active β-catenin on postnatal growth. Mice expressing an oncogenic form of β-catenin demonstrated hyperplasia and resulting hepatomegaly as young as 3-4 weeks after birth [130]. Other reports have described

hepatomegaly soon after birth in mouse strains where a dominant stable form of β-catenin was activated by adenoviral inoculation [131]. Finally, we have recently generated transgenic mice overexpressing wild-type β-catenin and observed a 15% increase in liver size in these mice compared to normal wild-type aged-matched controls [132]. This study also identified EGFR, a receptor for a known hepatocyte mitogen, EGF, as a transcriptional target of Wnt/β-catenin signaling.

1.4 BETA-CATENIN IN REDOX REACTIONS AND OXIDATIVE STRESS

1.4.1 Oxidative stress in the liver

Reactive oxygen species (ROS), reactive nitrogen species (RNS), and free radicals are critical for physiology. They are a normal byproduct of aerobic metabolism and also serve important functions as messenger molecules both inside and outside the cell. Nitric oxide (NO), for example, is important in neural signaling, cardiac and vascular function, and platelet regulation [133]. Reactive oxygen species generated by growth factors and cytokines are implicated in mediating cellular responses through signal transduction [134]. However, when the balance between ROS generation and anti-oxidant protection is disrupted, cells undergo oxidative stress [135]. Oxidative stress can cause damage through inflammation, ischemia, apoptosis, and necrosis.

Radicals from ROS and RNS are generated by neutrophils, Kupffer cells, cytochrome P450s, and mitochondria [136]. Free radicals have an unpaired electron and are either nitrogen- or oxygen-based. Examples of some biologically relevant free radicals include NO (nitric

oxide), O_2^- (superoxide), and HO (hydroxyl radical). They are produced as by-products of biochemical reactions such as oxidative phosphorylation, which generates energy through the electron transport chain. During this process, a small percentage of electrons prematurely leak to oxygen and are released from the mitochondria as superoxide [137, 138]. Hydrogen peroxide, another free radical, is produced from the dismutation or reduction of superoxide. ROS are also formed from the Fenton reaction, in which a transition metal (usually iron) is recycled by superoxide and in the process converts hydrogen peroxide into oxygen and hydroxyl radicals [139]. NO is created from nitric oxide synthetases (NOS), the most well-known being inducible NOS (iNOS). NOS creates NO from arginine, oxygen, and electrons and releases small amounts based on calcium levels. However, iNOS is capable of producing NO independent of calcium, resulting in large amounts of NO which can be either protective or damaging to the cell [133].

Free radicals are implicated in the pathophysiology of many different liver diseases. Free-radical by-products of ethanol metabolism can exacerbate alcohol-induced hepatitis through lipid peroxidation and induction of the cytochrome P450 isoform CYP2E1, which in turn creates more ROS [140, 141]. Acetaminophen-induced liver injury is caused by production of a reactive CYP2E1 metabolite, which binds to and depletes glutathione in the cell [142]. Viral hepatitis infections result in increased inflammation leading to oxidative stress through several mechanisms, including decreased anti-oxidants, increased ROS and cytokines, and iron overload [143, 144]. In fibrosis, activation of stellate cells by ROS results in increased collagen deposition [145]. ROS and RNS are generated during periods of hypoxia in hepatic ischemia/reperfusion injury concomitant with a decrease in anti-oxidants, reduction of mitochondria, and an increase in cytokines [146]. Free radicals are produced in several other diseases that invoke an inflammatory response, such as nonalcoholic steatohepatitis and sepsis.

In sepsis, however, NO has been shown to be protective, perhaps by invading and damaging infecting pathogens or by increasing blood flow to injured tissue [147, 148]

Reactive oxygen species have also been shown to play an important role in hepatocyte apoptosis. In the setting of liver injury, macrophages or neutrophils release superoxide or hydrogen peroxide, which can trigger apoptosis [149]. Toxins such as alcohol, acetaminophen, or chemotherapeutic drugs can also release intracellular free radicals, which induce expression of the Fas ligand [150]. Reactive oxygen species can stimulate apoptosis through several other mechanisms as well, including lipid peroxidation [151], intracellular oxidant stress [152], and enhancement of pro-inflammatory gene transcription such as TNF-α [153]. In turn, TNF-α signaling can increase reactive oxygen formation in hepatocytes [154]. Free radicals have also been implicated in activation of caspases [155] and mitochondrial membrane permeability [156]. High concentrations of TGF-β can also stimulate apoptosis through production of reactive oxygen species, although the mechanism is not well-understood [157].

In the cell, ROS and RNS are neutralized by anti-oxidants, which are small molecular weight molecules that can prevent oxidative damage by detoxifying free radicals [158]. Glutathione (GSH) is a peptide whose main function is to reduce peroxidases, although it also acts as a general free-radical scavenger [159, 160]. Catalases and peroxidases also neutralize hydrogen peroxide [161]. Superoxide dismutases (SODs) are a family of intracellular and extracellular enzymes that catalyze the breakdown of superoxide to hydrogen peroxide and water [162]. Metal-binding proteins such as transferrin, albumin, and hemoglobin can also serve as anti-oxidants [133]. Because they contain high concentrations of GSH, SOD, and catalase, hepatocytes are generally resistant to oxidative stress [158]. However, in some pathological conditions, such as those mentioned above, the endogenous cellular machinery is insufficient to

suppress or prevent oxidative stress. In these cases, exogenous anti-oxidants, like carotenoids or plant phenols, may be therapeutically beneficial. The most commonly utilized dietary anti-oxidant is vitamin C, whose potent free radical scavenging ability has been well-documented.

1.4.2 Regucalcin and oxidative stress

Regucalcin, also known as SMP30, was first identified as a calcium-binding protein located in the cytoplasm of hepatocytes. As such, it plays a critical role in calcium (Ca^{2+}) homeostasis in the liver [163]. Regucalcin activates the Ca^{2+}/Mg^{2+}-ATPase plasma membrane pump, thus allowing for influx of Ca^{2+} and maintenance of intracellular calcium levels [164, 165]. It also stimulates uptake of calcium from the cytoplasm into the mitochondria and microsomes to maintain cytoplasmic Ca^{2+} levels [166, 167]. Moreover, regucalcin acts to exclude Ca^{2+} from the nucleus by suppressing uptake and stimulating its release [168, 169]. In turn, injection of calcium chloride in rats *in vivo* stimulates regucalcin mRNA expression [170, 171]. Regucalcin can also translocate to the nucleus, where it inhibits both protein tyrosine phosphatase and protein kinase activity [172].

Regucalcin is expressed in large amounts in the liver [173], and therefore much of the characterization of this protein has occured in the context of liver physiology and pathophysiology. Regucalcin has been shown to be inhibitory for calcium-calmodulin dependent enzyme activity, including fructose 1,6-diphosphatase, which is involved in gluconeogenesis [174]. It also inhibits cAMP degradation and therefore plays a role in regulating signal transduction [175]. In regenerating rat liver, regucalcin plays an important role in the regulation of Ca^{2+} signaling from the cytoplasm to nuclei in proliferating cells [163, 176], which may partially account for the increased levels of regucalcin mRNA observed in the first 5 days after

PHx [177]. Interestingly, unlike other proteins that are upregulated during this time, regucalcin appears to have a suppressive rather than a stimulatory effect on regeneration. Regucalcin inhibits DNA [178], RNA [179], and protein synthesis [180] in rat nuclei isolated from regenerating rat liver. Therefore, because regucalcin functions as an inhibitor of several critical cellular processes, it is thought to have an important role in preventing hyper-proliferation of hepatocytes during regeneration [176]. This anti-proliferative effect was reported in hepatoma cells as well, where overexpression of regucalcin suppressed cell proliferation, possibly through decreased c-myc expression [181].

Regucalcin also plays an important role in preventing oxidative stress and apoptosis. Regucalcin increases superoxide dismutase activity in hepatocytes [182] and decreases NO synthetase activity [183], thus reducing the production of reactive oxygen species in the cell. Transfection of SMP30 decreases the formation of reactive oxygen species in HepG2 cells [184]. Conversely, loss of SMP30/regucalcin leads to production of reactive oxygen species in the brains and lungs of deficient mice [185-187]. DNA fragmentation, a hallmark of apoptosis, is inhibited by regucalcin through prevention of cytosolic calcium influx [188]. Overexpression of regucalcin in rat hepatoma cells protects them from LPS- or TNF-α induced apoptosis, although the mechanisms are not well-understood [189, 190]. Transfection of HepG2 cells with SMP30 suppresses cell death after TNF-α treatment through regulation of Akt activity [191]. The role of regucalcin in preventing apoptosis was confirmed *in vivo* in SMP30 knockout mice, which show enhanced susceptibility to TNF-α and Fas-mediated apoptosis [192]. Regucalcin/SMP30 was also recently discovered to function as a gluconolactonase, which is essential to vitamin C biosynthesis [193]. Therefore, many of its anti-oxidant and apoptotic effects may be derived from its ability to synthesize vitamin C to prevent cellular damage.

1.4.3 Regucalcin expression and the Wnt/β-catenin pathway

Our laboratory has previously reported the characterization of the β-catenin conditional knockout (KO) animals, which is an excellent resource for identification of novel targets of β-catenin in the liver [94]. Regucalcin was identified as one of the genes most affected by the loss of β-catenin. Gene array analysis of β-catenin KO mice generated using three different Cre lines reveals a 38-fold decrease in gene expression for regucalcin in KOs as compared to their wild-type (WT) counterparts (Table 1) [53, 94, 96]. To confirm the results of our gene array analysis, we isolated mRNA from 1, 3, and 6 month WT and KO livers. Figure 2 shows that regucalcin mRNA is present in WT animals but absent in the KOs; thus regucalcin expression is directly dependent upon the presence or absence of β-catenin. Based on this information, we hypothesized that regucalcin is a downstream target of β-catenin in the liver.

Table 1: Absolute expression of regucalcin and L-gulonolactone oxidase in β-catenin knockout mice generated using three different Cre lines.

Conditional β-catenin KO	Stage (age)	RGN Gene Expression WT	RGN Gene Expression KO
$Ctnnb1^{loxp/loxp}$; FoxA3-Cre	E16	5362	289
$Ctnnb1^{loxp/loxp}$; Alb-Cre	3 months	25218	4418
$Ctnnb1^{loxp/loxp}$; Afp-Alb-Cre	3 months	41038	1366

Conditional β-catenin KO	Stage (age)	L-gulonolactone oxidase Expression WT	L-gulonolactone oxidase Expression KO
Ctnnb1$^{loxp/loxp}$; FoxA3-Cre	E16	1214	595
Ctnnb1$^{loxp/loxp}$; Alb-Cre	3 months	4761	336
Ctnnb1$^{loxp/loxp}$; Afp-Alb-Cre	3 months	6698	309

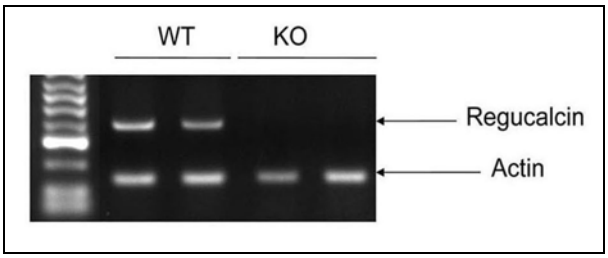

Figure 2: Semiquantitative RT-PCR using mRNA from WT and KO livers at 4-7 months of age shows regucalcin expression in WT livers only.

1.5 BETA-CATENIN IN HEPATOCYTE SURVIVAL

1.5.1 Apoptosis and cell survival mechanisms

Apoptosis, also known as programmed cell death, is a critical component in liver injury and can occur via two major pathways: the death receptor (extrinsic) pathway or the mitochondrial

(intrinsic) pathway [194]. The death receptor pathway is activated by binding of death receptor ligands to receptors on the cell surface, while the mitochondrial pathway is initiated by intracellular stresses such as DNA damage or changes of intracellular Ca^{2+}. Of these, the death receptor pathway has been extensively studied in hepatocytes, since hepatocytes express an abundance of death receptors [195]. This is presumably due to evolutionary pressure which favored enhanced death receptor expression in the liver to help eliminate pathogens and cells damaged by toxins [196]. Thus, death receptor-mediated apoptosis in the liver is a critical component of homeostasis, as it targets damaged or abnormal cells for elimination without disrupting liver physiology [196, 197]. Of the six identified death receptors, Fas and TNF-α are considered to have pathologic significance in the liver [198]. Activation of the Fas-mediated apoptotic pathway has been linked to liver diseases such as hepatic inflammation, viral hepatitis, alcoholic hepatitis, non-alcoholic steatosis, cholestasis, and Wilson's disease [199-203]. Likewise, TNF-α mediated activation of apoptosis has been implicated in alcoholic hepatitis, ischemia/reperfusion, and fulminant hepatic failure [204-207].

1.5.2 The Fas pathway

Fas (also known as CD95) is a member of the TNF receptor family which is primarily activated by Fas ligand (FasL), a membrane-spanning protein typically expressed on the surface of activated T-cells, although soluble forms of FasL are also present in serum [195, 208]. Alternatively, the Fas pathway can be induced in mice via intravenous injection of Jo-2 antibody, a Fas agonist which causes death of the animal through massive hepatocyte apoptosis [209]. Activation of the receptor by FasL or Jo-2 causes trimerization of the receptor, which leads to formation of the death-inducing signaling complex (DISC) [210-212]. This complex consists of

the Fas-associated death domain (FADD), which contains a death effector domain (DED) [213] that is required for caspase-8 recruitment. Caspase-8, also called the initator caspase for its ability to activate executioner caspases such as caspase 3, 6, and 7, is activated at the DISC [214, 215]. In Type II cells such as hepatocytes, additional activation of the mitochondrial pathway by caspase-8 is required for apoptosis [216]. Caspase-8 cleavage of Bid, a proapoptotic protein that translocates to the mitochondria, triggers the release of cytochrome c, which activates caspase-9 and amplifies the apoptotic signal [217, 218].

Hepatocytes can be protected from the effects of Fas by the anti-apoptotic Bcl-2 family of proteins, including Bcl-2, myeloid cell leukemia factor-1 (Mcl-1), and Bcl-x_L, which inhibit the mitochrondrial pathway [219-222]. Alternatively, apoptosis can be inhibited at the level of caspase-8 by c-FLIP, a structural mimetic of caspase-8 that lacks proteolytic activity and binds directly to either caspase-8 or FADD [223-225]. Other inhibitors of Fas-mediated apoptosis include X-linked inhibitors of apoptosis (IAPs) and serine protease inhibitors (serpins) such as antitrypsin [226, 227].

Recently, it has been shown that Met sequestration of the Fas receptor can also prevent Fas-mediated apoptosis in hepatocytes [228]. Mice lacking Met were hypersensitive to Fas-induced apoptosis [229]; similarly, high doses of HGF sensitize apoptosis-resistant bid-deficient hepatocytes to Fas-induced cell death through increased dissociation of Fas from Met [230]. Further, YLGA, a peptide derived from the Met α-chain, inhibits Fas-mediated apoptosis in fatty liver disease [228]. The Met/Fas interaction has been demonstrated to regulate apoptosis in endothelial cells as well [231]. Therefore, the stoichiometric association of Fas, a death receptor, and Met, a growth factor receptor, in regulating cell survival and apoptosis appears to be mediated by the amount of Met present at the cell surface. Interestingly, as mentioned

previously, Met also forms a complex with β-catenin on the surface of hepatocytes, allowing for cross-talk between the β-catenin- and HGF-mediated signaling pathways and synergistic activation of cell proliferation [51, 53]. However, the role of this interaction in cell survival, if any, is unknown.

1.5.3 The TNF-α pathway

TNF-α is a pleiotropic cytokine that is critical to many diverse cellular processes in the liver, including proliferation, inflammation, and cell death [232]. Execution of these functions occurs when TNF-α binds to its major receptor TNF-R1, causing a conformational change in the receptor which initiates DISC formation similar to that formed during Fas-mediated apoptosis. In the case of TNF-α-mediated signaling, this complex is composed of TNF/TNF-R1 trimers and the TNFR-associated death domain (TRADD) [233-235]. TRADD then recruits FADD, TNF-associated factor 2 (Traf-2), and receptor-interacting protein-1 (RIP-1) [236]. If anti-apoptotic signals are blocked, FADD activates caspase-8, which cleaves other downstream effector caspases like caspase-3 as well as activates pro-apoptotic members of the Bcl-2 family such as Bax and Bak [237]. Thus, TNF-α-mediated apoptosis utilizes the same downstream apoptotic machinery as the Fas pathway, albeit independently of Bid [238-240].

Although activation of TNF-α signaling is capable of causing apoptosis, the default pathway is survival, due to the robust activation of NF-κB. NF-κB is a dimeric transcription factor that consists of five members: p50, p52, p65 (also known as RelA), c-Rel, and RelB, with the prototypical NF-κB complex consisting of a heterodimer between p50 and p65 [241, 242]. Activation of NF-κB has pleiotropic effects in a wide variety of cellular programs, including promoting cell survival and suppressing apoptosis by regulating the transcription of anti-

apoptotic proteins [243-245]. In the absence of signal, NF-κB is retained in the cytoplasm by members of the inhibitory IκB complex [242]. Activation of the NF-κB pathway occurs when the IκBs undergo phosphorylation, ubiquitination, and degradation [246]. The kinases responsible for this phosphorylation are IκB kinases (IKKs), which are recruited to activated TNF-R1 by RIP-1 and Traf-2, which are part of the DISC complex mentioned previously [247]. Loss of the IκB complex after IKK phosphorylation liberates NF-κB, which then translocates to the nucleus [248]. Full activation of NF-κB requires further post-translational modification by phosphorylation of its various subunits through the action of kinases such as protein kinase A, casein kinase II, PKCζ, and GSK-3β [241, 249-252]. NF-κB is then able to provide survival signals to the cell through transcription of anti-apoptotic genes such as IAPs, c-FLIP, TRAFs, and Bcl family members [253-255].

A common means of inducing TNF-α signaling other than administration of TNF-α itself is through the use of lipopolysaccharide or LPS, an endotoxin derived from gram-negative bacteria [198, 256, 257]. The interaction of LPS with macrophages stimulates the release of TNF-α, which induces downstream signaling as described before. Because of the rapid induction of pro-survival signals by NF-κB, activation of the TNF-α-mediated apoptotic pathway both *in vivo* and *in vitro* requires hepatocyte sensitization through administration of transcriptional repressors, which suppress NF-κB-dependent gene transcription. D-galactosamine (GalN), one of the most commonly used sensitizing agents, suppresses transcription by depletion of UTP, thus inhibiting RNA synthesis and rendering hepatocytes susceptible to the effects of endotoxin [256, 258]. The half-life of GalN is approximately 3-4 hours [259, 260], which allows for significant and irreversible activation of the apoptotic machinery. Another type of repressor, actinomycin D (ActD), inhibits RNA synthesis by binding DNA duplexes and thus prevents transcription [261].

Thus, the toxicity of LPS or TNF-α is manifested as apoptotic injury only upon arrested transcription and functional translation of NF-κB-dependent pro-survival targets.

1.5.4 β-catenin in cell survival and apoptosis

β-catenin signaling appears to play a positive role in cell survival, as evidenced by experiments in which this pathway is either repressed or enhanced. Deletion of β-catenin or inhibition of Wnt signaling has been shown to induce apoptosis in several *in vivo* and *in vitro* models. Our laboratory has previously shown an increase in hepatocyte apoptosis in developing and adult livers following hepatocyte-specific deletion of β-catenin [89, 94]. Inhibition of β-catenin in cultured E10 livers by an antisense morpholino oligomer resulted in a higher number of apoptotic nuclei [89]. We have also shown that loss of regucalcin, a downstream target of β-catenin, promotes apoptosis in cultured human hepatoma cell lines [38]. There are several examples outside of liver as well. For example, overexpression of Dkk-1, an inhibitor of the Wnt/β-catenin pathway, induces apoptosis in human glioma cells through an altered balance in Bcl-2 and Bax protein levels [262].

Apoptosis can also be suppressed through activation of the Wnt/β-catenin pathway. Resistance to cancer therapy-mediated apoptosis in Wnt-1 expressing cells is mediated through activation of β-catenin signaling concurrent with inhibition of cytochrome c release [263]. Treatment of hepatic stellate cells with Wnt3a suppresses TRAIL-induced apoptosis and contributes to cell survival [264]. Additionally, constitutively active β-catenin inhibits the pro-apoptotic protein Bax in renal epithelial cells [265]. Thus the way in which β-catenin may impact cell survival may be highly tissue- and context-dependent.

The role of β-catenin specifically in hepatocyte survival warrants a comprehensive investigation. As discussed, β-catenin is known to associate with Met and Met in turn is known to associate with Fas. It will be critical to address if these interactions represent a functional cross-talk and thus may impact or regulate hepatocyte survival. However, somewhat paradoxically, activated β-catenin has also been shown to directly bind to and inhibit NF-κB by associating with the p65 subunit, thus preventing its translocation and target gene activation [266, 267]. Therefore, although β-catenin appears on the whole to contribute to the anti-apoptotic phenotype as indicated by an increase in basal hepatocyte apoptosis evident in KO livers, its role and regulation in hepatocyte survival remains to be elucidated in-depth, in light of diverse interactions at multiple levels within the death pathways.

1.6 CLINICAL IMPLICATIONS

1.6.1 Hepatocyte Transplantation

Orthotopic liver transplantation is currently the most effective and widely used method for treating liver insufficiency caused by chronic liver diseases such as fulminant hepatic failure, end-stage liver disease, and metabolic liver diseases. However, the relatively high risk of the surgery, combined with a requirement for lifelong immunsuppression and a shortage of healthy donor organs, has prompted clinicians to seek out promising alternatives. One such treatment is hepatocellular transplantation (HCT). HCT offers several advantages over whole-organ transplantation. First, HCT is a less-invasive procedure with fewer complications and lower morbidity [268]. Additionally, cells from one donor can be used to treat multiple patients, and

are obtained from livers that would be discarded or otherwise deemed unfit for transplantation. Finally, these cells can be manipulated by *ex vivo* gene therapy to incorporate genes that correct a metabolic disorder [269] or those that are aimed at preventing immune rejection. Donor hepatocytes are generally infused through the portal vein or the spleen [270], where they migrate to the liver and display normal architecture and morphology [271, 272]. HCT has been quite effective in human trials, treating inborn errors of metabolism such as Crigler-Najjar syndrome, alpha-1 antitrypsin deficiency, factor VII deficiency, ornithine transcarbamylase deficiency, and glycogen storage disease [273-278], as well as fulminant hepatic failure [268, 279-281]. However, issues of engraftment, donor cell viability, loss of function, and availability have limited the effectiveness and widespread use of HCT.

Differentiation of embryonic stem (ES) cells, and more recently induced pluripotent stem cells (iPSC), into hepatocytes has been seen by many as a viable source of cells for transplantation, as their successful differentiation would supply an unlimited amount of high quality hepatocyte-like cells [282-285]. Several groups claimed to have successfully differentiated ES cells into hepatocyte-like precursors [286, 287], although their use in transplant therapy has yet to be determined. Indeed, selective and careful differentiation can produce a hepatic gene expression profile in a fraction of ES cells [288, 289]. Some of these hepatocyte-like cells even express cytochrome P450 genes and demonstrate drug metabolic activity, which is necessary for proper liver function [290-292]. Other groups have had success in differentiating other cell types, such as human umbilical cord cells [293], hematopoietic stem cells [294], cord blood cells [295], fetal liver progenitor cells [296], murine oval cells [297], embryonic liver explants [298], and hepatoblasts transduced *ex vivo* with lentiviral vectors [299], into hepatocyte-like cells. However, these cells have a limited ability to repopulate the liver due to their

relatively immature phenotype [300], and often form teratomas following transplantation, even after partial differentiation in culture [301, 302]. Overall, although the use of ES cells and other precursor cells in treating hepatic insufficiency is promising, much more work needs to be done to determine the safety, functionality, and efficacy of these cells [283].

Another potential solution to the shortage of donor hepatocytes for HCT is the use of non-transformed hepatocyte cell lines. Conditionally immortalized hepatocytes have been shown to provide metabolic support in chronic liver insufficiency [303]. Similarly, successful genetic manipulation might also provide a source of hepatocytes with enhanced proliferative, engraftment, and/or survival capabilities, thus reducing or eliminating the bottlenecks associated with supply and quality of donor hepatocytes.

The role of β-catenin in hepatic differentiation, proliferation, and survival makes this an ideal candidate for gene therapy. Several studies have described the effects of constitutively active β-catenin in hematopoietic models. One group found that introduction of a stable form of β-catenin restores multipotency to committed hematopoietic progenitors, and promotes expansion of these progenitors in culture [304, 305]. Similarly, another group reported that expression of a mutant active β-catenin in normal hematopoietic progenitors impairs differentiation [306]. Finally, Reya et al. found that hematopoietic stem cells transduced with active β-catenin displayed more efficient reconstitution, higher levels of chimerism, and increased proliferation when transplanted into lethally irradiated mice [307]. Therefore, it is possible that overexpression of a mutated non-degradable, non-oncogenic form of β-catenin in hepatocytes, such as that seen in our S45D TG mice, will give these cells a growth and engraftment advantage in transplantation, similar to that seen in HSCs. Based on the roles of β-catenin in cell

proliferation, survival, and as a master differentiation factor for hepatocytes, it may be a desirable candidate to promote transplanted cell engraftment and function.

1.6.2 Oxidative stress, apoptosis, and liver disease

Upregulation of Fas-mediated apoptosis has been implicated in the progression of many human liver diseases. For example, apoptosis is increased in patients with alcoholic hepatitis, and the number of apoptotic cells correlates with disease severity [308]. As mentioned previously, one of the mechanisms contributing to apoptosis in alcoholic hepatitis is oxidative stress, which may cause cell death through mitochondrial dysfunction [309]. Additionally, both the Fas receptor and FasL are strongly expressed in patients with this disease, which suggests that FasL-positive hepatocytes can induce apoptosis in adjacent Fas-expressing hepatocytes [195, 308, 310]. Another disease in which apoptosis occurs primarily through Fas upregulation is non-alcoholic steatohepatitis [311]. Toxic bile salt accumulation in cholestatic liver disease results in induction of apoptosis in a Fas-dependent manner independent of FasL [312]. Fas signaling is also an essential component in hepatocyte injury and apoptosis in Wilson's disease [313]. In viral hepatitis, apoptosis, which is regulated either by the pathogen itself or the host cytotoxic T cell-mediated immune response, occurs primarily through the Fas pathway [314]. Fas receptor expression has been correlated with areas of inflammation and necrosis in hepatitis B [315], and is also present in livers of patients with hepatitis C [316].

The TNF-α pathway is also dysregulated in a wide range of liver diseases. In addition to Fas, TNF-R1 is upregulated in livers of patients with alcoholic liver disease [196, 317]. Serum levels of TNF-α are also increased in alcoholic steatohepatitis patients and correlate with disease progression and mortality, suggesting that this pathway plays a role in the pathogenesis of this

disease [318]. Oxidative stress caused by glutathione depletion can exacerbate the severity of alcoholic steatohepatitis by sensitizing hepatocytes to TNF-α [319]. TNF-R1 and TNF-α are upregulated in both hepatitis B and hepatitis C, probably as a result of inflammation due to viral infection, and are associated with disease progression and severity [320-323]. TNF-α-mediated apoptosis also plays a role in ischemia/reperfusion injury and fulminant hepatic failure, likely through overaction of the immune system and generation of reactive oxygen intermediaries [324].

Because apoptosis is a common feature in the pathology of many different liver diseases, therapeutic inhibition of this process has the potential to reduce injury and improve patient survival [195]. Several therapies aimed at reducing apoptosis are already being utilized in pre-clinical and clinical trials in the treatment of liver disease. Infliximab, a monoclonal antibody raised against TNF-α, has been shown to attenuate hepatic injury in a pilot study [325]. Administration of IDN-6556, a caspase inhibitor, lowered aminotransferase activity and decreased liver damage in hepatitis C patients [326-328]. Chronic cholestatic liver disease is commonly treated with ursodeoxycholic acid, which is believed to attenuate both oxidative stress and apoptosis [329, 330]. Exogenous anti-oxidant therapy has also been utilized to treat liver injury and apoptosis related to ROS/RNS; however, the results of these studies have been mixed [133].

Other novel anti-apoptotic therapies may include activators of pro-proliferative pathways, which would shift the balance towards cell survival and away from death. The Wnt/β-catenin pathway is one such example of a pro-survival pathway that may be amenable to manipulation. Although the precise contribution of β-catenin to cell survival is currently unknown, there is some evidence to suggest that β-catenin plays an anti-apoptotic role in liver, as outlined above.

However, it remains elusive how components of the Wnt pathway, specifically β-catenin, may interact with Fas- or TNF-α-dependent cell death pathways. Based on such interactions, it may become relevant to modulate this pathway for improving outcomes in apoptosis-mediated liver injury both experimentally and clinically.

2.0 LIVER REGENERATION ADVANTAGE IN MICE OVEREXPRESSING SERINE-45 MUTATED BETA-CATENIN

2.1 ABSTRACT

An important role for β-catenin in liver regeneration has been observed in mice, rats and more recently in zebrafish. In order to elucidate any impact of β-catenin stimulation on liver regeneration, we utilized transgenic (TG) mice that express Ser45 mutated β-catenin under an albumin promoter/enhancer. In cell culture, TG hepatocytes exhibit enhanced cell proliferation as measured by thymidine incorporation, which was increased almost 2-fold over wild-type (WT) mice at D5 in culture. Using the partial hepatectomy (PHx) model of liver regeneration, we found a dramatic increase in hepatocytes in S-phase in the TG and WT livers at 40 hours (40H) and 72H, respectively. Coincident with the earlier onset of proliferation, cytoplasmic stabilization of β-catenin and its nuclear translocation as well as a dramatic increase in total and nuclear cyclin-D1 protein is also evident at 40H post-PHx in TG and at 72H in the WT. In order to investigate if there is any difference in tyrosine (Y)-phosphorylation, an alternate mechanism of β-catenin activation, we examined Y654-β-catenin levels in regenerating livers in WT and TG mice. An increase in Y-654-β-catenin was evident at 40H and 72H in TG and WT mice, respectively. Finally, we tested if exogenous stimulation of β-catenin signaling via hydrodynamic delivery of Wnt-1 naked DNA could impact regeneration in WT mice. We

observed a significant increase in total Wnt-1, β-catenin, glutamine synthetase, cyclin-D1 and PCNA in the Wnt-1 and not the control plasmid-injected mice. In conclusion, stimulation of Wnt/β-catenin signaling is advantageous for liver regeneration through induction of the downstream target cyclin-D1, and thus might have implications in regenerative medicine.

2.2 BACKGROUND

The Wnt/β-catenin pathway is an evolutionarily conserved pathway that has proven to be essential in liver health and repair [331]. In adult resting liver, active β-catenin is limited to hepatocytes in the centrizonal area where it regulates expression of target genes such as glutamine synthetase (GS) and other genes involved in xenobiotic metabolism, and thus is implicated in liver zonation [124, 332]. However, in the rest of the unchallenged adult liver, Wnt/β-catenin signaling is essentially quiescent. The overall steady-state condition is characterized by the phosphorylation and subsequent degradation at key serine/threonine sites that is the hallmark of β-catenin turnover. Hence, β-catenin is localized predominantly at the cell membrane and is largely absent from the cytoplasm and nucleus in a mature hepatocyte.

At the cell membrane, β-catenin plays an important role in cell-cell adhesion, forming a bridge between the actin cytoskeleton and E-cadherin [40, 41]. Phosphorylation of β-catenin at tyrosine residue 654 decreases its interaction with E-cadherin [42, 43]. β-Catenin also forms a complex with HGF receptor Met [49-51]. Upon HGF signaling β-catenin becomes phosphorylated at tyrosine residues 654 and 670 and translocates to the nucleus to induce target gene expression [52, 53].

The partial hepatectomy (PHx) model, in which two-thirds of the rat or mouse liver is removed and the remaining lobes enlarge to restore the original liver mass [105], is an ideal environment to study the role of β-catenin in controlled growth after injury. Activation of β-catenin signaling during liver regeneration and delay in regeneration due to its loss has been reported in rat and mouse models [94, 126-128]. The decrease in hepatocyte proliferation due to the absence of β-catenin, as well as the role of β-catenin in regulating cell proliferation, has motivated us to examine the behavior of hepatocytes that express Ser45 mutated β-catenin. Ser45 is a key residue in β-catenin that regulates GSK-3β/Axin/CK1/APC dependent degradation of this protein. The mutation of β-catenin at S45 compromises this process and contributes to its overall stabilization [333]. Here, we report a growth advantage both in culture of hepatocytes expressing S45-mutated β-catenin and after partial hepatectomy in TG mice due to upregulation of cell cycle regulators such as cyclin-D1. We also demonstrate that tyrosine-phosphorylation of β-catenin may play an important role during initiation of the regenerative process. Finally, we show that induction of the β-catenin pathway through hydrodynamic delivery of Wnt-1 plasmid injection in wild-type mice increases β-catenin stabilization along with downstream targets and associated cell proliferation after partial hepatectomy. In conclusion, we demonstrate that exogenous activation of β-catenin through Wnt-1 may be a useful strategy to improve liver regeneration provided useful positive modulators of the pathway become available.

2.3 MATERIALS AND METHODS

2.3.1 Animals

Liver-specific transgenic mice overexpressing human β-catenin gene were described previously by our group [132]. Briefly, we generated β-catenin transgenic mice in a C57BL/6 background that express the human β-catenin gene under an albumin promoter/enhancer. Here, we utilize transgenic mice that were generated similarly but in FVB background and with the exception that the β-catenin gene was engineered to carry a mutation affecting serine-45, a site relevant in β-catenin degradation [333]. The transgenic mice express S45D-β-catenin mutant under an albumin promoter/enhancer and are henceforth referred to as TG mice. The TG mice used in the study (~3 months old males) display an overall increase in β-catenin protein at the hepatocyte membrane only and lack nuclear or cytoplasmic localization in the normal state, although it was observed temporally in TG mice less than 2 months of age.

2.3.2 Surgery

All animal usage was approved by the Institutional Animal Care and Use committee at the University of Pittsburgh. Around 3-month old male FVB WT and TG mice were subjected to partial hepatectomy. Briefly, three of the five lobes in a mouse liver are surgically removed [104]. The remaining 2 lobes regenerate and the liver mass is completely restored by the 14^{th} day after PHx [334]. Three-five WT and TG mice were sacrificed at time 0, 10 hours (H) 20H, 40H, 72H, 4 days (D4), D5, D9, D14, 1 month (M) and 3M. Livers were processed for paraffin embedding and protein isolation as described previously [94].

2.3.3 Primary hepatocyte culture

Hepatocytes were isolated from 3-month old WT and TG mouse livers (n=3) using a modified 2-step collagenase perfusion protocol [335, 336], resuspended in minimal essential media (MEM) supplemented with 10% fetal bovine serum (FBS), and seeded at a density of 200,000 live cells per ml onto either wet or dry collagen-coated plates. After 2 hours, media were replaced with a chemically defined MEM-based growth medium containing insulin/transferrin/selenium (ITS; 1g/L), dexamethasone (10^{-7}M), HGF (40ng/ml), and epidermal growth factor (EGF; 25ng/ml) as described previously [337]. Plates were incubated at 37°C in 5% CO_2 until harvested for proliferation and viability assays as described below.

2.3.4 Wnt-1 Plasmid injection

The recombinant Wnt-1 expression plasmid was purchased from Upstate Biotech (Catalogue # 21-121). The plasmid contains HA-tagged mouse *Wnt-1* cDNA under the control of the CMV promoter. For hydrodynamic delivery of naked DNA, 1 μg/gm of body weight of Wnt-1 plasmid or pcDNA3 control plasmid (Invitrogen, San Diego CA) was administered via hydrostatic tail vein injection to CD-1 mice weighing around 18 grams, as described previously [338]. Twenty-four hours after the injection, the mice were subjected to PHx. Mice were sacrificed at 30 hours after PHx and livers processed for total and nuclear protein extraction and immunohistochemistry.

2.3.5 Protein Extraction, Immunoprecipitation, and Western Blots

Whole-cell lysates from mouse livers were prepared by homogenization using RIPA buffer (9.1 mmol/L dibasic sodium phosphate, 1.7 mmol/L monobasic sodium phosphate, 150 mmol/L sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate [pH adjusted to 7.4]) containing fresh protease and phosphatase inhibitor cocktails (Sigma, St. Louis, MO) [94]. Nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit as per the instructions (Thermo Fisher Scientific, Rockford, IL). The concentration of the protein in all lysates was determined by the bicinchoninic acid assay using BSA as a standard.

For immunoprecipitation studies, 500 μg of cell lysate from WT and TG livers harvested at T0, 40H, and 72H after PHx (prepared in RIPA buffer in the presence of inhibitors) was precleared with goat IgG together with Protein A/G agarose for 30 m at 4^0C. After centrifugation, the supernatants were incubated with either Met antibody for 1 h at 4^0C followed by overnight incubation with Protein A/G agarose at 4^0C, or agarose-conjugated β-catenin antibody overnight at 4^0C. Pellets were collected the next day, washed in PBS containing inhibitors, resuspended in loading buffer, and subjected to electrophoresis, as described below.

Proteins were subjected to SDS-PAGE [126]. Briefly, 50 μg of protein from cell or liver lysate or 15-20 μl of eluate from IP studies was resolved on 7.5% or 12% precast SDS-PAGE gels (ISC BioExpress, Kaysville, UT) using the mini-PROTEIN 3-electrophoresis module assembly (Biorad, Hercules, CA), followed by transfer to either Immobilon-PVDF membranes (Millipore, Bedford, MA) or nitrocellulose membranes (Bio-Rad Labs, Hercules, CA). Membranes were stained with Ponceau-S solution to confirm equal loading and then blocked in either 5% nonfat dry milk in blotto solution (20mM TRIS-HCl pH 7.5, 150 mM NaCl, 0.1%

Tween 20), 10% Block Hen (Aves Labs, Inc, Tigard, OR) followed by 5% milk in blotto, or 5% BSA in blotto, followed by incubation with primary antibody diluted in either 5% milk/blotto or 5% BSA/blotto for 1-2 h or overnight. Membranes were washed and incubated in horseradish-peroxidase conjugated secondary antibodies (Chemicon, Temecula, CA) for 1h followed by washing. Secondary antibodies were goat anti-mouse (1:50,000), donkey anti-rabbit (1:25,000), donkey anti-goat (1:25,000) (Chemicon), and goat anti-chick (1:15,000) (Aves Labs, Inc., Tigard, OR). Proteins were detected by Super-Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and visualized by autoradiography. Primary antibodies used in this study were against β-catenin (1:1000) (BD Biosciences, San Jose, CA), glutamine synthetase (1:500), Wnt-1 (1:200), lamin B1 (1:200), Met (1:200), and c-myc (1:200) (all from Santa Cruz Biotechnology, Santa Cruz, CA), cyclin-D1 (1:500) (Neomarkers, Fremont, CA), HGF (1:500) (R&D Systems, Minneapolis, MN), Y654-Phospho-β-Catenin Antibody [339] (Aves Labs, Inc.), and actin (1:5000) (Chemicon). Blots were stripped with IgG elution buffer (Pierce, Rockford, IL) for 30 m before re-probing.

2.3.6 Immunohistochemistry

Immunohistochemistry was performed on mouse livers and primary hepatocyte cell cultures. Tissues fixed in 10% formalin and embedded in paraffin were sectioned at 4 μm onto Superfrost Plus glass slides (Fisher Scientific, Pittsburgh, PA). Sections were stained with hematoxylin and eosin (H&E) in order to determine the mitotic index. Additional sections were microwaved in either citrate buffer or 1% $ZnSO_4$, pretreated with 3% H_2O_2 to eliminate endogenous peroxidases, and blocked using protein blocking agent (Thermo Scientific, Waltham, MA) or Super Block (Scy Tek, Logan, UT). Alternatively, cell culture plates

containing hepatocyte cultures were fixed with 2% paraformaldehyde for 15 m, permeabilized with 2N HCl/1% Triton-X-100, and blocked in Ultra V Block (Lab Vision Products, Fremont, CA). Primary antibodies used for this project were anti-PCNA (1:5000) (Dako, Denmark), anti-β-catenin (1:50) (BD Biosciences), glutamine synthetase (1:50) (Santa Cruz Biotechnology), Y654-Phospho-β-Catenin Antibody (1:100) (Aves Labs, Inc.) and anti-cyclin-D1 (1:50) (Neomarkers). Secondary antibodies were biotinylated donkey anti-mouse (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), donkey anti-goat (Chemicon), or goat anti-chick (Aves Labs, Inc.), all used at a 1:500 dilution. Immunohistochemistry was performed using the Vectastain ABC Elite kit and developed using DAB (Vector Laboratories, Inc., Burlingame, CA). The slides and culture plates were counterstained with either Harris or Shandon hematoxylin and mounted with cytoseal or crystal mount (Biomeda Corp., Foster City, CA).

2.3.7 Cell Growth and Viability Assays

A [^{3}H]thymidine uptake assay was utilized as previously described to measure cell proliferation [340, 341]. Briefly, WT or TG primary hepatocytes were dosed with 2.5 μCi/ml of [^{3}H]thymidine (MP Biomedicals, Solon, OH) 48h before harvesting and incubated at 37°C in 5% CO_2. Plates were harvested at two time points – 48 and 120 hours post-plating - and assessed for thymidine incorporation as follows. 1.5 ml of cold 5% (w/v) trichloroacetic acid was added to each well after removal of media. The plates were placed at 4°C for 2 h and then were washed and allowed to air-dry. The precipitate was solubilized by the addition of 1 ml of 0.33 M NaOH, and an aliquot was added to Universol (MP Biomedicals). The counts/min of the solubilized precipitates were determined with a scintillation counter (Beckman Instruments, Palo Alto, CA).

Cell growth and viability was measured using the MTT assay as described previously [342]. Briefly, 10% vol/vol of 5 mg/ml (3,4.5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) was added to cultures of WT or TG primary hepatocytes for 30 m. The media were aspirated after 30 m followed by addition of 1 ml of isopropanol. After shaking the plate gently for 2 m, 200 µl of this solution was examined for conversion to colored formazan by spectrophotometric analysis (490 nm).

2.3.8 Statistical Analysis

All experiments were performed three or more times. Representative data from experiments is presented. Autoradiographs of various western blots were scanned and subjected to densitometry using the ImageJ software. Mean integrated optical density (IOD) was compared between various groups. Statistical assessment of significance was made by the Student's *t*-test (Kaleidagraph, Synergy Software). A *P* value of less than 0.05 or 0.001 was considered significant (*) or extremely significant (**), respectively.

2.4 RESULTS

2.4.1 Increased growth of TG hepatocytes in culture

Hepatocytes from WT and TG mice were isolated and cultured in the presence of HGF and EGF. The cells were examined under phase-contrast microscopy 48 hours and 120 hours after plating. WT and TG hepatocytes appeared morphologically indistinguishable at 48H of

culture. However, at 120H, TG hepatocytes appeared more flattened, spread out, and healthy as compared to WT suggesting more robust growth (Fig. 3A). Based on this observation, we compared the proliferation and viability of TG and WT hepatocytes in culture. The proliferative capacity of these hepatocytes was assayed by adding [3H] thymidine to the cultures as detailed in the Methods. TG hepatocytes exhibited around 2-fold ($P < 0.05$) increase in thymidine incorporation over WT after 120H (Fig. 3B), suggesting greater proliferation by the TG cells in culture. To confirm this result, we cultured WT and TG cells for 48 and 120 hours and stained them with proliferating cell nuclear antigen (PCNA), a marker of DNA synthesis and cell proliferation. Figure 3C shows that while the number of PCNA-positive cells is unchanged over time in cultures of WT hepatocytes, there is a 27% increase in PCNA positivity from 48 to 120 hours in TG cultures. Next, we examined the cell viability of WT and TG hepatocytes grown in the presence of HGF and EGF as measured by (3,4.5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) assay, which also showed significantly greater viability in hepatocytes from TG as compared to WT mice ($P < 0.05$) at 120H (Figure 3D). Thus, overall the TG hepatocytes exhibit *in vitro* growth advantages over WT hepatocytes in culture.

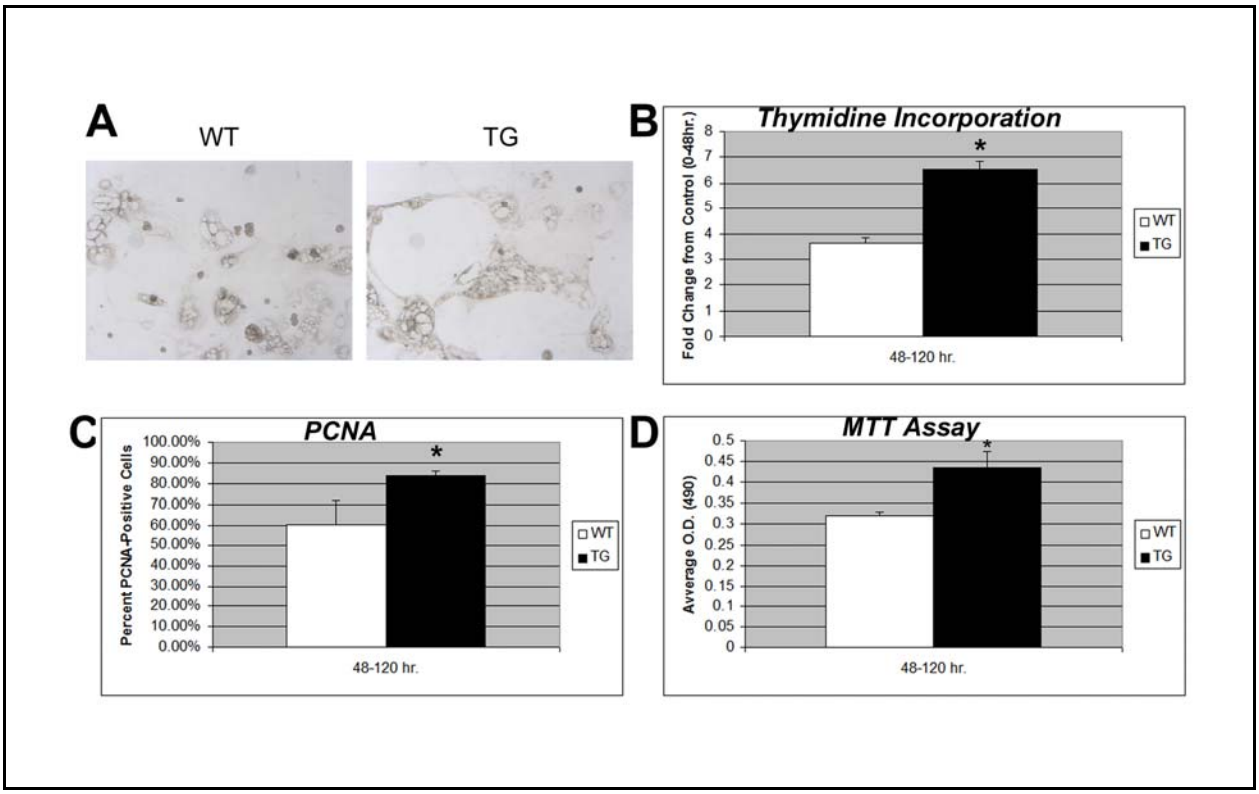

Figure 3: TG hepatocytes have a growth advantage over WT cells in culture. **(A)** WT and TG hepatocytes in culture are morphologically distinct at 120H. **(B)** Increased [3H] thymidine uptake by TG hepatocytes as compared to WT cells. The counts were normalized to values at 40H after culture for each group and presented as fold change. **(C)** While numbers of PCNA-positive hepatocytes remained constant between 40H and 120H in WT hepatocyte culture, a net and significant gain in numbers of PCNA-positive cells was evident between 40H and 120H in the TG hepatocytes. **(D)** A significant increase in cell viability as determined by MTT assay was observed in TG versus WT hepatocytes at 120H in culture.

2.4.2 β-catenin overexpression provides a regenerative advantage to TG mice over WT after partial hepatectomy

WT and TG mice were subjected to partial hepatectomy (PHx) in order to determine the proliferative status of β-catenin overexpressing livers after injury. Regenerating livers from WT and TG mice were harvested at various time points as indicated in the Methods. These time

points reflect DNA replication and peak mitosis reported at around 48 hours after PHx in C57BL/6 mice [343] and around 48-72 hours in FVB mice [344]. Since we identified an *in vitro* growth advantage of the TG hepatocytes, we also included the early time points for a more thorough analysis. Finally, we chose the later time points to determine any effect of excess β-catenin on termination of regeneration or identify any late consequences such as growth dysregulation.

WT livers at 40 hours after PHx exhibit very little active mitoses upon histological examination (average <1 cell per 20X field), suggesting that hepatocytes have not undergone cell division at this time. Interestingly, TG livers are undergoing active mitosis at this time (average 9 per 20X field) (Figure 4A and 4B). At 72 hours, the WT livers display 11-12 mitotic figures per 20X field, while the numbers in TG are in excess of 19-20 mitotic figures per 20X field.

Next, PCNA immunohistochemistry was performed to address the state of cell cycle progression in the WT and TG livers after PHx. A highly significant ($p<0.001$) increase in the numbers of PCNA-positive cells representing hepatocytes in S-phase was evident even at 40 hours in TG mice (448 +/-33.2), which is completely lacking in the corresponding WT livers at this stage (1.9 +/- 1.9) (Figure 4C and 4D). At 72 hours, both WT and TG livers display comparable numbers of PCNA-positive hepatocytes (329 +/- 78.6 vs. 323 +/- 45.3, respectively). At D4 and D5, the numbers of PCNA-positive cells were greater in WT than TG ($P < 0.05$) (223 +/- 106 vs. 64.9 +/- 68.4 for D4 and 43.1 +/- 14.2 vs. 25.5 +/- 8.4 for D5). By D9 and D14, the number of PCNA-positive cells has fallen to less than 20 cells per field in both WT and TG. Similarly, insignificant differences in the numbers of PCNA-positive cells are observed in TG and WT hepatocytes at 1 and 3 months after hepatectomy. Thus, β-catenin overexpression leads to earlier entry of hepatocytes into the cell cycle and more robust hepatocyte proliferation in TG

livers as compared to WT livers after partial hepatectomy. However, this increase in proliferation is transient and not sustained and thus does not lead to any phenotype of growth dysregulation in TG livers up to 3 months after PHx.

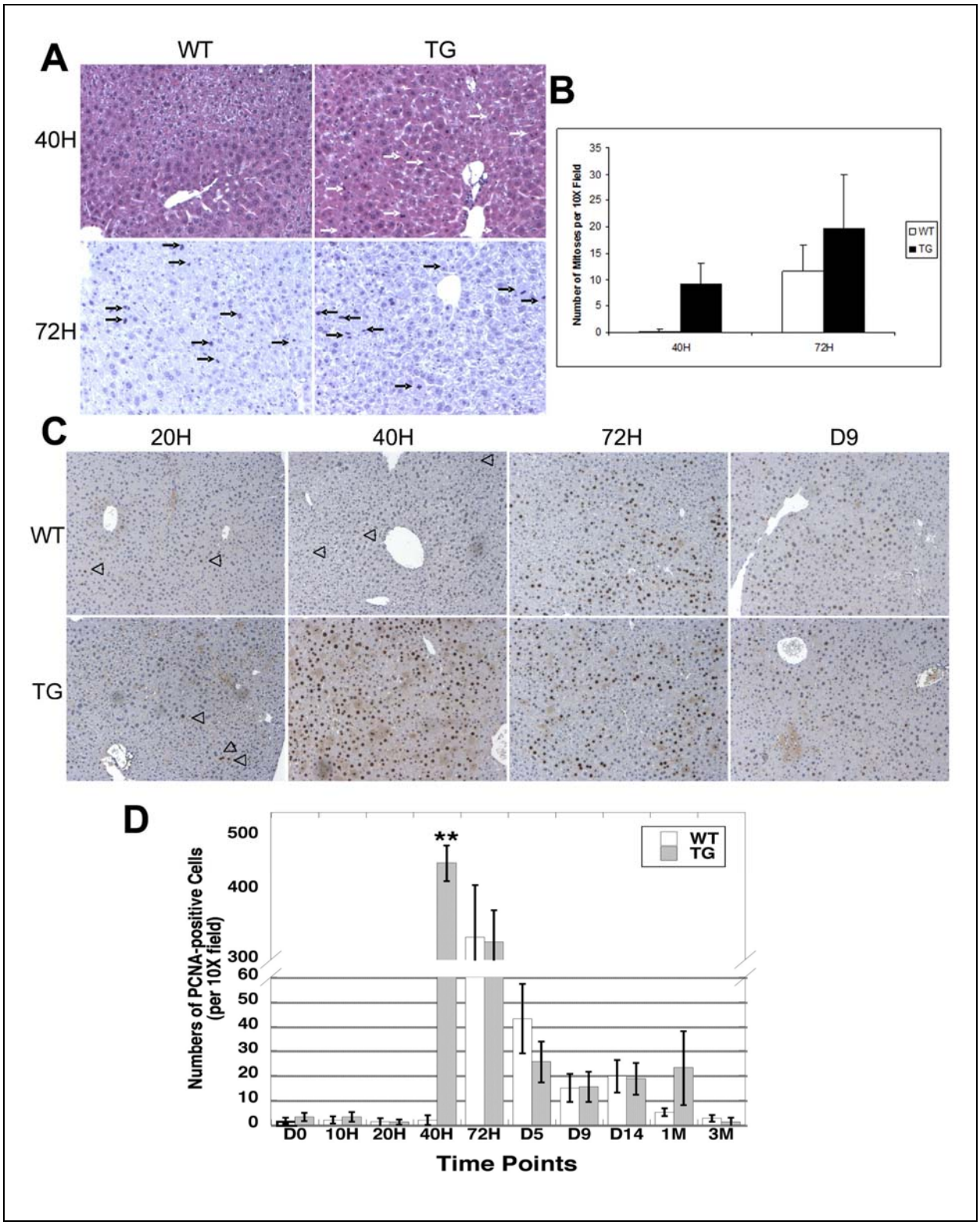

Figure 4: There is a dramatic upregulation of proliferation 40 hours after PHx in TG mice. (A) At 40 hours (40H), WT livers are quiescent; however, TG livers are clearly undergoing mitosis. Both WT and TG livers have

approximately the same number of mitotic figures at 72H. **(B)** Quantification of the number of mitotic figures in representative 10X fields (n=5) show TG livers to have significantly more cells in mitosis at 40H and 72H than corresponding WT livers. **(C)** At 40H TG livers show massive PCNA-positive hepatocytes in S-phase, while WT livers are PCNA-negative. WT livers show many PCNA-positive hepatocytes at 72H post-PHx. **(D)** Quantification of the number of PCNA-positive cells in 5 representative 10X fields from three WT and TG livers show significantly greater numbers of PCNA-positive hepatocytes in TG livers at 40H ($p<0.001$).

2.4.3 Early β-catenin activation in TG mice imparts a regenerative advantage over WT after PHx

Next, we examined β-catenin protein expression through western blot analysis to verify the contribution of β-catenin in the regenerative process in WT and TG and to specifically address the mechanism of enhanced hepatocyte proliferation in TG livers. TG livers show an increase in nuclear β-catenin at 40 hours, which corresponds to the peak of hepatocytes in S-phase of the cell cycle in these mice. Higher nuclear translocation of β-catenin is observed at 72 hours in the WT livers (Figure 5B).

We next investigated the expression of several known downstream targets of β-catenin to determine which, if any, were expressed earlier or were upregulated in TG mice after PHx. Cyclin-D1 protein, a downstream target of β-catenin [25, 26] that controls the critical G1/S transition in the cell cycle [345], is increased in total cell lysate in the TG livers at 40 hours (Figure 5A). This mirrored an increase in nuclear cyclin-D1 at 40 hours and also was evident at 72 hours in TG livers. No increase in total or nuclear cyclin-D1 was observed in WT liver at 40 hours, however its nuclear levels were increased over all preceding time points at 72 hours after PHx.

We also examined protein levels of other β-catenin targets, such as glutamine synthetase, which is involved in glutamine metabolism [34], and c-myc, a transcription factor best known for its oncogenic properties [27]. Interestingly, these remained unchanged between the TG and WT groups at all time points during the regenerative process (Figure 5A). Thus, the regenerative and proliferative advantage seen in TG mice is due to an early nuclear translocation of β-catenin in TG mice, which led to an upregulation of cyclin-D1, an important cell cycle regulator.

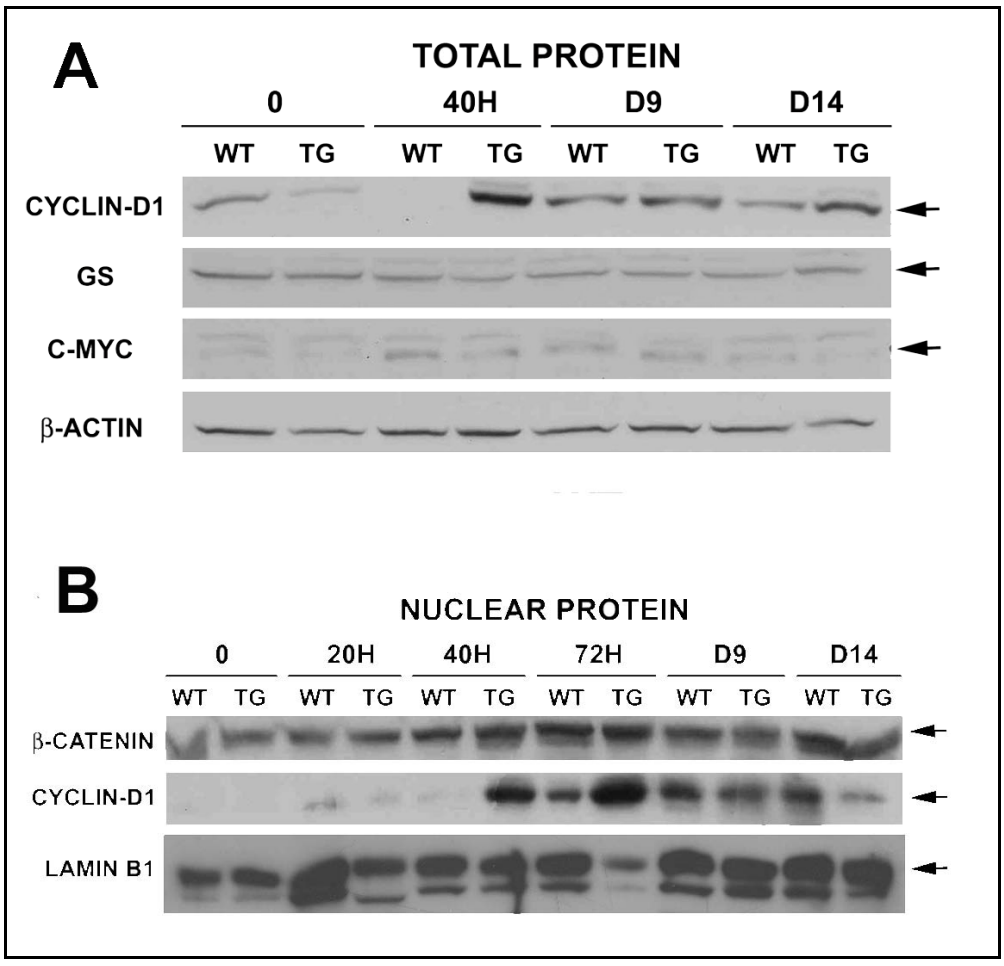

Figure 5: β-catenin and cyclin-D1 increase earlier in TG mice during regeneration as compared to WT mice. **(A)** The increase in cyclin-D1 in TG livers at 40H after PHx is seen in whole cell extracts, while levels of glutamine synthetase and c-myc remain unchanged. Actin verifies equal loading. **(B)** Nuclear extracts from regenerating

livers show an increase in both nuclear β-catenin and cyclin-D1 that begins at 40H in TG and 72H in WT livers. Lamin B1 verifies equal loading.

Finally, we investigated β-catenin redistribution by immunohistochemistry in the WT and TG livers in pre-hepatectomy and regenerating livers at 40 hours. The TG livers at Time 0 exhibit higher β-catenin at the hepatocyte membrane than the WT livers as expected. Forty hours after PHx, while β-catenin was still predominantly observed at the hepatocyte membrane in the WT, a noteworthy cytoplasmic stabilization and nuclear translocation is evident in TG liver sections (Figure 6). This is in agreement with the western blot analysis presented above and clearly demonstrated an early and robust β-catenin activation in the TG mice during the process of liver regeneration.

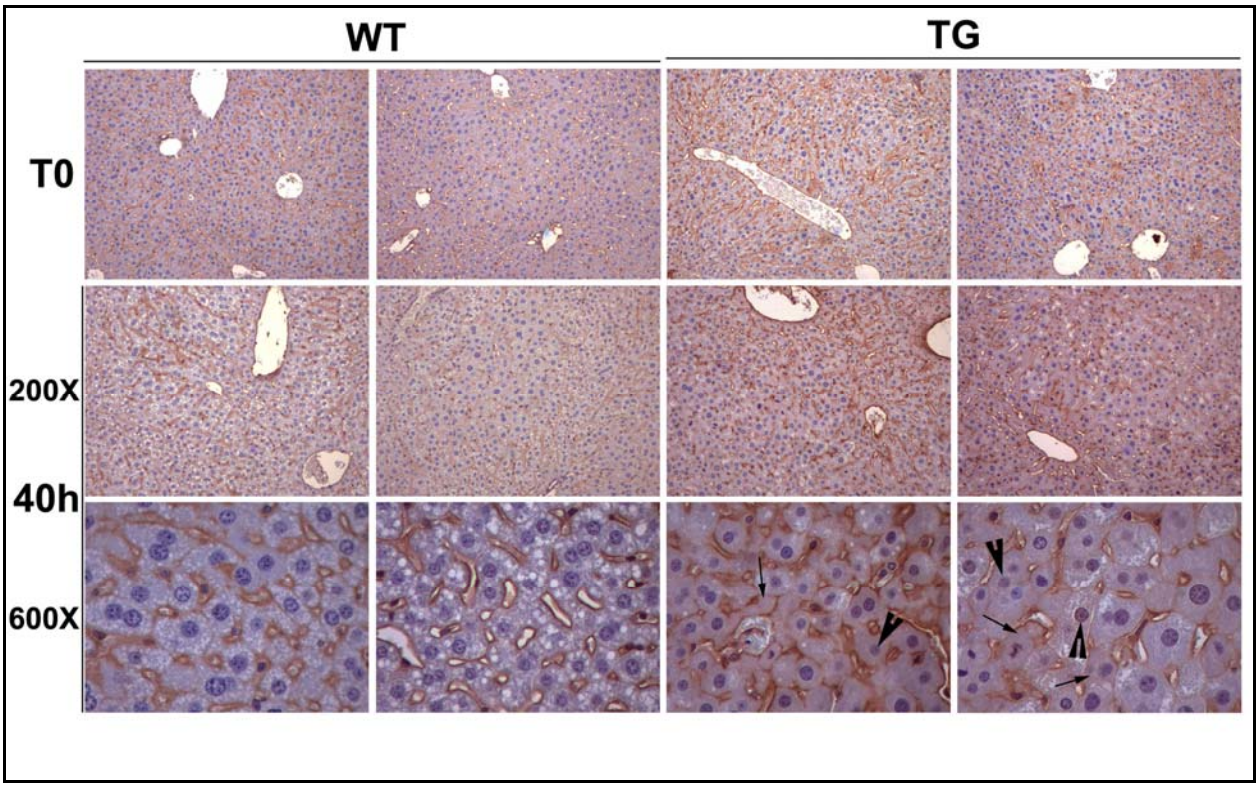

Figure 6: Immunohistochemistry for β-catenin demonstrates translocation to the nucleus in the TG livers at 40H as evidenced by an increase in cytoplasmic and nuclear staining. Arrows indicate cytoplasmic staining; arrowheads point to nuclear staining.

2.4.4 Early tyrosine phosphorylation of β-catenin in TG livers after PHx

Mutation of Ser45 in CTNNB1 is known to affect the degradation of β-catenin protein; however in our TG mouse model, this mutated form of β-catenin was deemed as being not constitutively active as seen by its predominant membranous localization and lack of nuclear staining in livers of TG mice greater than 2 months. This was an intriguing observation that shows an adaptive capability of hepatocytes to regulate a mutant form of β-catenin through its membranous sequestration. To address the mechanism which may have resulted in an increase

in the nuclear translocation and activation of β-catenin signaling in TG mice at 40 hours after PHx, we investigated the tyrosine phosphorylation status of β-catenin during liver regeneration in TG and WT mice. Phosphorylation at Y654 is a hallmark of HGF- and EGF-mediated β-catenin activation and translocation [52]. Western blotting for Y654 shows around a 20% decrease in Y654-phosphorylated β-catenin in TG livers as compared to WT at baseline (Figure 7A and 7C). While the difference is modest, hypophosphorylation of β-catenin at this residue may be an important mechanism of regulating S45D mutant β-catenin by enabling its enhanced membranous localization with E-cadherin and Met. However, there is an increase in β-catenin phosphorylation at 40 hours after PHx in TG mice and at 72 hours in WT mice (Figure 7A). This increase was around 40% at 40 hours in the TG livers as measured by densitometry (Figure 7C). Increased levels of total HGF protein were observed in WT and TG mice at 40 hours and 72 hours after PHx over pre-hepatectomy levels although HGF levels were modestly higher in WT compared to TG livers at 72 hours (Figure 7A).

Since HGF is known to phosphorylate c-Met, which in turn phosphorylates β-catenin and results in dissociation of the β-catenin-Met complex and nuclear translocation of β-catenin, we sought to determine Met-β-catenin association in TG and WT mice during the regeneration process [51]. Coprecipitation studies identified a decrease in β-catenin-Met association at 40 hours and 72 hours in TG and at 72 hours in WT mice supporting premature HGF/Met activation of β-catenin in TG mice after PHx (Figure 7B). Densitometry revealed a decrease of association from 37% to around 18% in TG livers at 40 hours after PHx, while it remained unchanged in WT mice (Figure 7D). The association dropped to around 9% in both TG and WT mice at 72 hours after PHx. While these changes appear modest, their functional implications would be marked

especially in TG livers, where β-catenin is mutated at S45 and hence cannot be degraded by the ubiquitin-proteasome pathway.

To conclusively address the fate of tyrosine-phosphorylated β-catenin, we next determined the localization of Y654-β-catenin in WT and TG livers during liver regeneration by immunohistochemistry **(**Figure 7E**)**. Scanty cytoplasmic staining for Y654-β-catenin is observed in in pre-hepatectomy WT and TG livers. However, a robust increase in nuclear and cytoplasmic localization of Y654-β-catenin is observed in TG livers at 40 hours and 72 hours. A similar increase is observed in WT livers at 72 hours only. Thus, β-catenin activation in TG mice at 40 hours is at least in part contributed by its tyrosine-phosphorylation, which might be HGF-dependent and a function of excessive β-catenin at the hepatocyte membrane in TG livers.

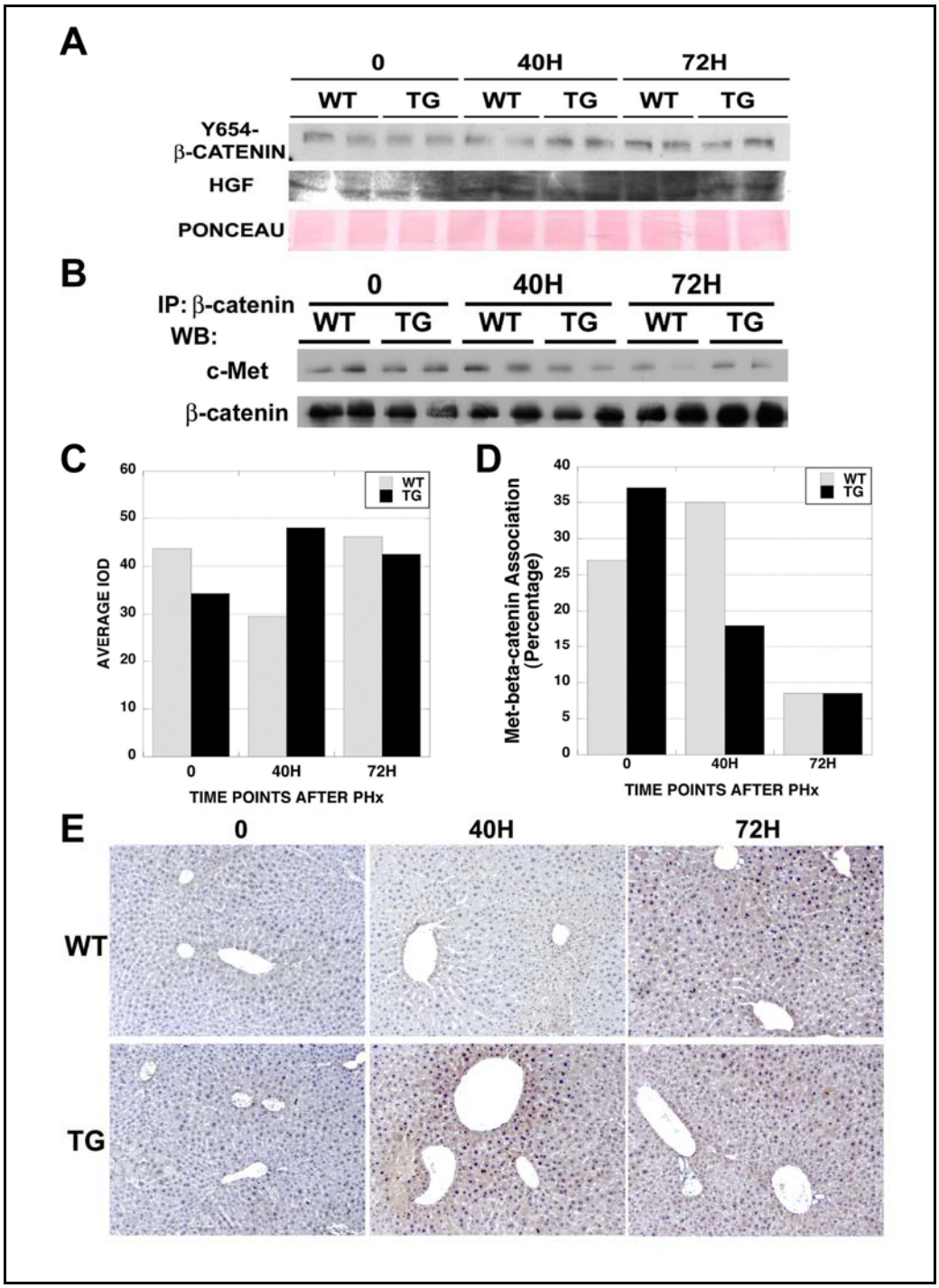

Figure 7: Tyrosine-654 (Y654) phosphorylation and dissocation of β-catenin from Met during regeneration occurs at 40H in TG mice and 72H in WT mice. **(A)** Western blot using total cell lysates detects increased phosphorylation of Y654 at 40H and 72H after PHx in TG and WT respectively. Levels of HGF increase in WT and TG livers at 40H and beyond. **(B)** Immunoprecipitation studies detect dissociation of β-catenin from Met at 40H in

TG livers, but at 72H in WT livers. **(C)** Denistometry of scanned representative western blots reveals around 40% increase in Y654-β-catenin in TG mice at 40H; a similar increase in Y654-β-catenin occurs in WT mice at 72H. O.D. was normalized to loading control. **(D)** Quantitative changes in c-Met-β-catenin association during liver regeneration in WT and TG mice reveal a decrease at 40H in TG and at 72H in both TG and WT. O.D. was normalized to loading control. **(E)** Immunohistochemistry for Y654 β-catenin reveals increased cytoplasmic and nuclear localization at 40H and 72H after PHx in TG and at 72H in WT livers.

2.4.5 Hydrodynamic injection of Wnt-1 naked DNA induces β-catenin activation and regeneration in wild-type mice

Finally, in order to determine if temporal activation of Wnt/β-catenin signaling could impact hepatocyte proliferation during regeneration, we systemically administered pCMV-Wnt-1 plasmid or empty expression plasmid vector pcDNA3 in wild-type CD-1 mice. Twenty-four hours after injection, these mice were subjected to partial hepatectomy and the livers were harvested at 30 hours after PHx. This time-point was selected because peak hepatocyte proliferation after PHx is reported at 48 hours in CD-1 mice [346] and we wanted to determine any premature entry into cell cycle as a sign of cell proliferation and enhanced regeneration in the Wnt-1-plasmid-injected group of mice. Western blot for Wnt-1 protein confirms an increase in expression at 30 hours after PHx in the Wnt-1 injected animals compared to the control group (Figure 8A). Concurrent with this increase, β-catenin translocation to the nucleus is also evident (Figure 8B). A simultaneous increase in the protein expression of β-catenin downstream targets such as glutamine synthetase and cyclin-D1 were observed at 30 hours after PHx in the Wnt-1-treated group by western blot analysis and/or immunohistochemistry (Figure 8A and 8C).

To determine the effect of Wnt-1 injection on cell proliferation, we examined both control and Wnt-1 injected livers for PCNA to identify cells in S-phase of cell cycle. Figures 8C and 8D show an almost three-fold increase in the numbers of PCNA-positive hepatocytes at 30 hours in the Wnt-1 injected mice than in the controls ($p<0.05$). These data suggest that like the TG mice, the Wnt-1 injected mice have a proliferative advantage over the pcDNA3-injected controls due to enhanced β-catenin activation after PHx, and thus exogenous stimulation of β-catenin signaling may be an attractive means to stimulate regeneration.

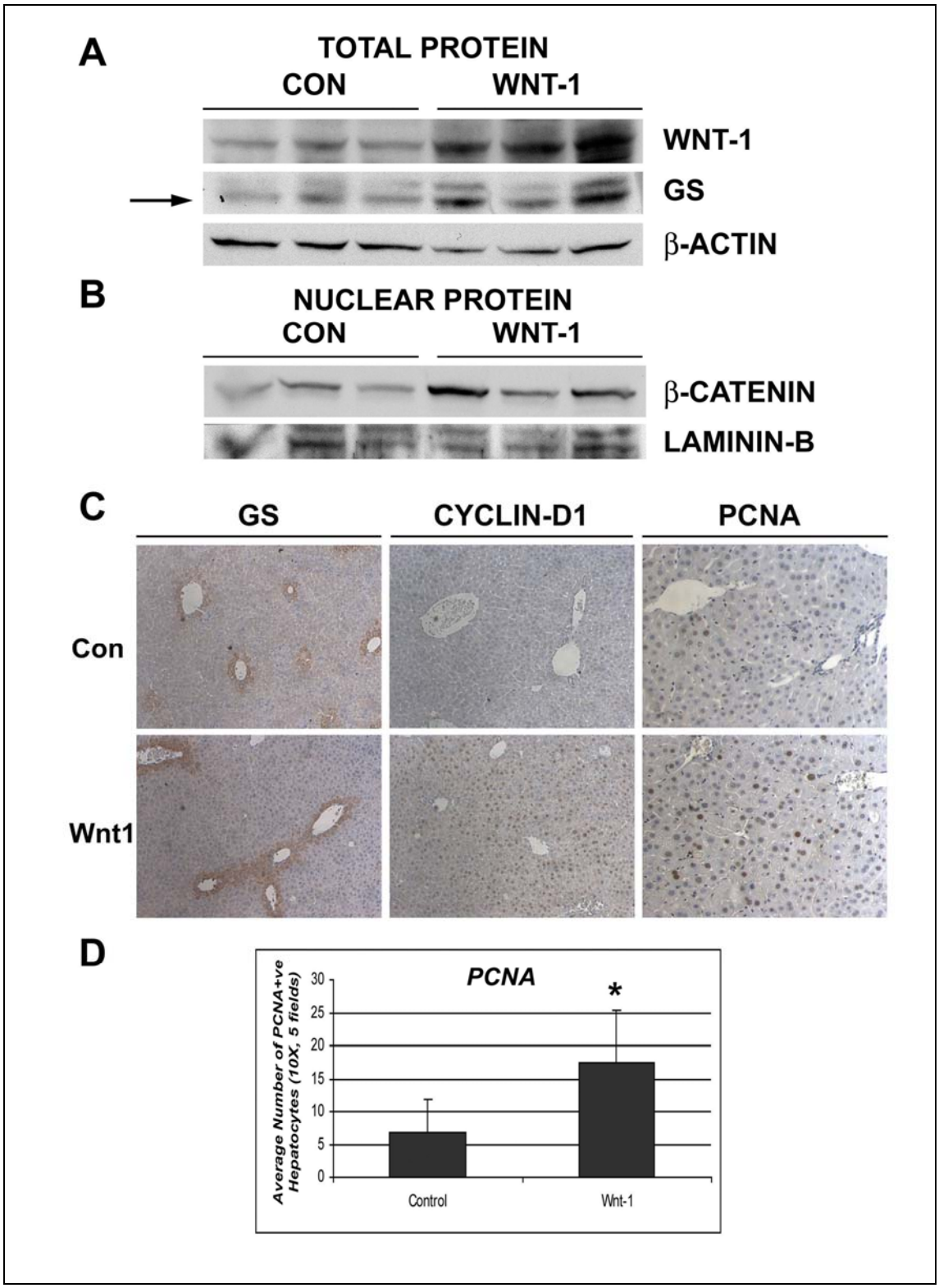

Figure 8: Hydrodynamic delivery of Wnt-1 plasmid and not pcDNA3 through tail vein induces Wnt/β-catenin activation in the liver. **(A)** Western blots utilizing livers from Wnt-1 or pcDNA3 plasmid injected mice at 30H

after PHx reveal increased protein levels of Wnt-1 and glutamine synthetase in the Wnt-1 injected group over the controls. **(B)** Western blots using nuclear lysates show increased β-catenin in the Wnt-1 injected livers as compared to pcDNA3-injected controls at 30H after PHx. **(C)** Downstream targets glutamine synthetase and cyclin-D1 are increased in Wnt-1 injected animals at 30H after PHx, as shown by immunohistochemistry. A concomitant increase in the number of PCNA stained cells is evident in the livers of Wnt-1 injected animals at 30H after PHx as compared to controls. **(D)** Quantification of the number of PCNA-positive cells in five representative 10X fields from Wnt-1 or pcDNA3-injected livers (n=3) show significantly greater numbers of proliferating cells at 30H after PHx in the experimental group ($p<0.05$).

2.5 DISCUSSION

β-Catenin is the crucial downstream effector of the canonical Wnt pathway, and its activation is essential in liver development and regeneration [331]. Mutations in the β-catenin gene that render the protein aberrantly active such as mutations affecting serine-45 have been commonly implicated in HCC [347]. Phosphorylation of β-catenin at serine-45 is essential for proteosomal degradation. Both non-phosphorylatable and phospho-mimetic mutations affecting this residue along with others in exon-3 have been shown to retard degradation of β-catenin protein [333]. In the current study we employed transgenic mice overexpressing mutant S45D-β-catenin under an albumin promoter/enhancer in order to investigate the impact on hepatocyte proliferation in primary cultures and during liver regeneration. These transgenic mice showed temporal activation during early postnatal liver development but at 2 months of age, β-catenin was limited predominantly to the hepatocyte membrane.

What was the mechanism of membranous sequestration of the oncogenic mutant β-catenin in the TG hepatocytes? An approximately 20% decrease in Y654-phosphorylated β-catenin in TG liver over WT, concomitant with enhanced membranous localization of β-catenin, indicates a rather novel means of directing mutant β-catenin to complex with Met at the hepatocyte membrane. This also suggests a role for a tyrosine phosphatase which might in fact be a part of this adaptive mechanism and may be potentially exploited for therapeutic benefit. Indeed, protein tyrosine phosphatases such as PCP-2 are known to inhibit β-catenin signaling by inducing its association with E-cadherin [348]. Despite successful sequestration of β-catenin at the hepatocyte membrane, TG hepatocytes show a growth advantage both *in vitro* and *in vivo*. This indicates that when suitable signals are provided, the excess β-catenin from its membranous pool is available for transactivation of target genes (Figure 9). Indeed in primary cultures, the growth advantage of TG hepatocytes is due to increased availability of β-catenin from the membrane due to the presence of growth factors such as HGF and EGF in the culture media, which are known to induce tyrosine phosphorylation-dependent β-catenin nuclear translocation and activation [51, 349].

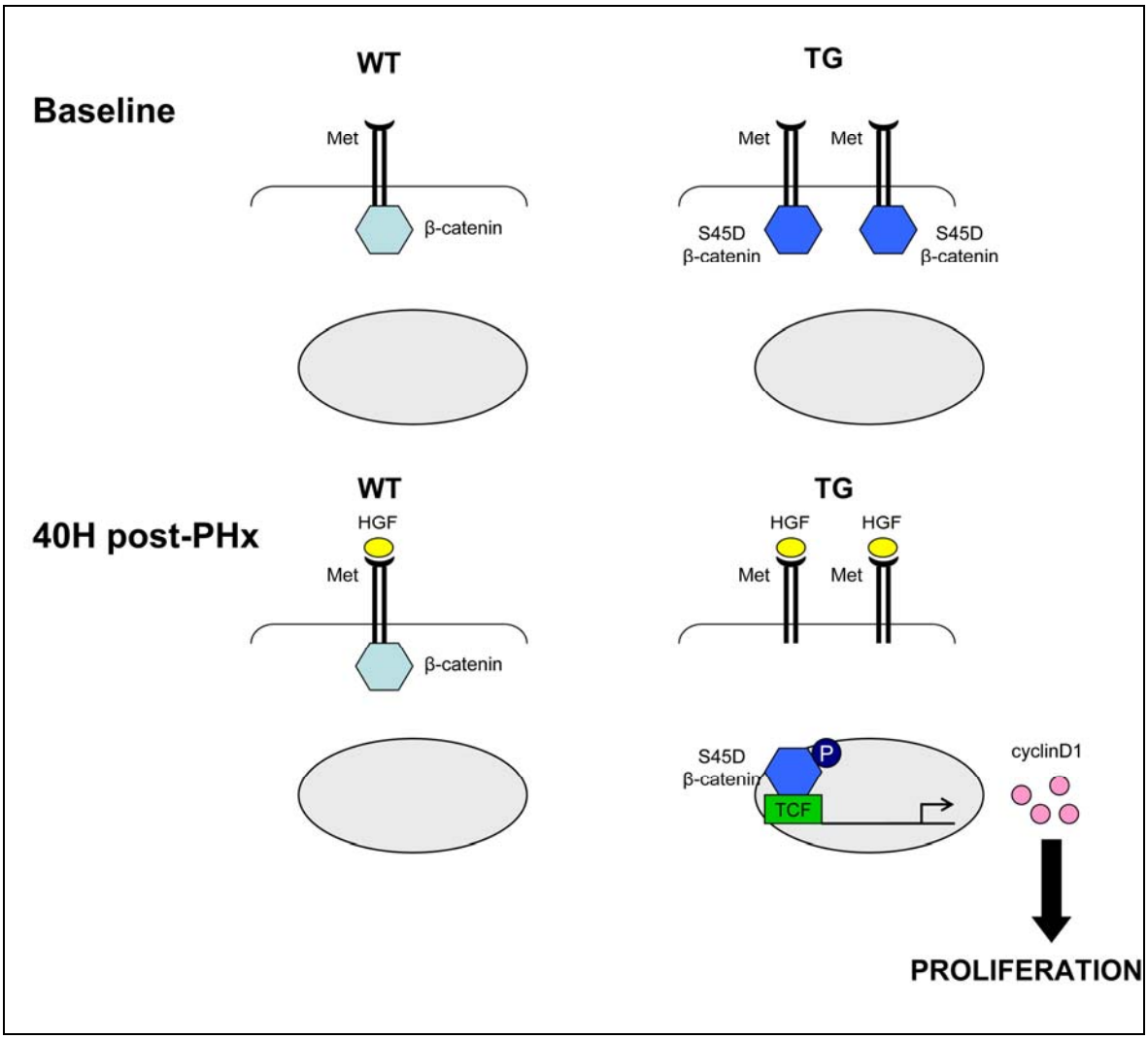

Figure 9: The growth advantage of S45D TG hepatocytes after PHx may be attributed to acceleration of β-catenin dissociation, phosphorylation, and nuclear translocation 40 hours after partial hepatectomy. At baseline, excess S45D-mutated β-catenin is sequestered at the cell membrane by complexing with the Met receptor. After partial hepatectomy, mutated β-catenin is rapidly tyrosine phosphorylated and translocates to the nucleus, where it turns on expression of cyclin-D1, thus inducing accelerated proliferation as early as 40 hours after PHx. In WT livers, this phosphorylation and translocation of β-catenin does not occur until 72 hours after PHx.

The positive role of β-catenin in liver regeneration has been identified in rats, mice, zebrafish and humans [94, 126-128, 350, 351]. Along those lines, we identify a regenerative advantage after PHx *in vivo* in the current TG mice, where hepatocyte proliferation was earlier

and robust. It is important to note that despite the earlier initiation of DNA synthesis and proliferation in the TGs after PHx, the regeneration process was completed unequivocally and without any adverse consequences. The differences in liver weight to body weight ratios between WTs and TGs are unremarkable at all time points after PHx; the number of apoptotic nuclei at D9 and D14 also do not differ significantly between WTs and TGs (data not shown). Additionally, there is no evidence of either dysplastic nodules, adenomas, or HCC as late as 3 months after PHx.

The regeneration advantage seen in our TG model was due to enhanced cyclin-D1 expression, which is necessary for G1 to S phase transition of the cell cycle [352]. While cyclin-D1 as the target of β-catenin signaling has been known for quite some time [25, 26], its true relationship to β-catenin in the liver has been controversial with precedence given to targets such as GS [353]. However, several studies support a key role of β-catenin in regulating cyclin-D1 in the liver especially during proliferation. In prenatal and postnatal liver development cyclin-D1 levels closely follow β-catenin activity in normal and β-catenin-conditional knockouts [96, 354]. Similarly, β-catenin regulates cyclin-D1 expression during regeneration after sublethal doses of acetaminophen [351]. Finally, conditional hepatic disruption of β-catenin compromises cyclin-D1 expression during both development and liver regeneration [94, 96]. Thus, it is of relevance to emphasize that genetic targets of β-catenin signaling are tissue-and context–dependent and while cyclin-D1 may be subject to Wnt-independent regulation, β-catenin is among the chief regulators of cyclin-D1 and, in turn, of cell proliferation in liver.

Our data also suggests that there may be an opportunity to stimulate liver regeneration from a therapeutic standpoint, through Wnt/β-catenin activation. Transfection of Wnt-1 plasmid was previously shown to induce nuclear localization of β-catenin in NIH3T3 [355] and

mammary epithelial cells [50]. Rat fibroblasts infected with Wnt-1 adenovirus or retrovirus showed proliferation in the absence of serum [356], which was mediated in part by β-catenin activation [357]. In our study, hydrodynamic delivery of Wnt-1 DNA led to β-catenin activation, increased cyclin-D1 expression and high hepatocyte proliferation after PHx. Thus our report for the first time provides evidence that exogenous canonical Wnt activation might be useful to augment liver regeneration, especially in the case of acute liver failure.

3.0 BETA-CATENIN REGULATES VITAMIN C BIOSYNTHESIS AND CELL SURVIVAL IN MURINE LIVER

3.1 ABSTRACT

Since the Wnt/β-catenin pathway plays multiple roles in liver pathobiology such as regulating cell proliferation, it is critical to identify additional gene targets that mediate diverse effects. Here, we report a novel role for β-catenin in controlling ascorbic acid biosynthesis and in turn regulating cell survival in murine liver through regulation of expression of regucalcin or senescence marker protein-30 and L-gulonolactone oxidase. RT-PCR, Western blotting and immunohistochemistry demonstrate decreased regucalcin expression in β-catenin-null livers and greater expression in β-catenin overexpressing transgenic livers, HepG2 hepatoma cells (containing constitutively active β-catenin), regenerating livers and in hepatocellular cancer tissues that exhibit β-catenin activation. Interestingly, coprecipitation and immunofluorescence studies also demonstrate an association of β-catenin and regucalcin. Significantly lower serum ascorbate levels were observed in β-catenin knockout mice as a consequence of decreased expression of regucalcin and also of L-gulonolactone oxidase, the penultimate and last (also rate-limiting) steps, respectively, in the synthesis of ascorbic acid, a known anti-oxidant. There was also a concomitant increase in basal hepatocyte apoptosis in these mice. To test if ascorbate deficiency secondary to β-catenin loss and regucalcin decrease was indeed contributing to

apoptosis, β-catenin-null hepatocytes or regucalcin siRNA- transfected HepG2 cells were cultured, and both cell types exhibited significant apoptosis that was alleviated by the addition of ascorbic acid. Thus, through regucalcin and L-gulonolactone oxidase expression, β-catenin regulates vitamin C biosynthesis in murine liver, which in turn is one of the mechanisms contributing to the role of β-catenin in hepatocyte survival.

3.2 INTRODUCTION

The Wnt/β-catenin signal transduction pathway, which is essential for normal development and tissue regeneration [2, 358], also has a well-characterized role in tumorigenesis and is implicated in cancers affecting several tissue types, including the colon, rectum, lung, skin, breast, and liver [359-362]. Mutations in β-catenin lead to aberrant signaling, which causes stabilization and nuclear translocation of β-catenin, along with subsequent activation of target genes such as c-myc and cyclin-D1 [25, 27, 363]. In the liver, mutations in β catenin gene are primarily involved in the pathogenesis of HCC [364-367]. Because the Wnt/β-catenin pathway plays an essential role in both liver biology and cancer [331], it is critical to identify other potential targets of this pathway.

Regucalcin, also known as SMP30 [165], is a Ca^{2+}-binding protein that has been implicated in maintaining cell homeostasis and function [163, 176]. Regucalcin activates plasma membrane Ca^{2+}-pumping ATPases and thus regulates the cytosolic calcium ion levels [164, 368]. Expression of regucalcin can be stimulated by the addition of calcium [170, 369-371]. Moreover, regucalcin may regulate the transcriptional process by binding protein and DNA in the nucleus [372]. Much of the characterization of regucalcin has occurred in the context of the

liver. Regucalcin plays an important role in the regulation of Ca^{2+} signaling from the cytoplasm to nuclei in the proliferative cells of regenerating rat liver [163, 176]. During regeneration, regucalcin has been suggested to suppress protein synthesis [180] and DNA synthesis [178, 373]. Regucalcin has also been implicated in the suppression of RNA synthesis in the nucleus [172, 374], NO synthase activity [183, 375], and protein phosphatase activity [376, 377]. The overexpression of regucalcin in hepatoma cells suppresses cell proliferation and the expression of several known oncogenes, such as c-myc [181]. Regucalcin overexpression also has a suppressive effect on apoptosis induced by TNF-α or thapsigargin [189]. Because regucalcin functions as an inhibitor of several cellular processes, it is thought to have an important role in suppressing the differentiation and proliferation of hepatocytes after liver injury [176].

Regucalcin may play a role in liver tumorigenesis as well. Regucalcin mRNA is decreased in chemically-induced rat hepatomas [378], as well as in the rat hepatoma cell line H4-II-E [377]. These data suggest that regucalcin may be implicated in the formation of some hepatomas through a decrease in expression, which can lead to an overabundance of hepatocyte proliferation. Interestingly, the human hepatoma cell line HepG2 also expresses regucalcin, and its expression could be further stimulated by insulin [379, 380], suggesting that glucose metabolism and homeostasis may play a role in regulating regucalcin expression.

More recently, regucalcin/SMP30 was reported to be a gluconolactonase [193]. Conversion of L-gulonate to L-gulonolactone is the penultimate step in the biosynthesis of L-ascorbic acid, the final step being oxidation to ascorbic acid by L-gulonolactone oxidase [381]. It should be noted that the inactivity of the latter enzyme due to mutations that has led to inability of humans, guinea pigs and other species to synthesize vitamin C. In summary, regucalcin is thought to have a diverse role in proliferation, survival and differentiation of cells [176].

In a previous study, our laboratory found that β-catenin conditional knockout animals exhibited altered gene expression for many potential and known targets of β-catenin [94]. Regucalcin was one of the genes that was most affected by the loss of β-catenin. Based on this information, we hypothesized that regucalcin is a downstream target of β-catenin in the liver. In order to establish the relationship between β-catenin and regucalcin in normal liver, we analyzed regucalcin expression in β-catenin conditional knockout mice, and in rats subjected to two-thirds partial hepatectomy. We then expanded this initial characterization by examining human hepatocellular carcinoma tissues to determine the clinical significance of this relationship. We also performed siRNA studies and immunoprecipitations to determine the biological function of regucalcin in liver. Lastly, we addressed the role of regucalcin as a target of Wnt/β-catenin signaling in hepatocytes, which along with L-gulonolactone oxidase, plays an important role in vitamin C biosynthesis and also regulates cell survival. Thus, through these approaches, we demonstrate that regulation of SMP30 and L-gulonolactone oxidase expression by β-catenin contributes to a broader role of Wnt/β-catenin in liver homeostasis.

3.3 MATERIALS AND METHODS

3.3.1 Tissues, Animals, and Cell Lines

A total of 19 human hepatocellular carcinomas from two different groups were used for this study. The first group (Group 1), containing nine samples, was obtained from HCCs that had known β-catenin mutations; the second group (Group 2), containing ten samples, was obtained from HCCs where the β-catenin status is unknown. Normal livers (from donor liver

tissue) were obtained from 4 cases. Tissues from the first group were processed for mRNA analysis, whereas samples from the second group were processed for protein and immunohistochemistry.

Mouse models used in this study include a conditional β-catenin knockout described previously [94], and a stable β-catenin overexpressing transgenic mouse similar to one described previously [132]. For the conditional knockout, homozygous floxed β-catenin mice were bred to albumin-cre mice (both on a C57BL/6 background); the resulting mice, after another backcrossing to homozygous floxed β-catenin mice resulted in conditional null mice with the genotype $Ctnnb1^{loxp/loxp}$; $Alb\text{-}Cre^{+/-}$ (referred to in this paper as knockout or KO mice) and controls with the genotype $Ctnnb1^{loxp/loxp}$; $Alb\text{-}Cre^{-/-}$ or $Ctnnb1^{loxp/Wt}$; $Alb\text{-}Cre^{-/-}$ (referred to in this paper as wild-type or WT mice). The mice were sacrificed at 1, 3, and 6 months time points, and the livers were harvested for protein and immunohistochemistry. The β-catenin TG mice (generated in an FVB background) show significantly elevated total β-catenin as compared to their wild-type littermates and were generated similar to the albumin/β-catenin transgenic mice characterized previously [132], except in this strain, β-catenin carries a point mutation at serine-45 rendering it more stable overall, as discussed in the preceding chapter.

Rat liver tissue was obtained from partial hepatectomy studies, as previously described [94]. Frozen livers from partial hepatectomy studies were harvested for protein.

For hydrodynamic delivery of naked DNA, 1 μg/gm of body weight of Wnt-1 plasmid (Millipore, Billerica, MA) or pcDNA3 control plasmid (Invitrogen, San Diego CA) was administered via hydrostatic tail vein injection to CD-1 mice weighing around 18 grams, as described previously and in the preceding chapter [53, 338]. Twenty-four hours after the injection, the mice were subjected to PHx. Mice were sacrificed at 30 hours after PHx and livers

were processed for total and nuclear protein. Cytoplasmic and nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit as per the instructions (Thermo Fisher Scientific, Rockford, IL).

Hep3B and HepG2 human hepatoma cell lines described previously [382] were obtained from American Type Culture Collection (Manassas, VA) and cultured in EMEM (Cambrex, East Rutherford, NJ) with 10% FBS (Mediatech, Herndon, VA) in a humidity-saturated incubator with 5% CO_2 maintained at 37^0C.

3.3.2 mRNA Isolation and Real-Time PCR

mRNA was isolated and purified from frozen livers using TRIzol (Invitrogen, Carlsbad, CA). For RT-PCR, the RNA (from 4-7 month old WT and AFP/Alb/Ctnnb1 KO mice) was diluted to a working concentration of 1 μg/μl in nuclease-free water with the addition of RNase inhibitor followed by removal of potential contaminating DNA by treatment with DNase I (Promega, Madison, WI). Two μg total RNA was used for reverse transcription in a 20 μl reaction buffer with random primers and Superscript III (Invitrogen) to generate first-strand cDNA. PCR was carried out using a standard PCR kit including Taq DNA polymerase (Invitrogen) with specific primer pairs and 1 μl cDNA. The forward (F) and reverse (R) primers for mouse regucalcin and L-gulonolactone oxidase were selected using Primer Express software (PE Applied Biosystems) and produce a 413-bp product and a 681-bp product, respectively. Regucalcin (F): 5'-GAT GCC CCA GTC AGT TCA GT-3'; Regucalcin (R): 5'-ATT CTG CGG TTG GAA ATC TG-3'. L-gulonolactone oxidase (F): 5'-AAA ACT GGG CGA AGA CCT ATG-3'; (R): 5'-CCT TGT TGG TGT GAT CTT GGT-3'. β-actin primers were used as an internal control, (F): 5'- CAG CTG AGA GGG AAA TCG TG -3'; (R): 5'- CGT TGC CAA

TAG TGA TGA CC -3' and produce a 150- bp product. PCR amplification was carried out as follows: initial denaturation at 94°C for 2 m, followed by 35 cycles or 28 cycles of denaturation at 94°C for 1 m, annealing 55°C for 1 m; 72°C for 1 m, and 10 m of final extension at 72°C [383].

For real-time PCR, following DNase treatment, mRNA was converted to cDNA using M-MuLV reverse transcriptase enzyme (Invitrogen, Carlsbad, CA) in a reverse-transcription (RT) master mix containing random primers, 5× RT buffer, dNTP mix, RiboLock, and M-MuLV reverse transcriptase enzyme [53].

Real-time PCR was performed as described previously [384]. TaqMan Gene Expression Assays for human regucalcin and β-actin were obtained from Applied Biosystems (Foster City, CA). The standard conditions used for real-time PCR were as follows: 50°C for 2 m and 95°C for 10 m followed by 40 cycles of 15 s denaturation at 95°C and 1 m annealing/elongation at 60°C. TaqMan signal (Applied Biosystems, Foster City, CA) was measured in each step. The 96-well plate contained a standard curve using normal human liver samples and β-actin primers. Changes in regucalcin mRNA were normalized to actin mRNA and presented as fold-change over the average from four normal livers. Mean fold gene expression was calculated with Applied Biosystems software. In hepatoma cell lines, the expression of regucalcin gene was calculated using the delta-delta CT method [385]. Each sample was run in duplicate.

3.3.3 Protein Extraction, Immunoprecipitation, and Western Blots

Whole-cell lysates from mouse livers, cell cultures, and human tumor tissues were prepared by homogenization using RIPA buffer (9.1 mmol/L dibasic sodium phosphate, 1.7 mmol/L monobasic sodium phosphate, 150 mmol/L sodium chloride, 1% Nonidet P-40, 0.5%

sodium deoxycholate, 0.1% sodium dodecylsulfate [pH adjusted to 7.4]) containing fresh protease and phosphatase inhibitor cocktails (Sigma, St. Louis, MO) [386]. The concentation of the protein in the lysates was determined by the bicinchoninic acid assay using BSA as a standard.

For immunoprecipitation studies, one thousand micrograms of cell lysate from HepG2 and Hep3B cell lines (prepared in RIPA buffer in the presence of inhibitors) was precleared with the appropriate control IgG (either mouse or goat) together with Protein A/G agarose for 30 m at 4^0C. After centrifugation, the supernatants were incubated with either regucalcin/SMP30 antibody or β-catenin antibody for 1 h at 4^0C, followed by overnight incubation with Protein A/G agarose at 4^0C. Pellets were collected the next day, washed in PBS containing inhibitors, resuspended in loading buffer, and subjected to electrophoresis.

Proteins were electrophoresed and blotted as described previously [49, 126]. Briefly, 50 μg of protein from cell or liver lysate or 15-20 μl of eluate from IP studies was resolved on 7.5% or 12% precast SDS-PAGE gels (ISC BioExpress, Kaysville, UT) using the mini-PROTEIN 3-electrophoresis module assembly (Biorad, Hercules, CA), followed by transfer to Immobilon-PVDF membranes (Millipore, Bedford, MA). Membranes were stained with Ponceau-S solution to confirm equal loading, then blocked in 5% nonfat dry milk in blotto solution (20mM TRIS-HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20), followed by incubation with primary antibody diluted in 5% milk/blotto for 1-2 h or overnight. Membranes were washed and incubated in horseradish-peroxidase conjugated secondary antibodies (Chemicon, Temecula, CA) for 1 h followed by washing. Proteins were detected by Super-Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and visualized by autoradiography. Primary antibodies used in this study were against regucalcin/SMP30 (K-18 used at 1:500; C-16 used at 1:150), β-catenin

(1:200), glutamine synthetase (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA) and actin (1:5000) (Chemicon, Temecula, CA). Secondary antibodies were goat anti-mouse (1:10,000), donkey anti-rabbit (1:10,000), and donkey anti-goat (1:2500) (Chemicon, Temecula, CA). Blots were stripped with IgG elution buffer (Pierce, Pierce, Rockford, IL) for 30 m at room temperature before re-probing. Densitometry analysis was performed using Image J version 1.38. All experiments were performed in triplicates and representative results are shown.

3.3.4 Immunohistochemistry and Immunofluorescence

Immunohistochemistry on paraffin-embedded sections was performed on mouse livers and human tumors [387]. Tissues fixed in 10% formalin and embedded in paraffin were sectioned at 4 μm onto Superfrost Plus glass slides (Fisher Scientific, Pittsburgh, PA) and heat-fixed overnight at 37°C. Sections were microwaved in citrate buffer, pretreated with 3% H_2O_2 to eliminate endogenous peroxidases, and blocked using protein blocking agent (Thermo Scientific, Waltham, MA). Primary antibodies used for this project were anti-β-catenin (1:200), anti-SMP30 aka regucalcin (1:100), anti-glutamine synthetase (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-β-catenin (1:50) (BD Biosciences, San Diego, CA). Secondary antibodies were biotinylated horse anti-mouse (Vector Laboratories, Inc., Burlingame, CA), goat anti-rabbit, and donkey anti-goat (Chemicon, Temecula, CA), all used at a 1:500 dilution. Immunohistochemistry was performed using the Vectastain ABC Elite kit (Vector Laboratories, Inc., Burlingame, CA) and developed using either DAB (Vector Laboratories, Inc., Burlingame, CA) or AEC substrate (Scytek Laboratories, Logan, UT). The slides were counterstained with aqueous Gill's hematoxylin and mounted with either crystal mount or cytoseal (Biomeda Corp., Foster City, CA). For β-catenin, GS and regucalcin expression in HCCs, each sample was

allocated an arbitrary IHC score on the basis of the intensity of the expression of β-catenin, GS and regucalcin in the nucleus and/or cytosol as compared to the normal liver samples. Each stain was scored as – (absent staining), + (positive, similar to normal) and ++ (strongly positive or greater than normal). For β-catenin IHC, even a subset of HCC cells showing nuclear and/or cytoplasmic localization were assigned a positive score.

Coverslips plated with either HepG2 or Hep3B hepatoma cell lines were processed for immunofluorescence as previously described [388]. Cells were washed with PBS, fixed in cold 2% paraformaldehyde in PBS for 15 m, and permeabilized in 0.1% Triton-X-100 in PBS for 10 m. Coverslips were then washed five times each in PBS and 1% BSA in PBS, and blocked for 1 h in 5% normal donkey serum in 1% BSA/PBS. Primary antibodies against β-catenin (1:100) and anti-SMP30 aka regucalcin (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA) were diluted in 1% BSA/PBS, added to the coverslips, and incubated overnight. Cells were washed five times in 1% BSA/PBS, then incubated for 1 h with secondary antibodies diluted in 1% BSA/PBS. Secondaries used for this project were donkey anti-goat Alexa 488 and donkey anti-mouse Cy3 at either a 1:500 or a 1:1000 dilution (Molecular Probes, Eugene, OR). Following five washes in 1% BSA/PBS and then PBS, coverslips were incubated with Hoescht counterstain (30 s), washed with PBS, and mounted on slides. All fluorescence labeling was imaged under Zeiss Axiovert 40 CFL microscopy (Carl Zeiss, Thornwood, NY) and photographs obtained using a Zeiss Axiocam MRC5 digital camera.

3.3.5 Primary Hepatocyte Culture

Hepatocytes from β-catenin KO mice were isolated using a modified 2-step collagenase perfusion protocol as previously described and seeded on chamber slides wet-coated with collagen at a density of 100,000 cells per ml in minimal essential media (MEM) supplemented with 10% fetal bovine serum (FBS) [335]. After 2 hours, media were changed to a chemically defined MEM-based growth medium containing insulin/transferrin/selenium (ITS; 1g/L), dexamethasone (10^{-7}M), HGF (40ng/ml), and epidermal growth factor (EGF; 25ng/ml) as described elsewhere [337]. The cells were cultured for 48 h in the presence or absence of ascorbic acid at a final concentration of 0.2 mM [389]. The apoptotic nuclei were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining using the ApopTag Peroxidase kit (Intergen Company, Purchase, NY) as previously described [89]. The numbers of positive cells per field were counted, for a total of 5 fields per condition.

Rat hepatocytes were isolated and plated as discussed elsewhere [337]. After 2 hours, plating media were replaced with basal medium, and either 5ng/ml recombinant mouse Wnt3a (R&D Systems, Minneapolis, MN) or PBS. Plates were harvested for nuclear protein 24 h after treatment. Nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit as per the instructions (Thermo Fisher Scientific, Rockford, IL).

3.3.6 Transfection of siRNA

Hep3B and HepG2 human hepatoma cell lines were seeded onto either 100-mm dishes or 6-well plates and maintained until the cells reached 50-60% confluence in EMEM (ATCC, Manassas, VA) with 10% FBS. Two independent siRNA's were utilized for transfection. Cells were transfected with siRNA purchased from either Dharmacon (Lafayette, CO) or Applied Biosystems (Foster City, CA). 100nM of control siRNA or siRNA against

human regucalcin mRNA from Dharmacon was transfected into cells using Oligofectamine transfection reagent (Invitrogen, Carlsbad, CA) in the absence of serum for 4 h. After incubation with siRNA, EMEM with 10% FBS was added to the cells without removal of the siRNA mixture. Media were changed 24 h after transfection, and fresh EMEM with 10% FBS was added. Plates were harvested for protein 48 h after addition of siRNA. Cells were transfected with 5nM of either control siRNA or siRNA against human regucalcin mRNA from Applied Biosystems using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) in the presence of serum. Media were changed 24 h after transfection, and fresh EMEM with 10% FBS was added. Plates were harvested for protein and RNA 48 h after addition of siRNA.

3.3.7 Cell Growth and Viability Assays

A [^{3}H]thymidine uptake assay was utilized as previously described to measure cell proliferation [340, 341]. Briefly, Hep3B and HepG2 human hepatoma cell lines transfected with control or human regucalcin siRNA were dosed with 2.5 μCi/ml of [^{3}H]thymidine (MP Biomedicals, Solon, OH) 24 h before harvesting and incubated at 37°C in 5% CO_2. The cells were harvested by adding 1.5 ml of cold 5% (w/v) trichloroacetic acid to each well. The plates were placed at 4°C for 2 h and then were washed and allowed to air-dry. The precipitate was solubilized by the addition of 1 ml of 0.33 M NaOH, and an aliquot was added to Universol (MP Biomedicals, Solon, OH). The counts/min of the solubilized precipitates were determined with a scintillation counter (Beckman Instruments, Palo Alto, CA). Plates were harvested 48 h after addition of siRNA.

HepG2 cells cultured on chamber slides were dosed with either control or human regucalcin siRNA in the presence or absence of ascorbic acid at 0.2 mM final concentration or in

the presence or absence of N-acetylcysteine (NAC) at 20mM final concentration in 10XPBS as reported elsewhere [389, 390]. 48 h after treatment, slides were fixed in a 3:1 solution of methanol/glacial acetic acid for 5 m and allowed to air-dry. Slides were processed for TUNEL analysis as described in the previous section.

Assays were done in triplicates and statistical assessment for significance was performed by the Student's *t*-test. A *P* value of less than 0.05 was considered significant.

3.3.8 Ascorbate and Lipid Peroxidation Assays

Fresh whole blood from age- and sex-matched WT and β-catenin KO mice (n=3) was obtained through retro-orbital bleeding and centrifuged at 4,000 rpm for 10 minutes to obtain serum. Serum was utilized to determine ascorbate levels with the Ascorbic Acid Assay Kit II (FRASC) Kit (Bio-Vision, Mountain View, CA) according to the manufacturer's instructions.

3.4 RESULTS

3.4.1 Regucalcin protein expression correlates with β-catenin expression

Livers from 1, 3, and 6 months old wild-type (WT) and β-catenin conditional knockout (KO) mice were used for isolation of protein and IHC. Deletion of β-catenin leads to a decreased expression of regucalcin protein in the KO livers at 3 and 6 months as compared to the WT and

coincides with loxP recombination driven by *albumin-cre recombinase* [391] (Figure 10A). This result correlates with the absence of regucalcin mRNA in the KOs as shown in Figure 2.

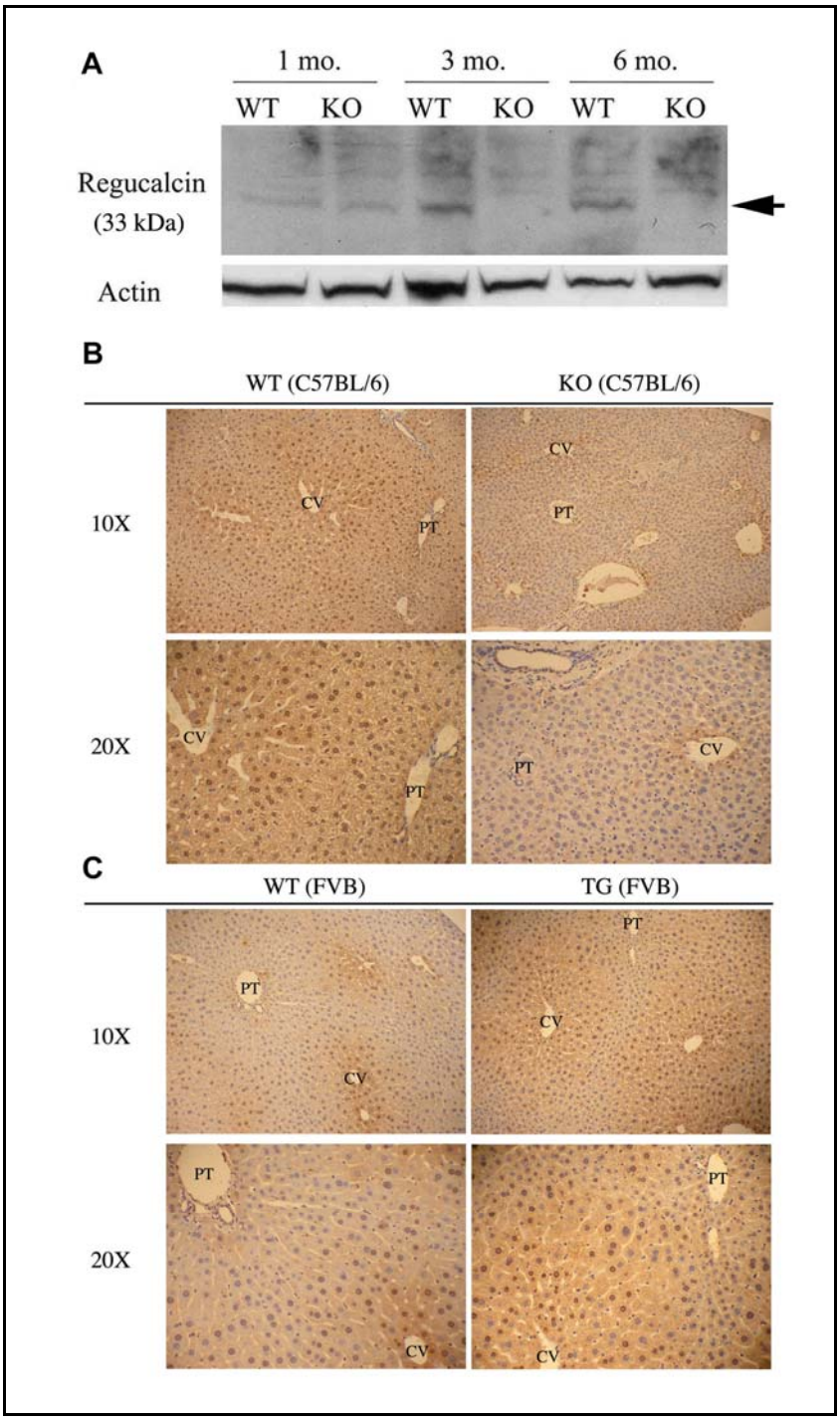

Figure 10: Regucalcin expression is regulated by β-catenin in the liver. (A) Western blot using whole cell lysates from WT and KO livers show regucalcin is present in all WT samples but is decreased at 3 and 6 months in the KO

mice. **(B)** Representative immunohistochemistry for regucalcin on 3-month old WT and KO livers (top four panels) shows cytoplasmic and nuclear expression of regucalcin protein in WT hepatocytes; in contrast, regucalcin expression was dramatically decreased in KO livers. PT-portal triad; CV-central vein. **(C)** Immunohistochemistry for regucalcin on 3-month old WT and stable-β-catenin overexpressing transgenic mice (bottom four panels) shows centrizonal regucalcin staining in WT FVB livers, whereas the transgenic livers show widespread nuclear and cytoplasmic regucalcin. PT-portal triad; CV-central vein.

IHC detected regucalcin protein in the nucleus and cytosol of WT hepatocytes (C57BL/6) in all zones, with a clear accentuation in centrizonal and sometimes midzonal areas (Figure 10B). However this expression was markedly diminished in the KO livers with only a few cells around the central vein showing leftover positivity for regucalcin (Figure 10B). Conversely, low levels of nuclear and cytoplasmic regucalcin were observed in normal FVB hepatocytes, and where regucalcin was clearly less pronounced than normal WT livers of C57BL/6 mice, it was mostly localized to centrizonal areas only and absent periportally (Figure 10C). However a noteworthy widening of regucalcin localization was observed around the centrizonal areas in β-catenin overexpressing transgenic (TG) livers with continued periportal sparing (Figure 10C). Thus regucalcin expression mimicked β-catenin levels in murine livers.

3.4.2 Regucalcin levels correlate with β-catenin target glutamine synthetase in the rat partial hepatectomy model

Previously, our lab has shown temporal changes in β-catenin expression during liver regeneration in rats after partial hepatectomy (PHx) [126]. A transient increase in β-catenin protein was accompanied by its nuclear translocation for up to 48 hrs after partial hepatectomy. We used this model of liver regeneration to determine if regucalcin expression correlated with

levels of glutamine synthetase, a known target of β-catenin [34]. Figure 11A shows an increase in regucalcin protein expression in the rat as early as 1 h following partial hepatectomy. Regucalcin then decreases at 12 h, but rises again at D1 post-PHx, suggesting a cyclic regulation of expression. This pattern coincides with glutamine synthetase during liver regeneration, although the protein levels of GS changed only marginally, and thus supports regulation of regucalcin by β-catenin in this model of liver growth.

3.4.3 Human hepatocellular carcinomas have increased levels of regucalcin compared to normal liver

Next, we examined human hepatocellular carcinomas for regucalcin expression in relation to β-catenin status. The two different groups used in the study are described in the Methods. We hypothesized that the samples containing known β-catenin mutations would have higher levels of regucalcin mRNA expression. Figure 11B shows the results of a representative real-time PCR reaction using mRNA from HCCs with mutations in exon-3 of β-catenin gene. Group 1 HCC samples with known activating mutations in β-catenin gene [339] showed a 40-1000-fold increase in regucalcin gene expression as compared to the normal livers (Figure 11B).

Next, paraffin tissues from group 2 HCC patients, where the β-catenin mutational status was unknown, were assessed for β-catenin, glutamine synthetase and regucalcin expression by IHC. We hypothesized that if we could determine the relative expression of β-catenin in these samples, we could establish a correlation between increases in β-catenin expression and an increase in regucalcin expression. Each tumor sample was allocated an arbitrary immunohistochemical score on the basis of the expression of β-catenin, GS and regucalcin in the

nucleus and/or cytosol (Figure 11C). Based on the IHC, normal human liver received a score of (-) for β-catenin in the nucleus and cytosol, and + for GS and regucalcin. As shown in representative HCC samples, increased cytoplasmic and nuclear β-catenin coincided with elevated GS and regucalcin staining in adjacent serial sections (Figure 11C). Overall, 9/10 HCC tissues exhibited some nuclear/cytoplasmic β-catenin in additional to its membranous localization, and 7/9 of these samples showed increased regucalcin, of which 6 also showed a concomitant increase in GS (Figure 11D). A small subset (T5 and T8) showed no evidence of increased GS or regucalcin despite exhibiting intense nuclear β-catenin staining.

3.4.4 Exogenous Wnt stimulation induces regucalcin expression *in vitro* and *in vivo*

Next, we investigated if exogenous Wnt stimulation had an effect on regucalcin protein expression. Rat primary hepatocytes were cultured in the presence of recombinant Wnt3a protein as described in the Methods. As expected, there was a significant increase in nuclear β-catenin in response to 24 h of Wnt3a treatment. There was also a corresponding increase in nuclear regucalcin levels after Wnt3a treatment (Figure 11E).

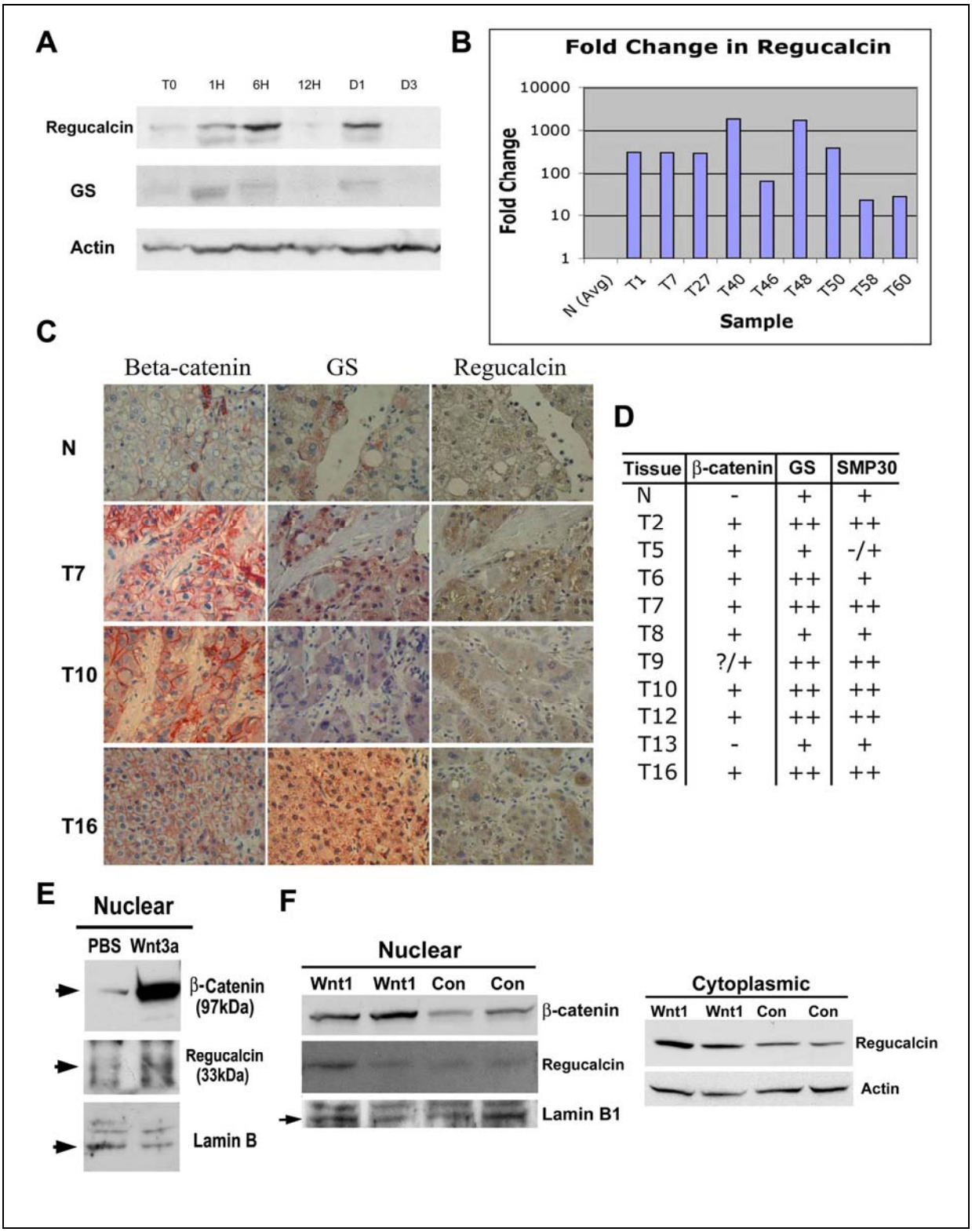

Figure 11: Regucalcin protein expression correlates with β-catenin activation during liver regeneration, in HCC and after exogenous Wnt administration. (A) Western blot with lysates from rat livers harvested at various

time points after partial hepatectomy display concomitant increase in regucalcin and known β-catenin target-GS expression at 1h, 6h and D1 during regeneration. **(B)** Real-time PCR analysis of mRNA harvested from human HCCs with known β-catenin mutations (Group 1) shows many fold increase in regucalcin mRNA expression, which was plotted for each tumor. Regucalcin mRNA expression was standardized to actin expression in each sample and normalized to average signal in four normal livers. **(C)** Immunohistochemistry for β-catenin, regucalcin, and GS on representative human HCCs where the mutational status of β-catenin is unknown (Group 2). Areas of increased β-catenin staining in the nucleus and cytoplasm often correlated to areas of high regucalcin and GS expression. **(D)** Arbitrary scoring of relative amounts of β-catenin, regucalcin, and GS in human HCCs with unknown mutational status of β-catenin (Group 2). Each tumor sample was given arbitrary immunohistochemical scores of (+) or (-) for presence or absence of nuclear and/or cytoplasmic β-catenin in hepatocytes. Regucalcin and GS samples were scored based on a comparison to the normal liver tissue; an increase above normal scored (++) or a level of expression similar to normal (+). Most biopsies that showed nuclear/cytoplasmic β-catenin also showed elevated levels of regucalcin and/or GS. **(E)** Western blot analysis utilizing nuclear fractions from rat hepatocytes cultured in the presence of Wnt3a or PBS for 24 hours shows an increase in β-catenin and regucalcin expression in Wnt3a-treated cultures only. Lamin B1 represents the loading control. **(F)** Western blot utilizing nuclear and cytoplasmic extracts from livers of two representative Wnt-1- or pcDNA3 (Con)-injected mice shows an increase in nuclear β-catenin and nuclear and cytoplasmic regucalcin expression in the experimental group.

As described in the previous chapter, we have previously employed hydrodynamic delivery of naked DNA as a means to induce expression of a gene in the liver [53]. Here, we utilized this approach to induce Wnt-1 expression in the liver after partial hepatectomy, as outlined in the Methods. An increase in the hepatic nuclear β-catenin level was observed in the Wnt-1- and not pcDNA3-injected mice (Figure 11F). A modest increase in levels of hepatic nuclear and cytosolic regucalcin accompanied the increased β-catenin expression in the Wnt-1-injected group as well (Figure 11F). Thus, Wnt/β-catenin activation induces regucalcin expression *in vitro* and *in vivo*, in both rat and mouse hepatocytes.

3.4.5 Association of regucalcin and β-catenin in human hepatoma cell lines

We next examined the human hepatoma cell lines Hep3B, which contains the wild-type β-catenin gene, and HepG2, which contains the β-catenin gene with a deletion in exon-3 rendering it non-degradable, for expression and functional analysis of regucalcin in liver biology [366]. Western blots demonstrate higher levels of regucalcin protein in HepG2 cells as compared to Hep3B cells (Figure 12A). This result was also confirmed by real-time PCR, which showed higher mRNA expression of regucalcin in HepG2 cells than in Hep3B cells (Figure 12B). Finally, immunofluorescence studies were concordant with the above results, with HepG2 cells displaying higher levels of regucalcin in the cytosol than Hep3B cells (Figure 12C). Interestingly, when cells were stained with both regucalcin and β-catenin, there appeared to be areas of signal co-localization in both cell lines, which was greater in HepG2 than in Hep3B cells (Figure 12C).

To determine whether this colocalization of β-catenin and regucalcin in HepG2 represented a physical association between the two proteins, we immunoprecipitated protein lysates from both HepG2 and Hep3B cells with regucalcin/SMP30 antibody and probed the blots for β-catenin and vice versa. Figure 12D shows coprecipitation of β-catenin and regucalcin in both HepG2 and Hep3B cells, although HepG2 cells show a stronger association. Also, following immunoprecipitation utilizing HepG2 cells, it was apparent that regucalcin associates with both the truncated (predominant species) and full-length form of β-catenin (minor species). Lysates incubated with normal IgGs instead of β-catenin or regucalcin antibodies did not pull down protein in either preparation (Figure 12D and data not shown). Thus, in addition to

regucalcin being a direct target of β-catenin signaling, these data demonstrate physical interactions between β-catenin and regucalcin**.**

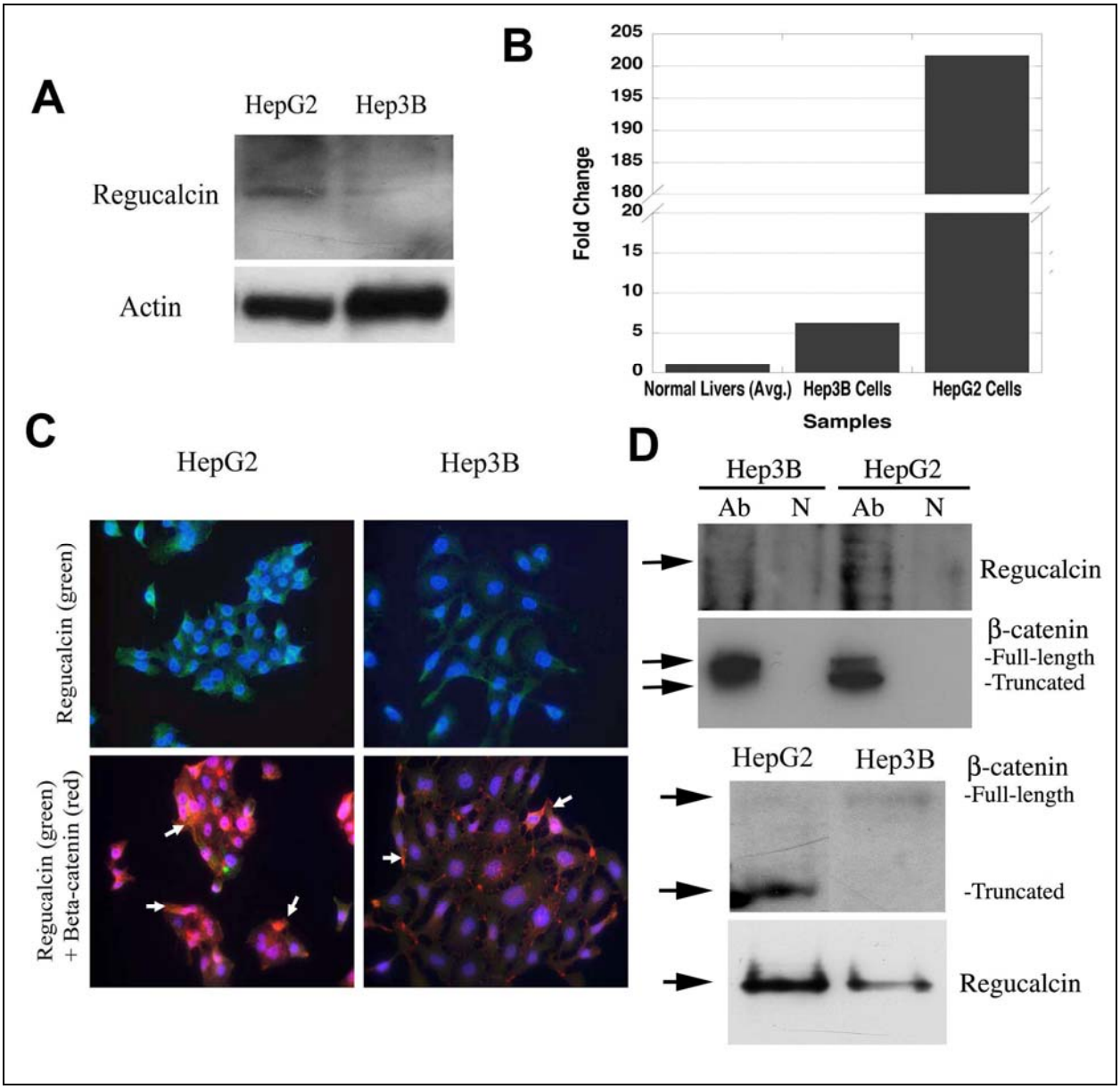

Figure 12: Regucalcin, which is expressed in both Hep3B cells and HepG2 cells, also associates with β-catenin in the cell. (A) Western blot of proteins harvested from HepG2 and Hep3B human hepatoma cell lines shows that HepG2 cells express higher levels of regucalcin than Hep3B cells. Western blot for actin serves as the loading control. **(B)** Real-time PCR analysis of mRNA harvested from HepG2 and Hep3B human hepatoma cell lines. Regucalcin mRNA expression was 6-fold higher in Hep3B cells but more than 200-fold higher in HepG2 cells as

compared to average of normal human livers. Regucalcin mRNA expression was standardized to actin expression. **(C)** Immunofluorescence on cultured HepG2 and Hep3B cells demonstrates higher regucalcin (green) and β-catenin (red) in HepG2 cells than Hep3B cells. Also, as seen in bottom panels, colocalization (white arrows) of β-catenin and regucalcin was evident especially in HepG2 cells. Images were taken at 600X magnification. **(D)** HepG2 and Hep3B cell lysates immunoprecipitated with β-catenin antibody and probed for regucalcin (top) show association of the two proteins. Non-specific IgG (N) did not pull down regucalcin. Similar lysates immunoprecipitated with regucalcin (SMP30) antibody and probed for β-catenin also show association of full length β-catenin with regucalcin in both cell types and truncated β-catenin-regucalcin association in HepG2 cells. Successful pulldown of β-catenin and regucalcin by their antibodies is verified in respective lower panels.

3.4.6 Regucalcin exerts a prominent effect on cell survival

Next, we attempted to address the biological role of regucalcin in liver. Since HepG2 cells exhibit higher levels of regucalcin, we investigated the effect of regucalcin inhibition on cell proliferation and survival in this cell line. HepG2 cells were cultured and treated with control and regucalcin siRNA constructs as outlined in the Methods. We found a 50-80% decrease in regucalcin mRNA expression over multiple experiments as shown in a representative real-time PCR, at 48 h after regucalcin siRNA-transfection (Figure 13A). This also coincided with decreased regucalcin protein production (Figure 13B).

Next, we measured the impact of regucalcin knockdown on cell proliferation by [^{3}H]thymidine incorporation. No significant difference in [^{3}H]thymidine incorporation was observed in HepG2 cells following regucalcin siRNA-transfection (Figure 13C). Although we were aware of the lower expression of regucalcin in Hep3B cells, we also examined the impact of regucalcin siRNA on proliferation in this cell line. Interestingly a marginal but significant and

consistent reduction in thymidine incorporation was observed following regucalcin siRNA transfection in Hep3B cells (Figure 13D).

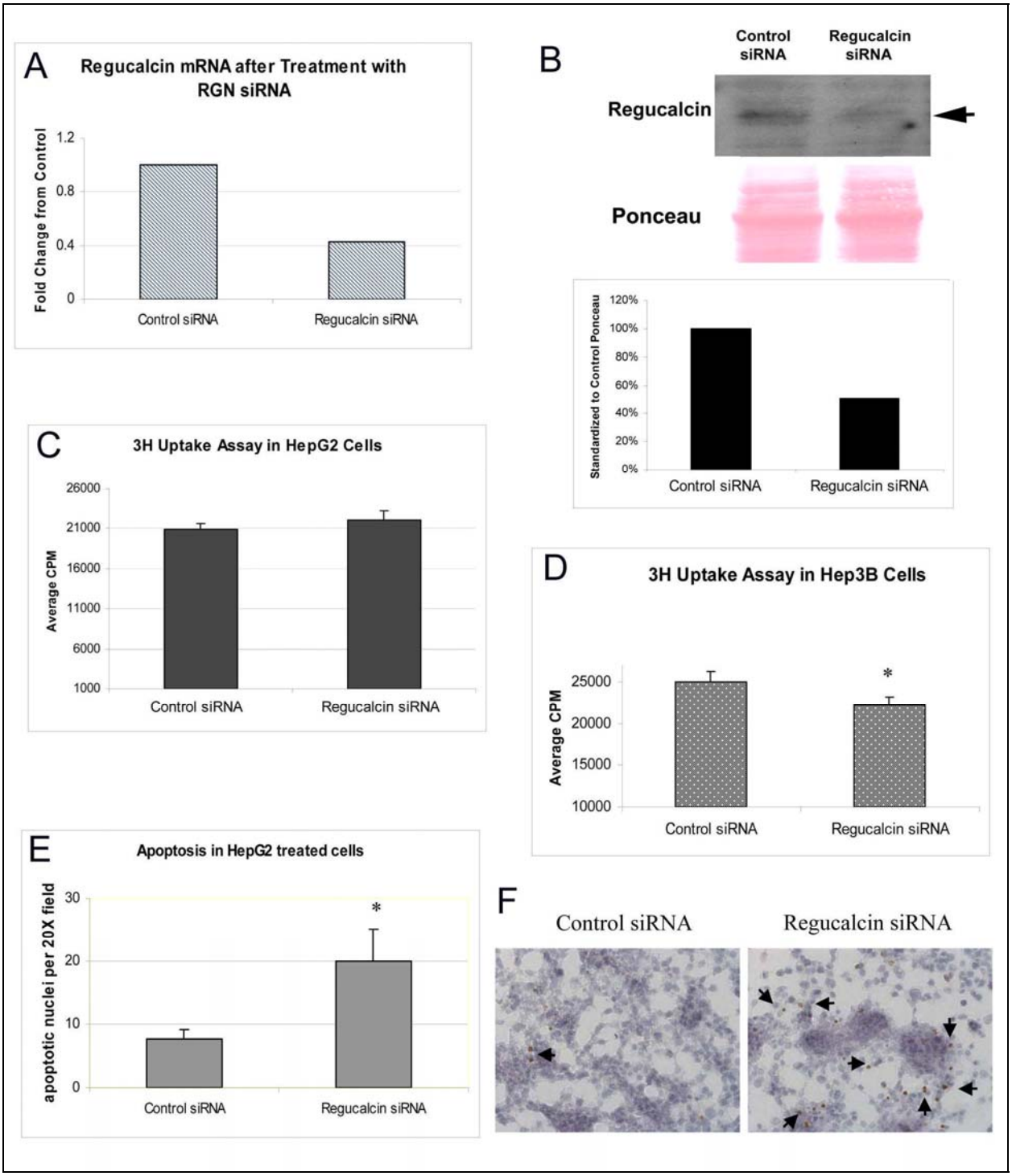

Figure 13: Regucalcin knockdown using siRNA affects survival of hepatoma cells. (A) Regucalcin siRNA decreases mRNA expression in hepatoma cells as shown in representative real-time PCR analysis of mRNA

harvested from HepG2 cells. The 2deltaCt of regucalcin mRNA was plotted for each cell line. In this representative plot, siRNA decreases regucalcin expression in HepG2 cells by 60% as compared to cells transfected with control siRNA. Regucalcin mRNA expression was standardized to actin expression. **(B)** A comparable decrease in regucalcin protein was also observed as shown in a representative western blot using lysates from Hep3B cells. Ponceau red staining verifies comparable loading in the western blot. Bottom panel is the plot for normalized integrated optical density from densitometric analysis of a representative western blot. **(C)** Unremarkable differences in thymidine uptake assay by control or regucalcin siRNA-transfected HepG2 cells. **(D)** A small but significant ($p<0.05$) decrease in thymidine uptake by regucalcin-transfected Hep3B cells as compared to control siRNA-transfected cells. **(E)** A greater than 2-fold increase in the numbers of TUNEL-positive apoptotic nuclei in regucalcin siRNA-transfected HepG2 cells as compared to control siRNA-transfected HepG2 cells. **(F)** Increased numbers of TUNEL positive apoptotic nuclei (arrowhead) are observed after regucalcin (right panel) and not control (left panel) siRNA transfection of HepG2 cells by representative immunohistochemistry. Images were taken at 200X magnification.

We then examined the effect of regucalcin suppression on cell survival in the HepG2 cells by TUNEL analysis. Transfection with regucalcin siRNA and not control siRNA led to a significant increase in the numbers of apoptotic nuclei in these cells (Figure 13E-F). Thus regucalcin seems to play an important role in cell survival in hepatoma cells.

3.4.7 β-Catenin regulates vitamin C synthesis through regulation of regucalcin expression

Regucalcin/SMP30 is an essential gluconolactonase in the liver and plays a pivotal role in regulating ascorbate biosynthesis [193]. Since SMP30 knockout mice fed a vitamin C-deficient diet have been shown to develop scurvy due to decreased levels of ascorbate, we investigated whether the β-catenin KO mice, which have decreased expression of regucalcin/SMP30, are also deficient in ascorbate production since these mice were being fed

normal mouse chow (Prolab Isopro RMH 3000), which lacks vitamin C. This became more relevant when gene array analysis of β-catenin-deficient livers also identified a significant decrease in the expression of L-gulonolactone oxidase, which is the final and rate-limiting step in ascorbic acid biosynthesis (Table 2). This decrease was also verified by RT-PCR (Figure 14A). Next, serum from WT and β-catenin KO animals was tested for ascorbate content (n=3). A 3.5-fold reduction in serum ascorbate levels was observed in β-catenin KO mice as compared to the WT littermates (Figure 14B). Therefore, β-catenin, through its role as a mediator of regucalcin and L-gulonolactone oxidase expression, plays an important role in regulating ascorbate levels.

3.4.8 Enhanced apoptosis of β-catenin-deficient hepatocytes is rescued by vitamin C supplementation

Since we previously reported a basal increase in the hepatocyte apoptotic index in β-catenin-conditional null mice, and an even greater increase in apoptosis during liver regeneration, we were interested to investigate if this could be contributed by regucalcin loss [94]. We cultured primary hepatocytes from β-catenin conditional null mice for 48 h. Greater than 90% of hepatocytes showed significant loss of viability resulting from apoptosis as shown by phase-contrast microscopy and TUNEL immunohistochemistry (Figure 14C-D). When β-catenin null hepatocytes were cultured in the presence of ascorbate, a dramatic and almost 100% rescue of cell viability was evident as reflected by negligible apoptosis assayed by TUNEL staining (Figure 14C-D).

3.4.9 Apoptosis in HepG2 cells due to regucalcin knockdown is rescued by vitamin C and NAC

Next, we wanted to explore if similar to mouse hepatocytes, apoptosis of HepG2 cells following regucalcin knockdown could be rescued by vitamin C. As expected and shown earlier (Figure 13E-F), a significant increase in apoptosis was evident in HepG2 cells 48 h after transfection of regucalcin and not control siRNA (Figure 14E). However, the presence of vitamin C in culture media led to a near-complete amelioration of the apoptosis brought about by regucalcin knockdown (Figure 14E).

It was interesting to note that ascorbic acid repletion led to the rescue of cell survival following siRNA-mediated regucalcin knockdown. Since human cells lack L-gulonolactone oxidase and are unable to synthesize vitamin C, we wondered if the rescue effect of ascorbate was specific to vitamin C deficiency brought about by regucalcin knockdown or through the general anti-oxidant effect of vitamin C [381]. To test this hypothesis, we utilized N-acetylcysteine (NAC), another well-known anti-oxidant, to determine its effect on regucalcin-siRNA mediated apoptosis of HepG2 cells [390]. A significant decrease in apoptosis was evident in NAC-treated HepG2 cells secondary to regucalcin knockdown (Figure 14F). These observations suggest that regucalcin might be important in cell survival in humans through regulation of oxidative stress, which may be independent of vitamin C biosynthetic function (Figure 15).

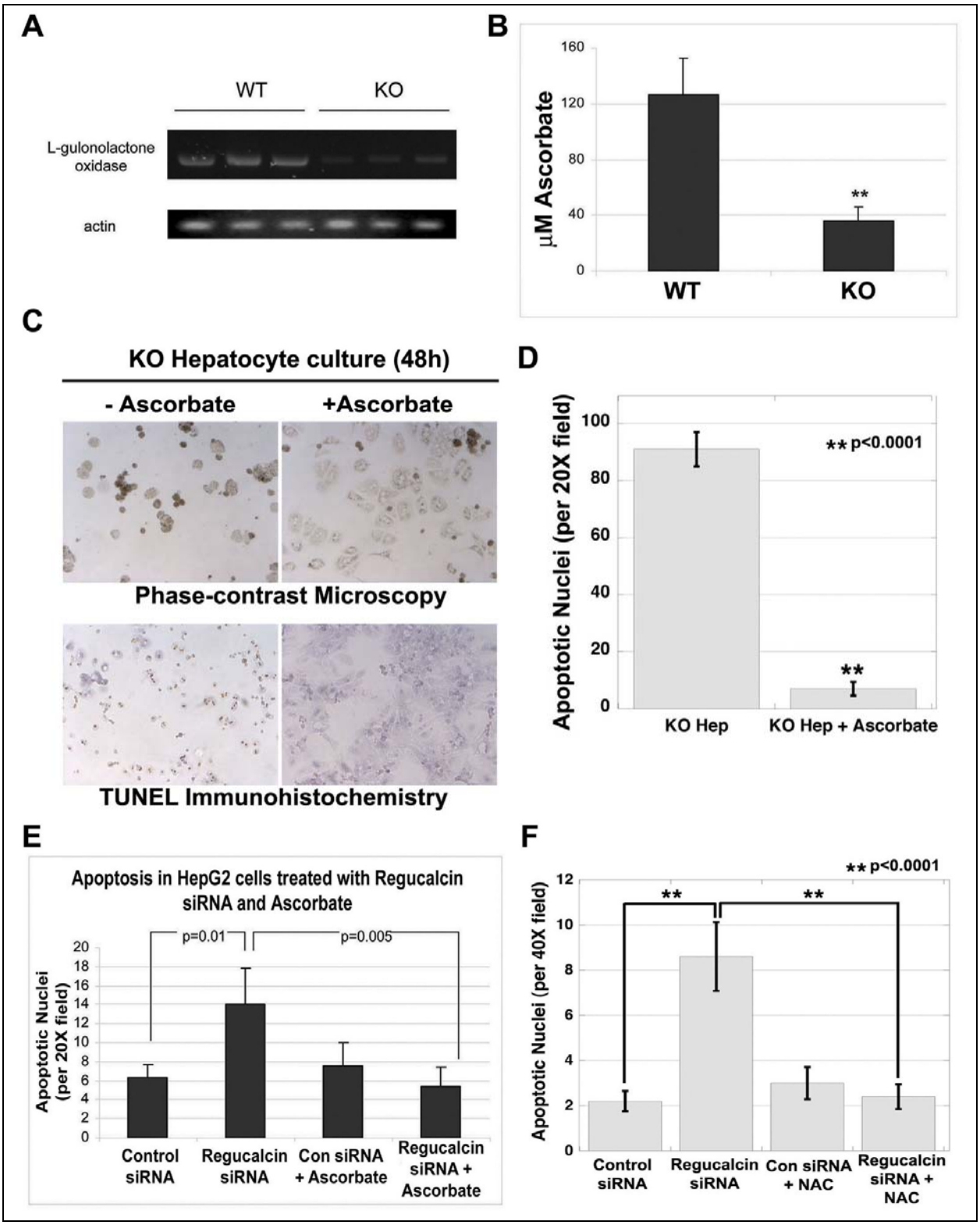

Figure 14: Decreased regucalcin expression in the absence of β-catenin negatively affects ascorbate levels and cell survival. (A) RT-PCR identified a dramatic decrease in L-gulonolactone oxidase expression in β-catenin-

deficient livers as compared to the WT livers. **(B)** A greater than 3-fold decrease in serum ascorbate levels was evident in the β-catenin KO mice (n=3) as compared to the WT littermates and the difference is statistically significant (**$p<0.001$). **(C)** β-Catenin-deficient hepatocytes (KO) exhibit loss of viability after 48 h in culture as shown by phase contrast microscopy (upper left panel) and increased TUNEL-positive nuclei (bottom left panel). Inclusion of vitamin C in the culture prevented loss of hepatocyte viability (upper right) along with a dramatic decrease in the TUNEL-positive hepatocytes (lower right). **(D)** Quantitative analysis of TUNEL immunohistochemistry identifies an extremely significant decrease in apoptosis in KO hepatocytes in the presence of vitamin C ($p<0.0001$). **(E)** Regucalcin siRNA induces significant apoptosis in HepG2 cells over the control-siRNA-transfected cultures ($p=0.01$). Addition of 0.2mM ascorbic acid significantly reduces apoptosis observed in response to regucalcin knockdown only ($p=0.005$) and does not impact the basal apoptosis in control siRNA treated HepG2 cells. **(F)** A similar rescue of apoptosis in HepG2 cells brought about by regucalcin knockdown is observed in the presence of 20mM of NAC ($p=0.01$).

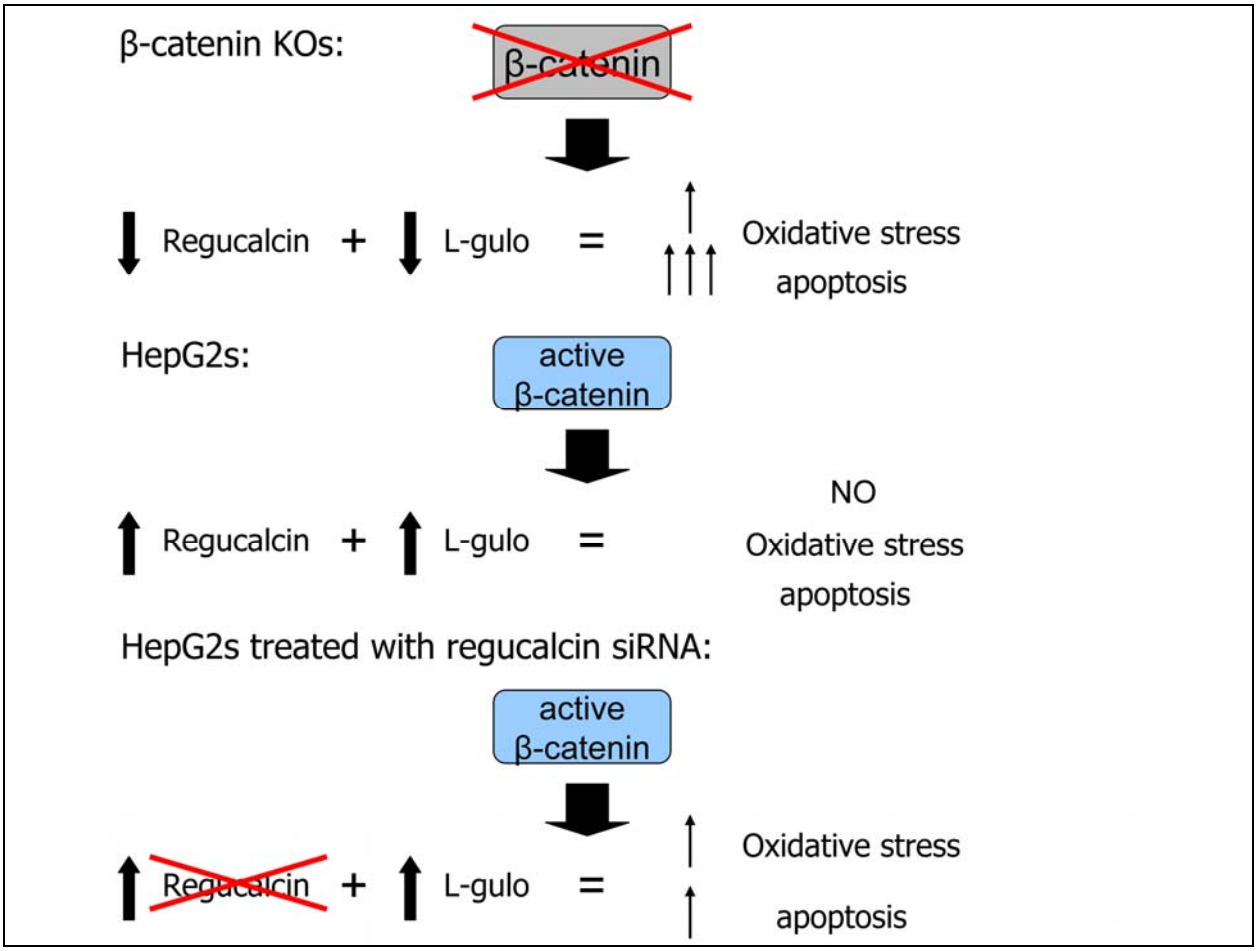

Figure 15: Regucalcin regulates oxidative stress and apoptosis in β-catenin conditional KO mice and in HepG2 cells. In the absence of β-catenin, both regucalcin and L-gulonolactone oxidase (L-gulo) expression is decreased in hepatocytes. This leads to a deficiency in ascorbate production, resulting in increased oxidative stress and apoptosis, which can be rescued by the addition of ascorbate to the culture media. The HepG2 human hepatoma cell line contains a mutated consitutively activated form of β-catenin and consequently has increased levels of regucalcin and (presumably) L-gulo expression. However, when HepG2 cells are treated with regucalcin siRNA, a modest but significant increase in both oxidative stress and apoptosis is observed, which can be rescued by the addition of anti-oxidants to the culture media.

3.5 DISCUSSION

β-catenin is the crucial downstream effector of the canonical Wnt pathway, and plays many roles in development and adult tissue homeostasis in various organs. Since it is a transcriptional co-activator, it is critical to identify the target genes that in turn are responsible for the pleiotropic roles of the pathway in diverse cellular and tissue processes. β-catenin has critical roles in regulating cell proliferation, differentiation, apoptosis, and adhesion in the liver, which in turn makes it relevant in liver development, regeneration, zonation, metabolism and cancer [34, 96, 124, 331, 392]. In the current study, we establish regucalcin as a novel target of β-catenin in the liver. Furthermore, we identified an important role of β-catenin in vitamin C biosynthesis and cell survival through the regulation of regucalcin and L-gulonolactone oxidase expression.

The first evidence of regulation of regucalcin/SMP30 by β-catenin came from the gene array analysis using livers from β-catenin conditional KO and wild-type littermates [94]. This was directly investigated in the current study, which showed that decrease in regucalcin correlated with β-catenin loss in KO livers. Similarly, β-catenin TG mice, which display elevated levels of β-catenin, also showed elevated levels of regucalcin protein in the nucleus and cytosol of hepatocytes as compared to controls. It is important to highlight the observed difference in regucalcin during steady state in the adult livers between the two wild-type samples that belonged to C57BL/6 and FVB strains. Clearly, a wider regucalcin expression in all zones was observed in C57BL/6 livers although it was more pronounced around central veins, as compared to the FVB livers, which showed a narrow centrizonal regucalcin expression only. This coincides with β-catenin signaling that is typically limited to centrizonal areas in normal

adult livers [124]. Strain-specific differences in the basal regucalcin expression adds to the category of a growing list of genes and proteins that exhibit such phenomena and might eventually be the basis of diverse responses or phenotypes observed in response to a similar stimuli [393].

To further reaffirm the regulation of SMP30 by β-catenin we examined a series of models that are known to exhibit β-catenin activation. Results of the liver regeneration studies show an increase in regucalcin protein expression as early as 1h after partial hepatectomy in the rat. This expression remains increased over T0 (with the exception of 12h post-PHx) until approximately D3. This expression corresponds to that of β-catenin during liver regeneration after partial hepatectomy, where nuclear translocation of β-catenin is observed within 5 min after hepatectomy and is retained in the hepatocyte nucleus until around 48h [126]. Moreover, regucalcin expression mirrored the expression pattern of GS, a known target of β-catenin, during liver regeneration [34]. Others have found that liver regucalcin mRNA levels are clearly increased 1-5 days after hepatectomy, in comparison with that of sham-operated rats [177]. Human hepatocellular carcinomas containing activating β-catenin mutations were also investigated for regucalcin expression, which was clearly elevated in these samples [339]. Similarly, those tumors with unknown β-catenin gene mutation status, but exhibiting β-catenin staining in the nucleus and/or cytosol, also showed an increase in both regucalcin and GS expression. Finally, we were interested to examine any impact of exogenous Wnt stimulation on regucalcin expression. Indeed, treatment of rat hepatocytes with Wnt3a induced β-catenin nuclear translocation as well as regucalcin expression. In an *in vivo* mouse study, we successfully delivered Wnt-1 plasmid DNA hydrodynamically through the tail vein [53], followed by partial hepatectomy, and harvested livers at 30 h. This led to an increase in nuclear β-catenin and

regucalcin expression in the livers of Wnt-1-injected and not pcDNA-injected mice. Thus, higher regucalcin levels are observed in the event of Wnt/β-catenin activation in mice, rats, and HCC patients. It has been previously suggested that regucalcin may function as an inhibitor of proliferation during liver regeneration in rats. Thus the role of regucalcin in cell proliferation will need to be investigated further as a primary or perhaps a secondary event due to its ability to regulate cell viability.

It was interesting to note that not only was the expression of regucalcin regulated by β-catenin, but that it associated with β-catenin in Hep3B and HepG2 cells, as well as in resting wild-type mouse livers (data not shown). This is not unusual in Wnt signaling since several regulators of the pathway are in fact transcriptional targets of β-catenin/TCF4, including TCF-1 [29], Axin-2 [394] and DKK1 [395]. Whether regucalcin itself is able to have an impact on Wnt signaling remains under investigation.

An important role of regucalcin has been reported in vitamin C biosynthesis [193]. Indeed β-catenin KO mice show significantly lower ascorbate levels secondary to decreased expression of both regucalcin and L-gulonolactone oxidase, both critical in vitamin C synthesis in murine hepatocytes [381]. It is also relevant to note that β-catenin KO livers show an increase in basal hepatocyte apoptosis, which is aggravated during liver regeneration [94]. To demonstrate a direct role of β-catenin in regulating apoptosis through vitamin C homeostasis, we cultured hepatocytes from β-catenin KO mice. These cells displayed massive apoptosis within 48 hours of culture. When cultured in the presence of vitamin C, there was a complete rescue of apoptosis of the β-catenin-deficient hepatocytes. This is in agreement with previous studies where regucalcin overexpression has been shown to suppress cell death [189]. Our results are also concordant with previous studies where mice containing a germline null mutation of SMP30

have been shown to be highly susceptible to both TNF-α and Fas-mediated apoptosis, thereby indicating that SMP30 plays a role in protection from apoptosis and contributes to cell survival [191, 192].

A significant increase in apoptosis was also evident following regucalcin suppression in HepG2 cells, although the increase in apoptosis was not nearly as pronounced as in the murine hepatocytes. Moreover, the apoptosis in regucalcin siRNA-transfected hepatoma cells was rescued by supplementation with ascorbic acid or NAC. Since both molecules possess strong anti-oxidant properties, these results suggest that decreased regucalcin in human hepatoma cells might be inducing cell death through oxidative stress. Thus, regucalcin may be regulating cell survival through regulation of redox state of the cell and independent of vitamin C biosynthesis in human cells. However, in murine cells, regucalcin might be regulating cell survival through distinct, yet unexplored apoptotic pathways as well as through control of oxidative stress, and both might be significantly contributed by its role in vitamin C biosynthesis. In fact, previous publications from our lab have documented increased oxidative stress and apoptosis in prenatal β-catenin KO livers [96], MCD-induced liver injury [396], and in adult livers from β-catenin conditional KOs [94, 397]. Altogether, these studies suggest that one mechanism by which β-catenin might be promoting cell survival is through regulation of regucalcin expression.

4.0 BETA-CATENIN KNOCKOUT MICE ARE PROTECTED FROM TNF-ALPHA MEDIATED APOPTOSIS

4.1 ABSTRACT

Wnt/β-catenin signaling plays an important role in hepatic homeostasis. Loss of β-catenin signaling in hepatocyte-specific conditional knockout (KO) mice led to a basal increase in the number of apoptotic hepatocytes. Additionally, β-catenin loss during liver development or in hepatoma cells often manifests as enhanced cell death. Knowing the role of TNF-α in regulating cell death, we explored the susceptibility of KO and their wildtype littermate controls (WT) to intraperitoneal (i.p.) injection of lipopolysaccharide (LPS) or TNF-α after D-galactosamine (GalN) or actinomycin D (ActD) pre-treatment. Paradoxically, KO mice are refractory to GalN/LPS, ActD/LPS and GalN/TNF-α and showed significantly lower morbidity than the WT animals. Furthermore, gross and histological examination of KO livers verified a dramatically lower injury than in the WT. Analysis of liver enzymes, TUNEL staining and caspase activity confirmed the presence of massive hepatic injury in the WT but not in the KO. To determine the possible mechanism of protection from TNF-α-induced injury, we analyzed the anti-apoptotic transcription factor NF-κB. Levels of NF-κB (p65 and p50) were elevated in the KO along with its downstream targets as compared to WT that show lack thereof. We also observed a basal increase in NF-κB signaling in KO reflected by elevated phosphoserine-536-

p65, along with greater expression of targets such as Fas and Traf-1. We next addressed the mechanism of basal increase in NF-κB activation, which may be responsible for protection against TNF-α injury in KO. Higher expression of TLR4, a receptor for LPS that is normally present in portal circulation, was observed in KO livers. Increased numbers of CD45+ve inflammatory cells that are a source of cytokines such as TNF-α and IL-6 were also evident in KO livers along with higher expression of several TNF-α-induced gene transcripts. In addition, p65-β-catenin association which has been shown to prevent NF-κB activation was lacking in KO hepatocytes. Phosphorylated p65 is seen in the nucleus as early as 1 hour after LPS administration in KOs but not in WTs, suggesting that the absence of β-catenin lowers the threshold of NF-κB activation by facilitating p65 nuclear translocation. Thus, we observed a paradoxical protection from TNF-α-mediated apoptosis in β-catenin conditional KO animals owing to preexisting priming of the liver through NF-κB activation which is due to multiple mechanisms. Therefore, β-catenin through its interactions with NF-κB may be an important component of balancing hepatocyte life and death and therefore regulating liver homeostasis.

4.2 INTRODUCTION

Hepatic inflammation due to a number of etiologies, such as viruses, alcohol, and others, is a common cause of acute hepatic injury and liver cell death. In fact, the liver is one of the organs most affected by septic shock, and treatment with various agents to recapitulate such injury is a useful method to study the role of cell death, or apoptosis, in this system [398]. Liver has a particular susceptibility to apoptosis, due to a rich expression of death receptors such as TNF-R1

and Fas [195]. For these reasons, much of our current knowledge on the subject of hepatocyte apoptosis has been derived from experimental induction of liver failure. Apoptosis can be induced through two major death-receptor mediated pathways in the liver: the Fas pathway and the TNF-α pathway. Fas-mediated apoptosis can be stimulated by intravenous injection of the anti-Fas antibody Jo-2 into mice. Jo-2 binds to the Fas receptor to directly induce apoptosis, which causes acute liver failure leading to lethality within hours of administration [399]. However, unlike Fas-mediated apoptosis, stimulation of the TNF-α pathway requires hepatocyte sensitization, which is usually accomplished by pretreatment with either D-galactosamine (GalN) or actinomycin D (ActD) before administration of either TNF-α ligand or lipopolysaccharide (LPS). GalN is a hepatotoxic agent that depletes UTP, thus inhibiting de novo RNA synthesis and sensitizing hepatocytes to the effects of endotoxin [256]. ActD is a different class of transcriptional inhibitor that binds DNA duplexes and prevents synthesis of protective proteins [261]. Treatment with LPS after GalN or ActD sensitivity causes hepatotoxicity and lethality, which is primarily mediated by release of TNF-α from macrophages that accumulate in response to LPS administration [257]. In addition, administration of recombinant TNF-α directly after sensitization causes hepatotoxicity identical to that seen in LPS-treated mice, which also suggests that TNF-α is the major mediator of endotoxin lethality [256]. Cell death caused by activation of the TNF-α pathway is believed to occur primarily by apoptosis, followed by necrosis.

In addition to initiating the cell death machinery, binding of TNF-α to its receptor (or binding of LPS to TLR-4) stimulates formation of signaling complexes that prevent cell death, culminating in the activation of nuclear factor kappa B (NF-κB) [400]. NF-κB is a group of dimeric transcription factors that initiate a wide variety of cellular programs in response to

inflammation and injury. Inactive NF-κB is sequestered in the cytoplasm by members of the IκB complex, which become phosphorylated and degraded in response to TNF-α signaling [242]. Loss of the IκB complex liberates NF-κB, which then translocates to the nucleus, where it becomes phosphorylated and fully activated [241]. Recently, NF-κB has also been recognized as a key apoptosis inhibitor through its activation of genes including IAPs, c-FLIP, TRAFs, and Bcl family members, among others [253, 254]. Expression and activation of these proteins suppresses caspase activation and thus allows the cell to avoid undergoing apoptosis**.** Activation of NF-κB by LPS or TNF-α can directly antagonize the pro-apoptotic effects of the latter [254], as demonstrated in experimental models, where p65 is required for protection from TNF-α-mediated apoptosis [401-403]. However, treatment with LPS or TNF-α alone does not cause any hepatocyte injury and thus requires agents like GalN, whose pre-treatment prevents NF-κB-mediated pro-survival target gene expression for up to 3-4 hours. In summary, signaling through the TNF-α pathway can result in either life or death for the cell depending on physiological context and pathway crosstalk, which favors activation of one pathway over another [404].

In recent years, the Wnt/β-catenin pathway has been increasingly recognized as an essential component of liver biology. Despite the wealth of knowledge on β-catenin in such processes as liver development, regeneration, and cancer formation, to our knowledge, no study has been conducted to precisely address the role of β-catenin in cell survival. Nonetheless, evidence exists that ablation of β-catenin renders hepatocytes more susceptible to apoptosis. Our laboratory has previously shown an increase in apoptosis following inhibition of β-catenin by β-catenin antisense morpholino oligomer in developing (E10) livers [89]. We also observed a higher number of apoptotic nuclei in the livers of β-catenin conditional knockout mice as compared with the wild-type livers after partial hepatectomy (PHx) [94]. Another group has demonstrated

that apoptosis is induced in cells treated with Dkk-1, a Wnt/β-catenin pathway inhibitor, via a downregulation of the anti-apoptotic protein Bcl-2 and an upregulation of the pro-apoptotic protein Bax [262]. β-catenin can also directly promote survival in renal epithelial cells by inhibiting Bax through activation of Akt [265]. Further, canonical Wnt signaling exerts an anti-apoptotic effect in human hepatic stellate cells and thus enhances their survival [264]. However, the mechanisms underlying Wnt-mediated regulation of hepatocyte apoptosis are incompletely understood.

To determine the molecular mechanisms underlying the basal increase in apoptosis seen in our KO mice, and to elucidate the role of Wnt/β-catenin signaling in hepatocyte apoptosis, we utilized the β-catenin conditional knockout (KO) mice referred above and challenged these KOs and their wild-type (WT) counterparts with transcriptional inhibitors followed by activators of the TNF-α mediated apoptotic pathway. We chose to investigate activation of the TNF-α pathway because preliminary gene array analysis revealed an upregulation of several TNF-α dependent genes in the KO livers at baseline (Table 2), suggesting that this pathway may be stimulated basally in the KOs. We monitored the animals for morbidity following LPS or TNF-α administration after GalN or ActD pre-treatment and then harvested the livers and serum from both genotypes and analyzed them histologically and chemically for liver injury and apoptosis. Surprisingly, we found that KO animals are more resistant to damage from TNF-α mediated apoptosis than WTs and showed decreased morbidity over time. Examination of various time points after GalN/LPS injection reveals that KOs show a rapid onset and partial resolution of hepatic injury which is limited as opposed to WTs, which accumulate irreversible and progressive damage. Investigation of potential protective mechanisms in the livers of KO mice reveals that NF-κB is upregulated both basally and after GalN/LPS treatment, possibly as a result

of increased endogenous inflammation in the KO. Finally, we describe the kinetics of β-catenin/p65 dissociation after LPS administration and show that loss of β-catenin in KO mice may allow for a more rapid translocation and activation of p65 after injury, which appears to occur through multiple mechanisms. Through these studies, we demonstrate that β-catenin KO mice are protected from TNF-α mediated apoptosis through NF-κB pathway activation, and that β-catenin through its interaction with NF-κB components may be playing a key role in balancing hepatocyte survival.

Table 2: Changes in basal expression of TNF-α induced genes, anti-death genes, caspase genes, and others in β-catenin KO mice at baseline and after partial hepatectomy

RESTING LIVER	**Fold change**
LPS-induced TNF-alpha factor	1.35
LPS-induced TNF-alpha factor	1.38
BCL2-antagonist of cell death	6.94
BCL6	5.42
HGF	9.27
caspase 9, apoptosis-related cysteine peptidase	-6.30
CASP8 and FADD-like apoptosis regulator	-4.19
C-Rel	-3.08
40H Post-PHx	
LPS-induced TNF-alpha factor	1.34
LPS-induced TNF-alpha factor	1.79
TNF-alpha induced adipose related protein	2.13
TNF-alpha induced protein 2	2.41
Fas-activated serine threonine kinase	6.90

4.3 MATERIALS AND METHODS

4.3.1 Animals

Conditional β-catenin knockout mice were generated as described previously [94]. Briefly, homozygous floxed β-catenin mice were bred to albumin-cre mice (both on a C57BL/6 background); the mice carrying a floxed β-catenin allele and cre-allele were backcrossed to homozygous floxed β-catenin mice that resulted in conditional null mice with the genotype $Ctnnb1^{loxp/loxp}$; $Alb\text{-}Cre^{+/-}$ (referred as knockout or KO mice from here on) and controls with the genotype $Ctnnb1^{loxp/loxp}$; $Alb\text{-}Cre^{-/-}$ or $Ctnnb1^{loxp/Wt}$; $Alb\text{-}Cre^{-/-}$ (referred to as wild-type or WT mice). Mice were maintained under specific pathogen free conditions in the Animal Facility in the University of Pittsburgh and were used under the approval of the University of Pittsburgh's Institutional Animal Care and Use Committee and in accordance with National Institutes of Health guidelines.

4.3.2 Induction of liver injury through the TNF-α pathway

TNF-α-mediated liver injury was induced by administration of D-galactosamine (GalN; Sigma, St. Louis MO) and lipopolysaccharide (LPS; Sigma L-2630, *Escherichia coli* 0111:B4); actinomycin D (ActD) (Sigma) and LPS; or GalN and recombinant mouse TNF-α (R&D Systems, Minneapolis MN). For the GalN/LPS studies, both WT and KO animals (either male or female) were injected intraperitoneally with GalN at a dose of 700mg/kg of body weight. Thirty minutes later, they were treated with LPS intraperitoneally at 50μg/kg of body weight [238, 405]. For the ActD/LPS studies, WT and KO female animals were injected

intraperitoneally with ActD at a dose of 800mg/kg of body weight, followed 30 minutes later by intraperitoneal injection of LPS at a dose of 50μg/kg of body weight [406]. For the GalN/TNF-α studies, WT and KO female animals were were injected intraperitoneally with GalN at a dose of 700mg/kg of body weight, followed 1 hour later by intravenous injection of 2μg/kg TNF-α as described previously [405]. In all treatment groups, mice were closely monitored and sacrificed at the first signs of morbidity (hunched posture, decreased core temperature, and decreased movement). Livers were harvested and frozen at -80C^0 for subsequent analysis. A portion of the liver was fixed in 10% formalin for paraffin embedding. Kaplan-Meier survival analysis was performed using XLSTAT (Addinsoft, New York, NY).

4.3.3 Measurement of serum AST and ALT levels

Whole blood from mice injected with GalN/LPS was collected through retro-orbital bleeding and spun at 4000 RPM for 10 minutes to separate the serum. To assess hepatocellular injury after LPS treatment, serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured by the In Situ Hybridization Laboratory within the Department of Pathology [407].

4.3.4 Histology, Immunohistochemistry, and TUNEL staining

Liver tissue fixed in 10% formalin and embedded in paraffin were sectioned 4 μm in thickness onto Superfrost Plus glass slides (Thermo Fisher Scientific, Pittsburgh, PA) and heat-fixed overnight at 37°C. The sections were stained with hematoxylin and eosin (H&E). Apoptotic nuclei were detected by the terminal deoxynucleotidyl transferase-mediated dUTP

nick-end labeling (TUNEL) staining using the ApopTag Peroxidase kit (Intergen Company, Purchase, NY) as previously described [89].

Immunohistochemistry on paraffin-embedded sections of mouse livers was performed as previously described [387]. Briefly, 4 μm thick tissue sections were microwaved in citrate buffer, pretreated with 3% H_2O_2 to eliminate endogenous peroxidases, and blocked using Ultra V Block (Lab Vision Products, Fremont, CA). Primary antibodies used for this project were anti-NF-κB p65 (A) (used at a 1:10 dilution and incubated 30 minutes) and anti-CD45 (used at a 1:100 dilution and incubated 1 hour) (both from Santa Cruz Biotechnology, Santa Cruz, CA). Biotinylated goat anti-rabbit secondary (Chemicon, Temecula, CA) was used at either a 1:200 or 1:500 dilution. Immunohistochemistry was performed using the Vectastain ABC Elite kit (Vector Laboratories, Inc., Burlingame, CA) and developed using DAB (Vector Laboratories, Inc., Burlingame, CA). The slides were counterstained with Shandon's hematoxylin (Thermo Fisher Scientific, Pittsburgh, PA) and mounted with cytoseal (Biomeda Corp., Foster City, CA).

4.3.5 Protein Extraction and Western Blots

Whole-cell lysates from mouse livers were prepared by homogenization using RIPA buffer (9.1 mmol/L dibasic sodium phosphate, 1.7 mmol/L monobasic sodium phosphate, 150 mmol/L sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate [pH adjusted to 7.4]) containing fresh protease and phosphatase inhibitor cocktails (Sigma, St. Louis, MO) [52]. Nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit as per the instructions (Thermo Fisher Scientific, Rockford, IL). The concentration of the protein in all lysates was determined by the bicinchoninic acid assay using BSA as a standard.

Proteins were subjected to electrophoresis and blotted as described previously [126]. Briefly, 50-100 μg of protein from liver lysate was resolved on 7.5%, 12%, or 4-15% precast SDS-PAGE gels (ISC BioExpress, Kaysville, UT) using the mini-PROTEIN 3-electrophoresis module assembly (Biorad, Hercules, CA), followed by transfer to Immobilon-PVDF membrane (Millipore, Bedford, MA). The membrane was stained with Ponceau-S solution to confirm equal loading and then blocked in either 5% nonfat dry milk or 5% BSA in blotto solution (20mM TRIS-HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20), followed by incubation with primary antibody diluted in either 5% milk/blotto or 5%BSA/blotto for 2 hours. The membranes were washed and incubated in horseradish-peroxidase conjugated secondary antibodies for 1 hour followed by washing in blotto. Proteins were detected by Super-Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and visualized by autoradiography. Antibodies used in this study were as follows: NF-κB p65 (A, F-6, and C-20 clones), NF-κB p50, Fas, Stat3, Traf-1, GAPDH, TNF-R1, TLR-4, TRADD, FADD, GSK-3β, Ser-9 GSK-3β, IL-6, E-cadherin, EGFR, Met, Glutamine Synthetase, Axin2/Conductin (Santa Cruz Biotechnology, Santa Cruz, CA; all used at 1:200 dilution); β-catenin (used at 1:500 dilution; BD Biosciences, San Jose, CA); Bcl-2 and pser536 p65 (both used at 1:1000 dilution; Cell Signaling Technology, Danvers, MA); and cyclin-D1 (used at 1:500 dilution; Neomarkers, Fremont, CA). Secondary antibodies were horseradish peroxidase-conjugated goat anti-mouse (1:35,000), donkey anti-rabbit (1:35,000), and donkey anti-goat (1:15,000) (Millipore, Temecula, CA).

4.3.6 Analysis of Caspase Activities

Caspase activities were analyzed as described in a previous study [218, 405]. Briefly, RIPA extracts from whole liver lysates (50-100 ug) were subjected to SDS-PAGE followed by

Western blotting with an anti-caspase 8, anti-caspase 3, or anti-cleaved caspase 3 (p17/p19 subunits) antibodies (Cell Signaling Technology, Danvers, MA). In addition, caspase activities were analyzed by incubating RIPA extracts (15 μg) with 20 μM site-specific fluorescent tetrapeptide substrates (DEVD-AFC for caspases 3, EMD Chemicals, Gibbstown, NJ) at 37 °C for 4 hours. Fluorescence was detected by a fluorescence spectrometer (PerkinElmer Life Sciences LS50-B) at 400 nm excitation and 505 nm emission wavelengths. The signals representative of caspase activities were standardized relative to wild-type baseline controls.

4.3.7 cDNA plate array

mRNA was isolated from WT and KO livers using TRIzol (Invitrogen, Carlsbad, CA) as previously described [408]. mRNA was then analyzed for NF-κB-regulated gene expression with a mouse NF-κB-regulated cDNA plate array (Signosis, Sunnyvale, CA) as per the manufacturer's instructions. Briefly, mRNA was reverse transcribed using a biotin-labeled NF-κB primer mix, mixed with hybridization buffer, and added to a plate containing 21 target genes. The plate was then incubated at 45^0C overnight, washed the next day, blocked, and developed with substrate. The plate was read on a BioTek HT (BioTek, Winooski, VT) with no filter to detect luminiescence.

4.4 RESULTS

4.4.1 β-catenin conditional knockout mice are resistant to TNF-α induced apoptosis

We wanted to determine if loss of β-catenin contributed to a basal increase in apoptosis through activation of the TNF-α pathway. To do this, we administrated a transcriptional inhibitor, D-galactosamine (GalN), followed 30 minutes later by bacterial lipopolysaccharide (LPS), a major inducer of endotoxic shock, to both WT and KO mice. The GalN/LPS model is an established and well-documented method of inducing hepatocyte apoposis through the TNF-α pathway [258, 260]. Animals were monitored closely for signs of morbidity (ruffled fur, inactivity) and sacrificed upon observation of these indicators. Table 3 summarizes the observations after GalN/LPS administration in WT and KO animals. Nine of nine (9/9) WT mice became lethargic and moribund approximately 6 hours after GalN/LPS administration, which represents the average time to sickness in WT animals [260, 409, 410]. Surprisingly, most (14/15) of the KO mice survived past 6 hours, with some KOs seemingly uncompromised and healthy as late as 12 hours post-treatment (Figure 16A). These mice displayed normal physical activity, did not assume a hunched posture, and responded well to external stimulation. Thus, although stimulation of the TNF-α pathway caused predictable morbidity in WT mice, KO mice showed a clear decrease in morbidity and mortality.

Table 3: Status and time to morbidity of WT and KO mice after GalN/LPS injection

Genotype	Sex	Time to Sac	Status	LW/BW
KO	F	6 hr.	moribund	7.24%
KO	F	7.5 hr.	alive	3.03%
KO	F	7.5 hr.	alive	4.24%
KO	F	7.5 hr.	moribund	5.14%

KO	F	7.5 hr.	moribund	5.86%
KO	F	7.5 hr.	alive	3.81%
KO	M	5.5 hr.	alive	4.03%
KO	M	6 hr.	alive	4.29%
KO	M	5.5 hr.	alive	3.77%
KO	M	5.75 hr.	alive	5.11%
KO	F	12 hr.	alive	3.48%
KO	F	7.5 hr.	moribund	6.26%
KO	F	10.5 hr.	moribund	5.19%
KO	F	12.5 hr.	alive	3.55%
KO	F	8 hr.	dead	7.97%
WT	F	6 hr.	moribund	7.44%
WT	F	6.5 hr.	moribund	8.22%
WT	F	6 hr.	moribund	9.04%
WT	F	6 hr.	moribund	8.18%
WT	F	6 hr.	moribund	7.13%
WT	F	5 hr.	moribund	8.63%
WT	F	5 hr.	moribund	8.37%
WT	M	5.25 hr.	dead	7.69%
WT	M	5.75 hr.	dead	6.11%

To further substantiate these findings we used actinomycin D (ActD), a different class of transcriptional inhibitor that intercalates DNA [261] instead of GalN, followed 30 minutes later by LPS treatment. As with GalN/LPS, the KO mice continued to exhibit resistance to the effects of ActD/LPS while WT mice displayed morbidity around 5 hours after LPS (Table 4).

Table 4: Status and time to morbidity of WT and KO mice after ActD/LPS injection

Genotype	Sex	Time to Sac	Status	LW/BW
KO	F	5 hr.	alive	4.14%
KO	F	4.45 hr.	alive	4.86%
KO	F	5 hr.	dead	5.42%
KO	F	5.5 hr.	alive	4.32%
KO	F	5.5 hr.	alive	4.06%
WT	F	5 hr.	dead	6.02%
WT	F	4.75 hr.	dead	7.21%
WT	F	5 hr.	dead	8.68%
WT	F	4.5 hr.	moribund	8.56%
WT	F	5.25 hr.	alive	5.27%
WT	F	5.5 hr.	dead	7.73%
WT	F	5.5 hr.	moribund	6.41%

Finally, to confirm the specificity of TNF-α apoptotic pathway activation in these animals, we administered GalN followed by intravenous injection of soluble TNF-α, a major mediator of LPS-induced hepatotoxicity [411, 412]. The KO mice were refractory to GalN/TNF-α treatment as well; while 3/3 WT animals were moribund or dead by 6.5 hours after treatment, 2/2 KO animals lacked any morbidity at the time of harvest (Table 5).

Table 5: Status and time to morbidity of WT and KO mice after GalN/TNF-α injection

Genotype	Sex	Time to Sac	Status	LW/BW
KO	F	6 hr.	alive	3.30%
KO	F	6.5 hr.	alive	4.43%
WT	F	6.25 hr.	moribund	6.93%
WT	F	6.5 hr.	dead	8.25%
WT	F	6 hr.	moribund	5.98%

4.4.2 Histological and biochemical analysis of WT and KO livers after GalN/LPS treatment shows that KO mice are protected from apoptosis and necrosis

Livers from WT and KO animals injected with GalN/LPS were harvested at the time of sacrifice. Morphologically, the WT livers appear dark-red in color and enlarged. In contrast, the KO livers, which are typically small and pale, maintain these characteristics and look nearly normal after treatment (Figure 16B). Serum analysis from sacrificed WT mice revealed a 40-fold increase in serum alanine aminotransferase (ALT), indicating severe liver damage [413], as compared to minimal injury in the KO mice (average 8,605 IU/L for WT vs. 169 IU/L for KO, normal <40). Additionally, there was a 20-fold increase in serum aspartate animotransferase (AST), another circulating marker of liver damage, in WTs as compared to KO mice (average

5,918 IU/L for WT vs. 234 for KO, normal <40) (Figure 16C). Histologically, the WT livers after GalN/LPS had areas of intense inflammation, massive cell death, and blood cell accumulation and sequestration, with only a few spared periportal cells. In contrast, KO livers showed very little damage; most of the hepatocytes appeared healthy, there was almost no red blood cell sequestration in the sinusoidal spaces, and the liver architecture was for the most part intact, with some sinusoidal dilation (Figure 16D). Assaying the livers of both genotypes for apoptosis revealed that GalN/LPS-treated WT livers had sustained widespread damage and contained numerous apoptotic hepatocytes. The KO livers, however, had dramatically fewer hepatocytes displaying TUNEL-positive apoptotic nuclei (Figure 16E). Finally, WT and KO livers were assessed for the presence of activated caspases by both Western blot (Figure 16F) and by fluorometric assay measuring cleavage of the caspase-3 peptide substrate DEVD-AFC at 6 hours post-GalN/LPS (Figure 16G). WT livers have significantly more caspase-3 and caspase-8 activation 6 hours after LPS than do KO livers, confirming the increased induction of the apoptotic machinery in WTs and lack thereof in KOs at this time.

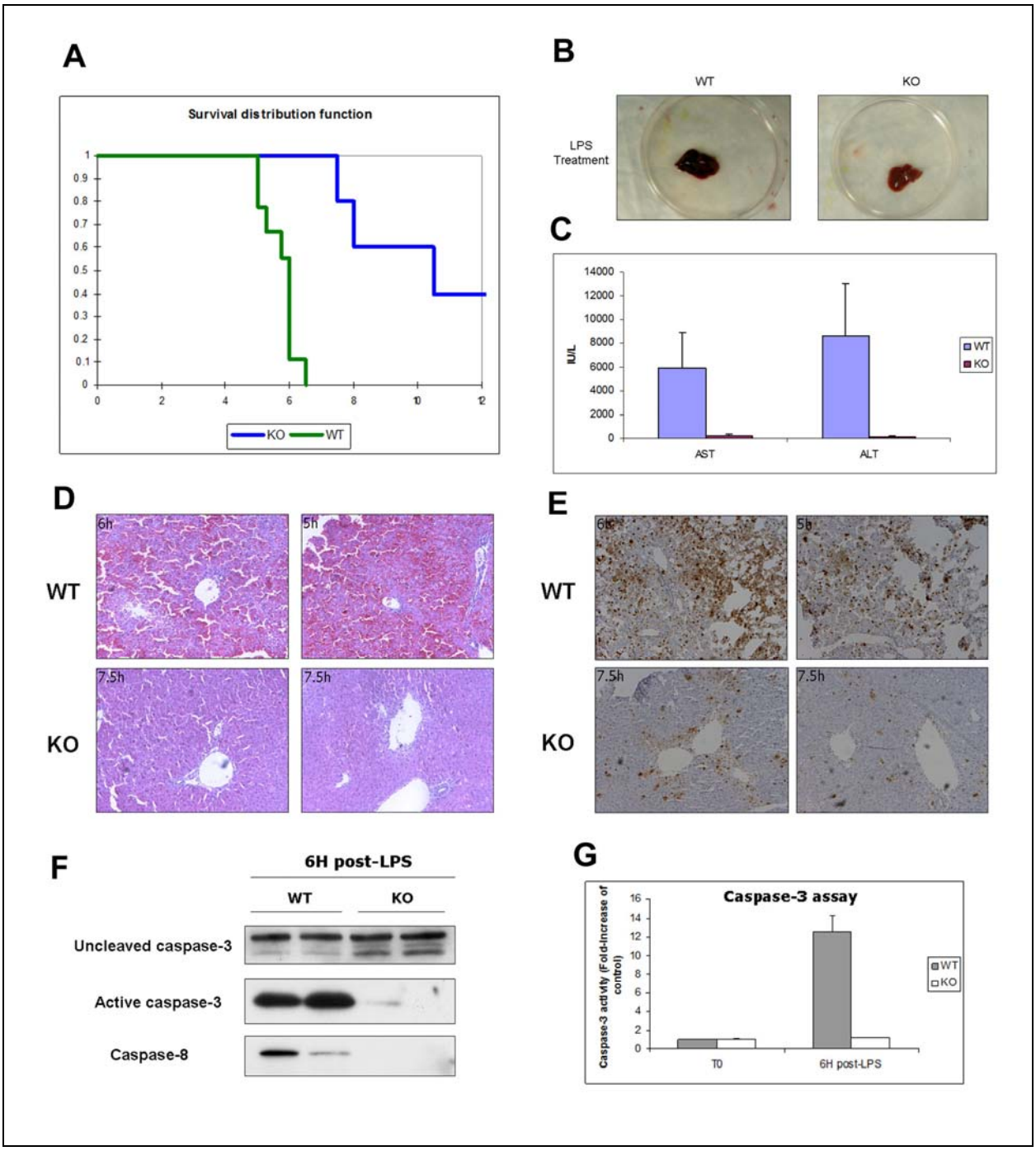

Figure 16: β-catenin KO mice are protected from injury induced via the TNF-α pathway. (A) Kaplan-Meier survival analysis of WT and KO mice after treatment witth GalN/LPS shows that KOs have prolonged survival compared to their WT counterparts. **(B)** Gross liver specimens from WT and KO mice injected with GalN/LPS demonstrate that WT livers become engorged with blood 6 hours post-injection, while the KO livers still appear relatively normal at the time of sacrifice. **(C)** Serum AST and ALT levels are higher in WT mice than in KO mice

after GalN/LPS treatment, indicating that KO mice have less hepatic injury after injection of GalN/LPS as compared to WT mice. **(D)** Injection with GalN/LPS results in a necrotic inflammatory histology in WT livers but not in β-catenin KO livers, as shown by hematoxylin and eosin staining. **(E)** The number of apoptotic hepatocytes after GalN/LPS injection is decreased in KO mice as compared to WT controls. Apoptotic cells were detected by TUNEL assay on paraffin embedded sections. **(F)** Cleaved caspase-3 and caspase-8 is increased in WTs compared to KOs after GalN/LPS treatment, as assessed by Western blotting. **(G)** The level of active caspase-3 activity is increased in WTs treated with GalN/LPS compared to KOs as measured by fluorometric assay.

4.4.3 KOs show an earlier peak of mild injury which is self-limited whereas WTs accumulate irreparable damage and succumb over time after GalN/LPS

Next, we injected WT and KO animals with GalN/LPS and harvested at various time points to compare the kinetics of injury escalation in the WTs and KOs. Livers from WT and KO animals were harvested at 3, 4, and 5 hours after GalN/LPS treatment and processed for histological analysis. TUNEL assay shows few apoptotic cells in either WTs or KOs 3 hours after GalN/LPS. Interestingly, at 4 hours post-treatment, KO animals display more TUNEL+ nuclei than WTs, which was opposite to the results seen at 6H, the time of morbidity in WT mice. However, at 5 hours post-GalN/LPS, the liver injury in KOs has not progressed or maybe even shown some resolution, while extensive apoptosis is evident in WTs, just like the observation at 6H (Figure 17A). H&E staining confirms the presence of significant hepatic damage in the WT at 5 hours after LPS while limited injury is evident in KOs (Figure 17B). AST and ALT measured from serum taken at the time of harvest shows an increase in AST in the KO animals at 4 hours after GalN/LPS, concurrent with the increase in apoptosis at this time. However, by 5 hours post-GalN/LPS – the time at which the WTs begin to show increased

TUNEL positivity and histologic injury – both AST and ALT are significantly higher in WTs than in KOs (Figure 17C). The AST levels jumped around 15-fold (from 100 to 1500) from 4H to 5H in WT, whereas there was only a 2-fold increase (from 250 to 500) in KO showing a dramatic deceleration of injury. These observations suggest that the decrease in liver damage seen in KOs after GalN/LPS is preceded by rapid onset and resolution of injury, while in the WTs, liver damage accumulates slowly and irreversibly.

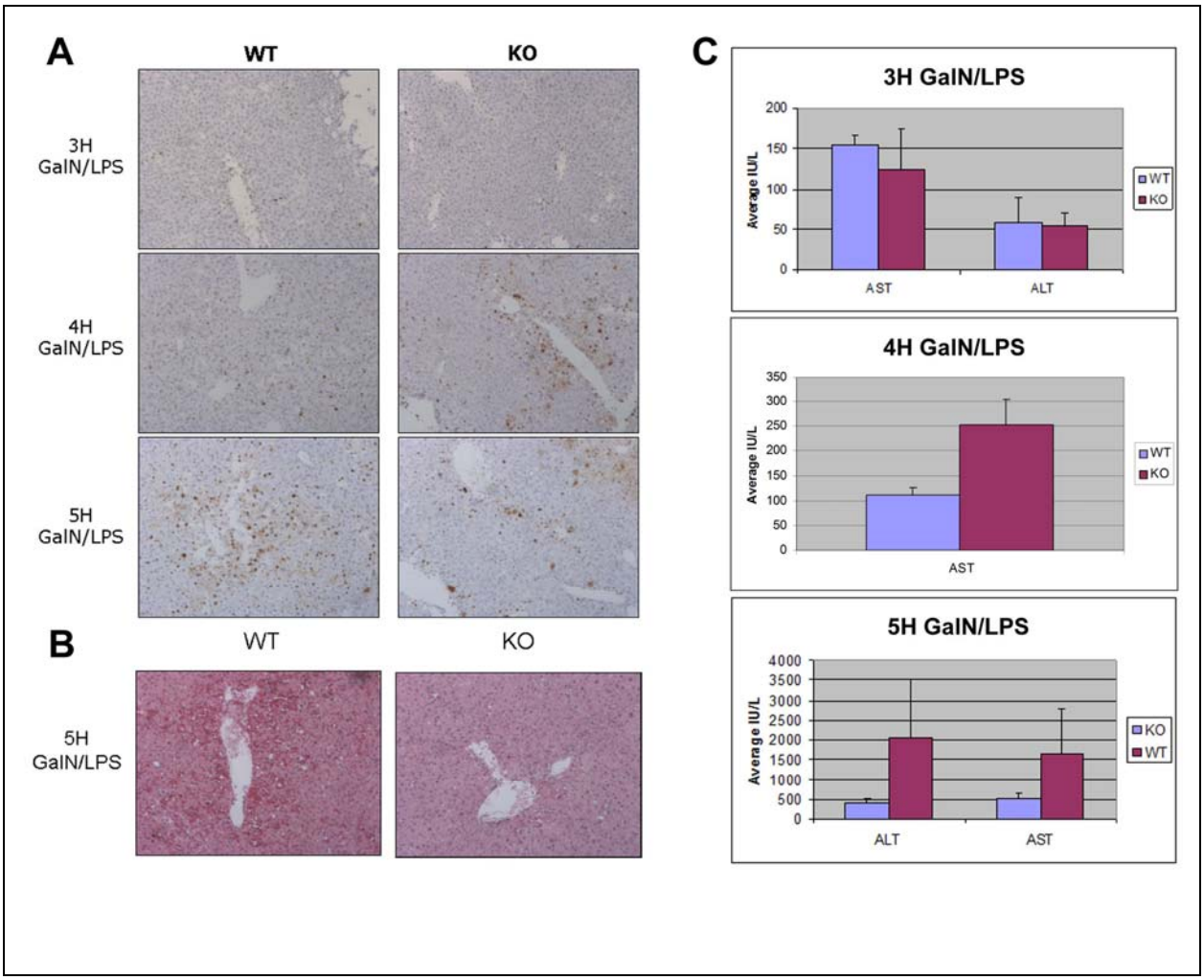

Figure 17: Accumulated damage to WT livers after GalN/LPS is irreversible, while damage in KO livers is arrested after an early onset. (A) TUNEL assay shows more positive cells in KOs as compared to WTs at 4H post-GalN/LPS. At 5 hours post-GalN/LPS, however, there is a rapid increase in the number of apoptotic cells in the

WTs compared to the KOs, which show levels of TUNEL positivity similar to or less than at 4H post-GalN/LPS. **(B)** H&E staining shows that WTs have significantly more damage than KOs as early as 5 hours after GalN/LPS. **(C)** Measurements of serum AST and ALT levels in WTs and KOs at various time points after GalN/LPS treatment shows increased liver injury in KOs at 4H, while at 5H the liver injury in WTs has caught up to and far surpassed that seen in the KOs.

4.4.4 NF-κB and its downstream targets are upregulated in KO livers 6H post-GalN/LPS

To determine the mechanism behind the protection from TNF-α mediated apoptosis seen in β-catenin KO animals, we first examined the expression of NF-κB in both WT and KO mice 6 hours after treatment with GalN/LPS. NF-κB was chosen as a potential inhibitor of cell death in our model because it is a transcription factor whose rapid translocation to the nucleus and subsequent activation of cytoprotective genes is known to play an important role in protection against pathogenic signals [414, 415]. Indeed, 6 hours after GalN/LPS treatment, there is a clear increase in the amount of total p65 (a subunit of NF-κB) in KOs as compared to WTs, as analyzed by Western blotting (Figure 18A). This increase may represent either an inhibition of p65 protein degradation or a re-synthesis of p65, since the effects of GalN are ameliorated approximately 3-4 hours after administration [259]. Cell fractionation demonstrates the presence of p65 protein in the hepatocyte nuclei of KO livers but not in WT livers, and its phosphorylation at the Ser-536 residue indicates that it is indeed transcriptionally active [416] (Figure 18B). Immunohistochemistry on WT and KO livers 5 hours after GalN/LPS treatment confirms the presence of extensive cytoplasmic and nuclear p65 in KO but not in WT livers (Figure 18C). Additionally, the presence of GSK-3β, a known NF-κB activator [417], as well as its Ser-9 phosphorylated form, is increased in KO as compared to WTs 6 hours after GalN/LPS treatment (Figure 18A,B), suggesting a possible mechanism for the phosphorylation of p65.

Examination of several downstream targets of NF-κB activation, such as Traf-1 [253] and Fas [418], as well as Stat3, which is itself a target of the NF-κB target gene IL-6 [419], shows that expression of these proteins is also higher in KO livers after GalN/LPS as compared to WT livers (Figure 19A). Indeed, many of the components of the LPS-induced NF-κB protective machinery, including toll-like receptor 4 (TLR-4; the receptor for LPS) [420], and tumor necrosis factor receptor type 1-associated DEATH domain protein (TRADD; an adaptor molecule that mediates cell death and NF-κB activation) [235] are increased in KOs compared to WT, while other proteins, such as TNF-R1 (the TNF receptor) [233] and Fas-associated death domain protein (FADD; an adaptor molecule that recruits caspases to the activated TNF-R1 or Fas receptors) [211] are unchanged between WTs and KOs after GalN/LPS treatment. Further, additional NF-κB targets, including inflammatory mediators and cytokines, were analyzed by cDNA array and found to be upregulated in KOs in comparison to WTs 6 hours after treatment (Figure 19B).

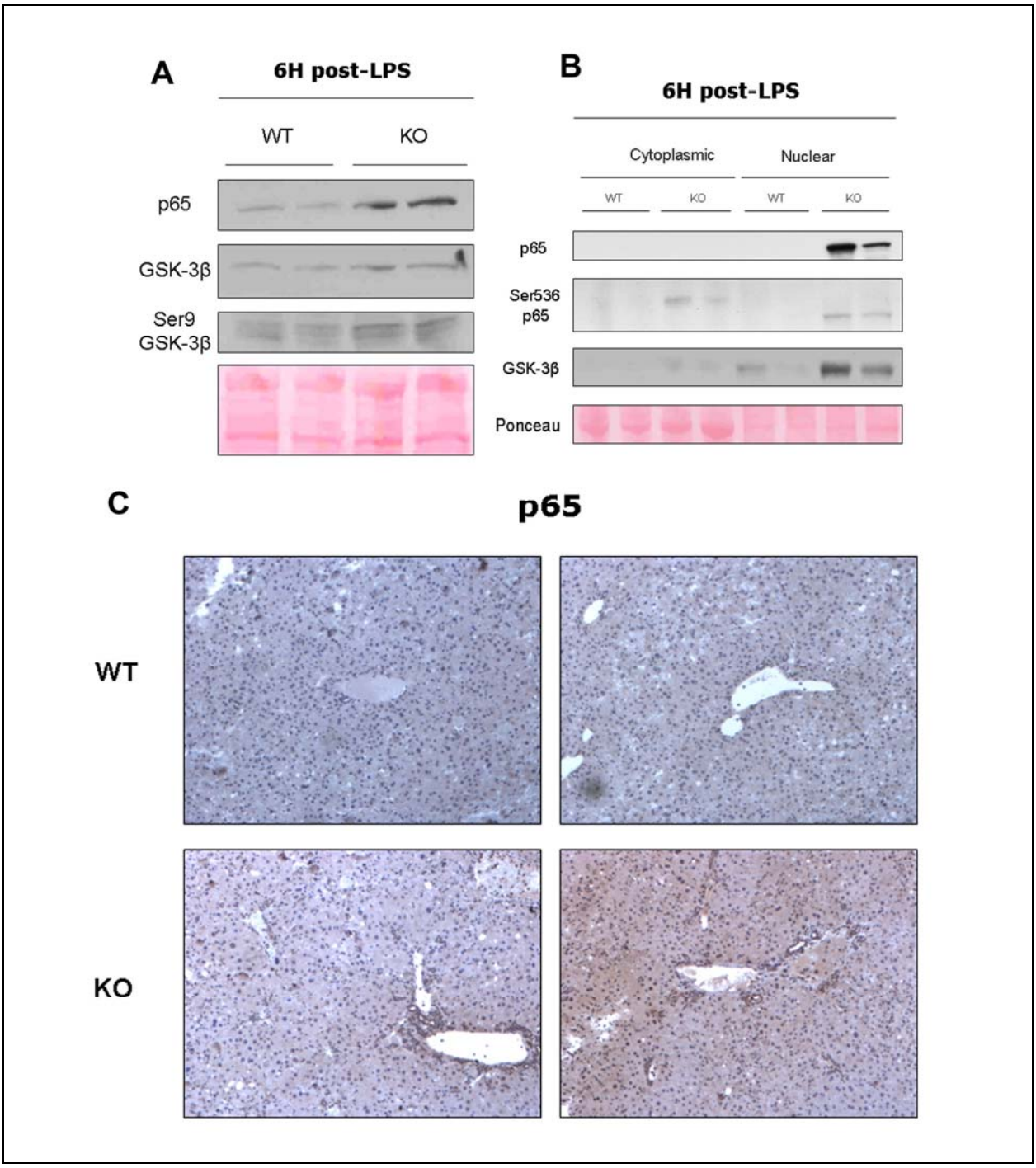

Figure 18: Cytoprotective proteins are higher in KO livers than in WT livers 6H post-GalN/LPS. (A) Western blot analysis shows that both p65 and GSK-3β are increased in whole-liver lysates from KOs as compared to WTs. **(B)** Both p65 and GSK-3β are localized mainly to the nuclei of the KO livers, as demonstrated by Western blot of cell fractionation extracts. Additionally, p65 is present in its active, phosphorylated form in both the

cytoplasm and nucleus of KO livers. **(C)** Immunohistochemistry for p65 shows a marked increase in p65 expression in the KOs as compared to WTs 5 hours after GalN/LPS administration.

To further confirm that NF-κB is playing a protective role in the KO animals after LPS injury, we examined p65 nuclear expression in KO animals that displayed a range of susceptibility to GalN/LPS. As shown in Table 3, around 7.5 hours after GalN/LPS, 6/15 KO animals displayed susceptibility, whereas 9/15 were resistant to LPS-induced apoptosis. Analysis from representative samples is shown in Figure 19C. The animals protected from GalN/LPS (that is, the ones that are still alive at 7.5 hours after treatment) have higher levels of nuclear p65 than those animals who exhibited susceptiblity, indicating a direct correlation between p65 nuclear expression and protection from apoptosis seen in some KO animals.

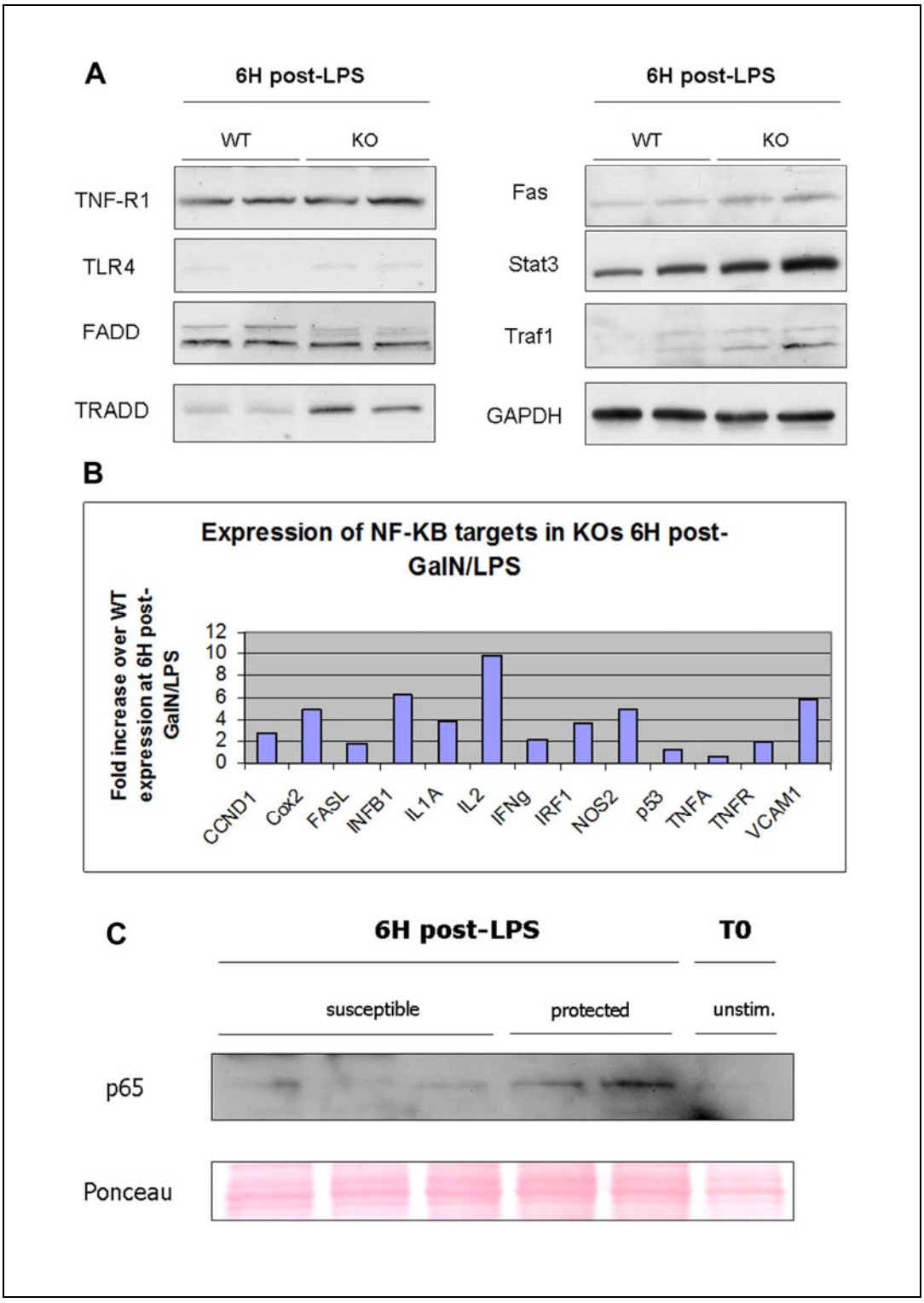

Figure 19: Expression of downstream NF-κB targets is increased in KO animals compared to WT animals after GalN/LPS. **(A)** Western blot of TNF-α/LPS pathway members and NF- κB target genes shows that expression of targets and some effectors is higher in KOs compared to WTs after GalN/LPS. **(B)** cDNA analysis of

selected NF-κB targets after treatment with GalN/LPS shows that the KOs have a several-fold increase in the expression of many NF-κB-induced genes compared to WTs. **(C)** Nuclear extracts from KO animals displaying differential susceptibility to GalN/LPS at 7.5 hours after treatment show that p65 is higher in KO animals that are protected from apoptosis as compared to those that are susceptible.

4.4.5 NF-κB is activated basally in KO animals

Since the KOs were still resistant to LPS even after administration of ActD, which has a prolongsed half-life compared to GalN, we wanted to address if any basal differences in NF-κB activity may be responsible for incurring prolonged protection in KOs. We examined whole-cell extracts from unstimulated WT and KO livers for the expression of NF-κB subunits and downstream targets. As shown in Figure 20A, there is no difference in total p65 expression between WTs and KOs at baseline. However, there is increased Ser536 phosphorylation in the KOs, indicative of activated p65. Additionally, p50, another NF-κB subunit, was higher in KOs as well, as are the NF-κB downstream targets Traf-1 and Fas. Immunohistochemistry confirms the presence of active p65 in KOs around the periportal and centrizonal areas, while in WTs, activation of p65 is absent (Figure 20B).

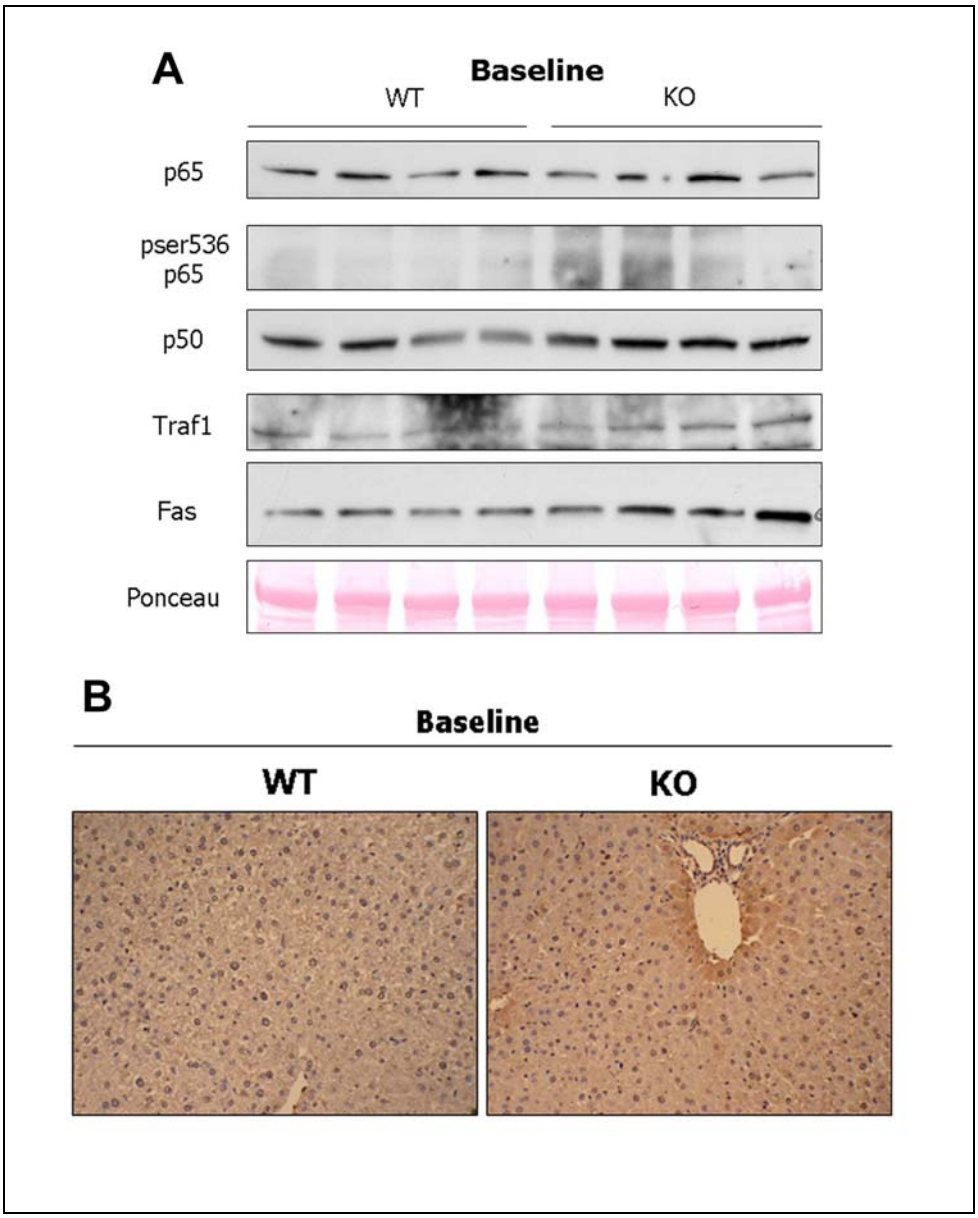

Figure 20: p65 is activated in KO livers at baseline. **(A)** RIPA extracts from unstimulated WT and KO livers show that although total p65 is unchanged, Ser536 p65 is increased in KOs, as are downstream targets Traf-1 and Fas. **(B)** Immunohistochemistry for phosphor-p65 confirms the presence of active p65 in the periportal and centrizonal regions of KO livers but not in WTs.

4.4.6 Basal increase in NF-κB activation occurs in KOs through several mechanisms

NF-κB, a common pro-survival pathway, is usually activated in response to injury or inflammation through diverse mechanisms [421]. To determine the cause of NF-κB activation in KO mice, we explored several inflammatory markers under unstimulated conditions. Expression of toll-like microbial pattern recognition receptor-4 or TLR-4, which is an important part of the innate immune response, is increased in KO as compared to WT at baseline (Figure 21A). In addition, increased TLR-4 (which is also a receptor for LPS) makes the hepatocytes more sensitive to endogenous LPS that is present in portal circulation emanating from the gut flora. LPS binding to TLR-4 induces NF-κB signaling through phosphorylation-induced degradation of IκBα, which is known to prevent nuclear translocation of NF-κB subunits. Immunohistochemical staining for CD45, a cell-surface marker for leukocytes [422], revealed greater numbers of macrophages including Kupffer cells in the unstimulated KO livers (Figure 21B). Taken together, these data indicate that KOs have a more robust endogenous inflammatory response, perhaps due to the presence of chronic inflammation, which in turn is known to stimulate activation of the NF-κB pathway.

TLR-4 activation is also known to trigger cell apoptosis [423]. Another method of stimulating apoptosis is through disruption of the interaction between Fas and the HGF receptor, Met. Since it is known that Met sequestration of the Fas receptor prevents hepatocyte apoptosis [424], we compared Met protein expression in WTs and KOs by Western blotting. We found a significant reduction in the levels of Met receptor in KOs under unstimulated conditions, simultaneous with an increase in the expression of Fas (Figure 21C). Interestingly a Fas-β-catenin association was identified in WT livers and was dramatically lower in KO (Figure 21D).

Previous reports have shown that another receptor tyrosine kinase, epidermal growth factor receptor (EGFR), which plays a key role in liver regeneration [425], can also prevent Fas-induced liver injury [426]. As shown in Figure 21C, expression of EGFR was found to be decreased in the KOs at baseline as well. The reduction in these two key growth factor receptors, together with a concurrent increase in TLR-4 and Fas receptor expression and a decrease in Fas-β-catenin interactions, may render the KO animals more susceptible to apoptosis.

As shown in Figure 21E and mentioned previously, the KO livers display a basal increase in apoptosis, as demonstrated by an increase in caspase-3, active caspase-3, and caspase-8 proteins. Previous work has shown that NF-κB can be directly activated during the induction of apoptosis [427]. Thus, the presence of a modest yet significant activation of the apoptotic machinery may lead to a compensatory upregulation of NF-κB in the KOs, which provides yet another mechanism of baseline NF-κB activation in these animals.

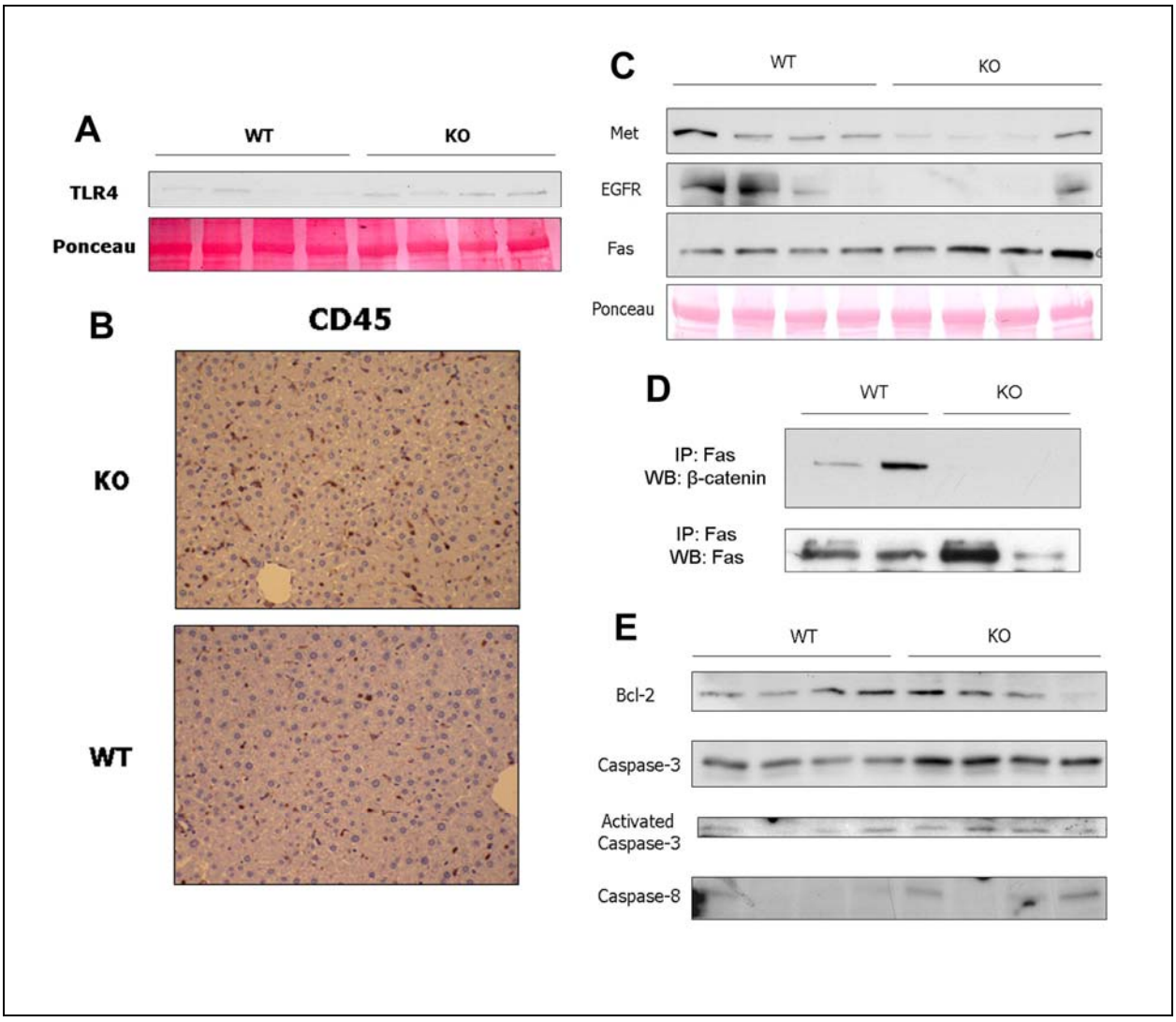

Figure 21: Activation of NF-κB in KO livers may occur through multiple mechanisms. **(A)** Western blot shows a basal increase in TLR-4 in KOs. **(B)** KO livers contain higher numbers of inflammatory macrophages and/or Kupffer cells, as shown by immunohistochemistry for CD45. **(C)** Expression of Met and EGFR is decreased in KOs compared to WTs at baseline, while expression of Fas is increased, as assessed by Western blot. **(D)** Immunoprecipitation shows that Fas and β-catenin associate strongly in WT livers at baseline and that this association is absent in KO livers. **(E)** Caspase expression and activation is higher in KOs than in WTs at baseline, although the levels of Bcl-2 are unchanged.

4.4.7 p65 associates with β-catenin in WT livers but not in KOs and may be part of an innate response to injury

In addition to its well-known interaction with the IκB complex, p65 has also been shown to physically associate with β-catenin in the context of colon, breast, and liver cancer. This association has been shown to inhibit p65 transactivation and prevent NF-κB target gene expression [266, 267]. To determine if the p65/β-catenin complex is present in hepatocytes as well, we immunoprecipitated protein lysates from both WT and KO livers with p65 and probed the blots for β-catenin. Figure 22A shows coprecipitation of β-catenin and p65 in both WT and KO livers. However, the association in KO livers is dramatically reduced and may represent association in the non-parenchymal cell compartment in which β-catenin is still present.

Because dissociation of p65 and β-catenin is a critical step in activation of NF-κB, we wanted to know if the p65/β-catenin complex contributes to the injury response in our model. We hypothesized that KO livers, which lack β-catenin, would have a lower threshold of p65 activation after apoptotic stimulus and thus respond more rapidly when challenged. To test this, we treated WT and KO animals with LPS only (no GalN), and harvested 1 hour after treatment. NF-κB nuclear translocation is known to peak approximately 1.5 hours after LPS stimulation [415, 428, 429], so any differences between WT and KO p65 activation should be evident by 1 hour. Figure 22B shows an increase in both p65 and p50 in the nucleus of KO livers as compared to WTs 1 hour after LPS treatment. Further, expression of IL-6, an acute downstream target of NF-κB [430], is also increased in the cytoplasmic fraction of KOs at this time.

To determine the kinetics of β-catenin/p65 dissociation, nuclear translocation and activation, we harvested WT livers at baseline as well as 1 hour and 2 hours after LPS stimulation and examined them by immunoprecipitation, Western blot, and

immunohistochemistry. Dissociation of β-catenin and p65 occurs as early as 1 hour after LPS in the WT (Figure 22D), with p65 nuclear translocation occurring simultaneously (Figure 22C). However, p65 does not seem to be transcriptionally active until 2 hours after LPS treatment, at which time the phosphorylated form appears in the nuclear fraction (Figure 22E and 22F). While increase in total p65 is evident in the nuclei 1 hour post-LPS injection, its peak of maximal phosphorylation is evident at 2 hours in WT, suggesting a small lag between its translocation and phosphorylation-dependent activation. Interestingly, p65/β-catenin co-precipitates again 2 hours after LPS treatment, and nuclear localization of p65 also decreases to near-baseline levels, indicating that the p65/β-catenin complex may be re-forming after initial dissociation. Nuclear β-catenin remains unchanged from baseline at 1 hour after LPS, indicating that p65 and β-catenin are not translocating to the nucleus simultaneously. In contrast to the WT kinetics, in the KOs p65 is phosphorylated and active as early as 1 hour after LPS treatment, with approximately 50% of hepatocyte nuclei staining positive for Ser536 p65 (Figure 22E). Thus, loss of β-catenin in the KOs lowers the threshold of p65 nuclear translocation and activation in reponse to stimulation which allows for more rapid protection in response to injury.

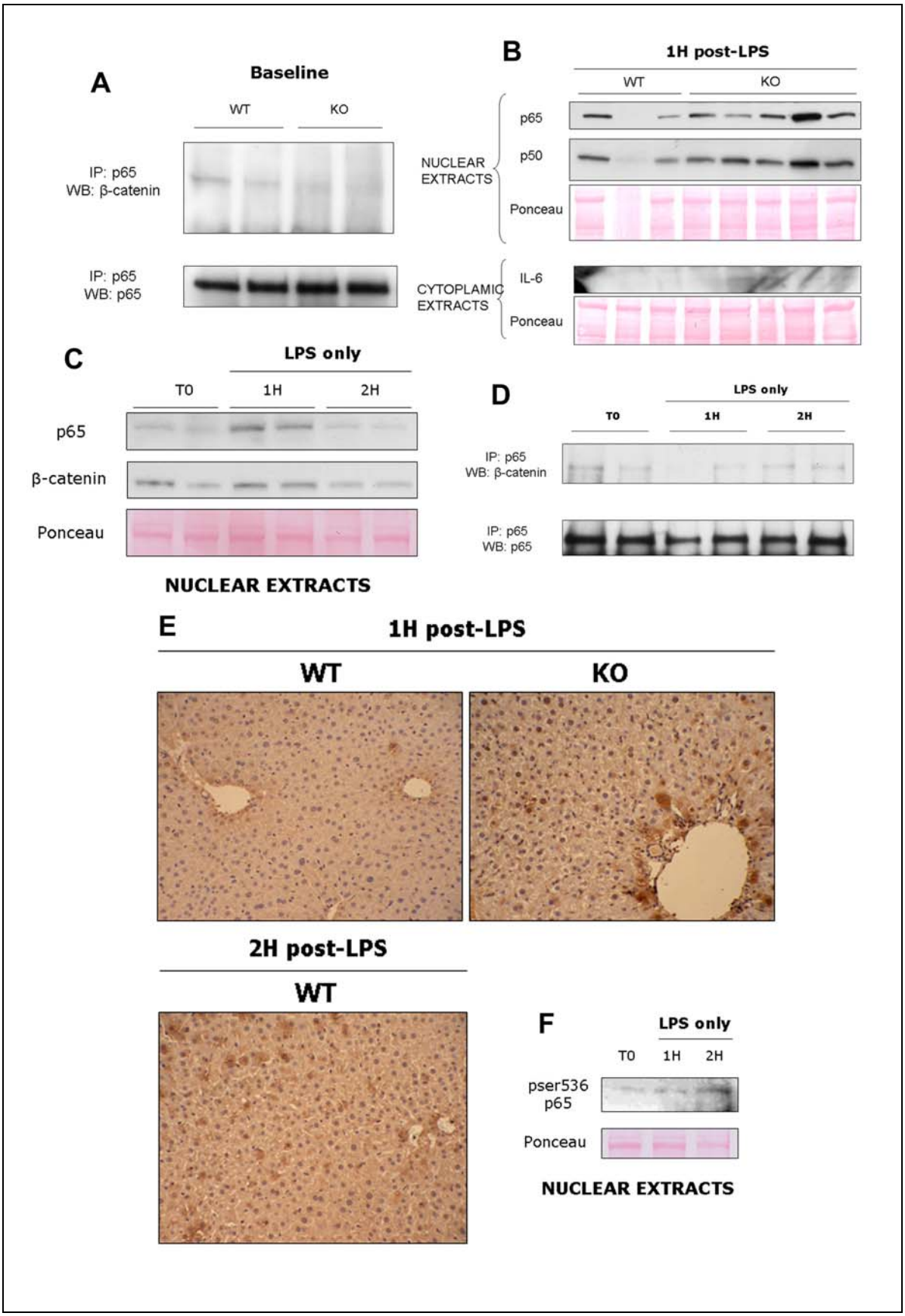
A
Baseline
WT
KO
IP: p65
WB: β-catenin
IP: p65
WB: p65
B
1H post-LPS
WT
KO
p65
p50
NUCLEAR
EXTRACTS
Ponceau
IL-6
CYTOPLAMIC
EXTRACTS
Ponceau
C
LPS only
T0
1H
2H
p65
β-catenin
Ponceau
NUCLEAR EXTRACTS
D
LPS only
T0
1H
2H
IP: p65
WB: β-catenin
IP: p65
WB: p65
E
1H post-LPS
WT
KO
2H post-LPS
WT
F
LPS only
T0
1H
2H
pser536
p65
Ponceau
NUCLEAR EXTRACTS

Figure 22: The decreased association of p65 and β-catenin in KOs may contribute to protection from injury after LPS treatment. **(A)** Immunoprecipitation shows that p65 and β-catenin associate strongly in WT livers at baseline but less so in KO livers. **(B)** Western blotting of nuclear extracts shows that NF-κB subunits and downstream targets are increased in KOs 1 hour after LPS as compared to WTs. **(C)** p65 translocates to the nucleus 1 hour after LPS injection in the WTs, while expression of nuclear β-catenin remains the same as baseline, as shown by Western blotting. **(D)** Association of p65/β-catenin decreases after administration of LPS in the WT, as assessed by immunoprecipitation. **(E)** NF-κB is active in the nucleus 1 hour after LPS treatment in the KOs but at 2 hours after LPS treatment in the WTs, as shown by immunohistochemistry for the phosphorylated form of p65. **(F)** Western blotting for the phosphorylated form of p65 indicates maximal activity in the nucleus 2 hours after LPS administration in WT livers.

4.4.8 β-catenin is degraded in WT livers in response to GalN/LPS treatment

Finally, to determine if Wnt/β-catenin pathway components may themselves undergo any changes after TNF-α mediated apoptosis, we examined expression of β-catenin and some of its downstream targets in WT livers at various time points, both before and after GalN/LPS treatment. Figure 23 shows that β-catenin decreases as early as 3 hours post-GalN/LPS. By 6 hours, β-catenin cleavage and degradation is pronounced, as shown by the appearance of a truncated form of β-catenin at the expense of the full-length form, and is most likely due to N-terminal truncation by caspases [431, 432]. GSK-3β, a kinase known to phosphoryate β-catenin and promote its degradation, was modestly downregulated. Although expression of some downstream targets such Axin2, and glutamine synthetase remain relatively unchanged over time, others, such as cyclin-D1, show a marked decrease in protein expression which coincides with β-catenin degradation starting 3 hours after GalN/LPS. In addition, expression of E-cadherin, which along with β-catenin is a critical component of cell-cell adhesion, decreases 5

hours after administration of GalN/LPS. This time point corresponds to the onset of hepatocyte necrosis and apoptosis in WT livers (Figure 17). Thus, β-catenin levels and activity decrease over the course of GalN/LPS treatment in WT animals and therefore does not appear to protect hepatocytes from apoptosis. On the contrary, degradation of β-catenin and reduced protein expression of some of its downstream targets, especially cyclin-D1, may contribute to the apoptotic phenotype.

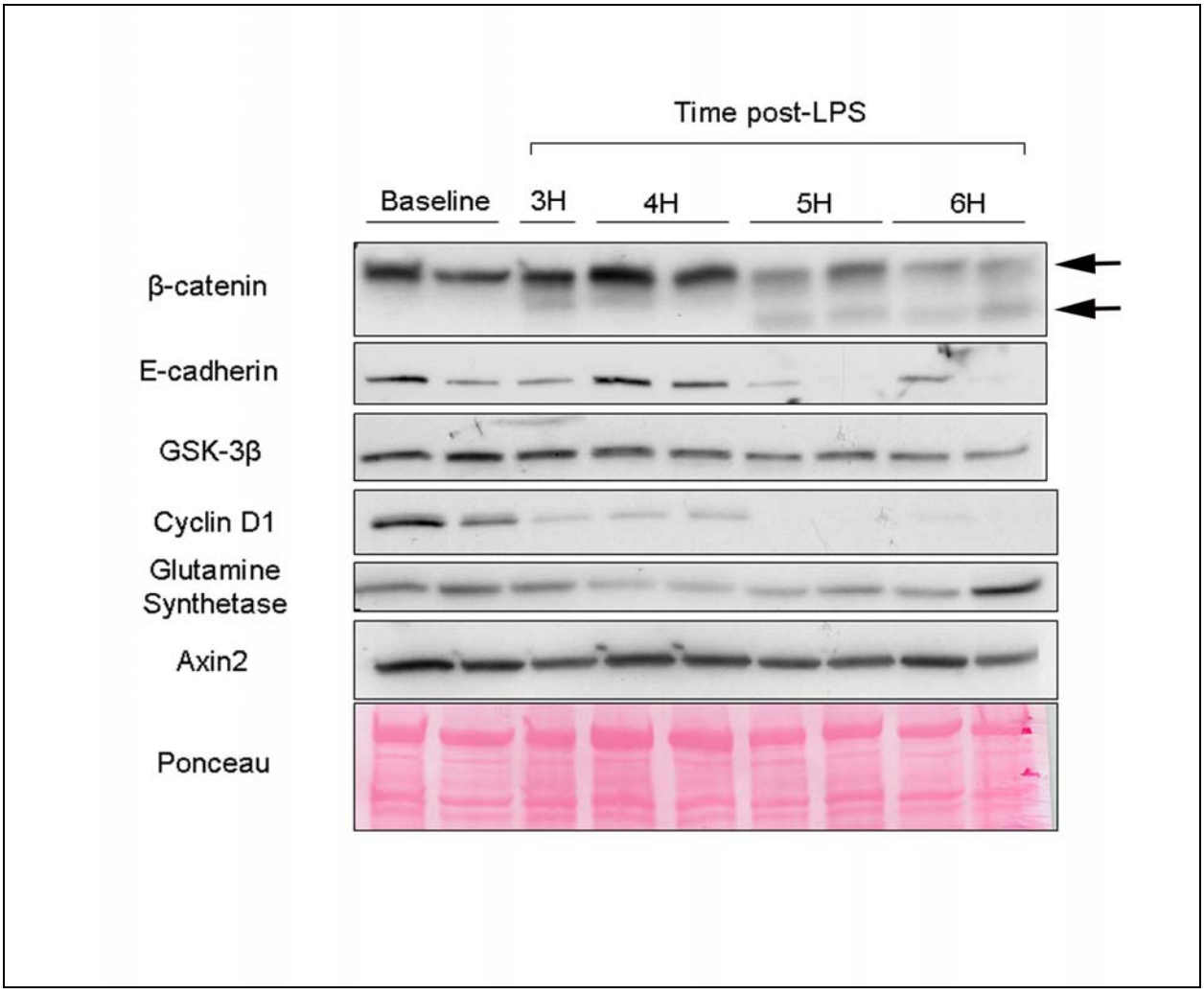

Figure 23: β-catenin is degraded over the course of GalN/LPS treatment, and expression of some of its downstream targets changes as well. Western blots show that β-catenin begins to be cleaved as early as 3 hours after GalN/LPS treatment in WT livers, with near-complete degradation occurring by 6 hours. Expression of some

β-catenin targets such as Axin2 remains unchanged over time, while others such as cyclin-D1 decrease with increasing β-catenin degradation.

4.5 DISCUSSION

β-catenin plays multiple roles in liver health and disease through regulation of processes such as proliferation, differentiation and adhesion. However its role in hepatic injury remains unexplored, albeit β-catenin conditional KO mice display a basal increase in TUNEL-positive apoptotic nuclei. To determine the role of β-catenin in hepatocyte survival, we compared injury and survival between KO and control animals after GalN/LPS, which is a major mode of hepatocyte apoptosis. Here, we demonstrate that, contrary to our expectations, loss of β-catenin in hepatocytes protects against TNF-α mediated apoptosis through a compensatory upregulation of the NF-κB pathway, which is activated basally and temporally after LPS-induced liver injury in the β-catenin KO livers.

The time to morbidity in WT C57Bl/6 mice following GalN/LPS treatment is generally 6-8 hrs [433]. In our system, we observed that, following LPS treatment, all (100%) of the WT mice were morbid by 6 hours. In contrast, nearly all (93.3%) of the KO mice were still alive at this time, with times to morbidity ranging from 7.5 hours to 10.5 hours after LPS (Table 3). In fact, a few KO mice remained in relative health until they were sacrificed at the outer limit of the predicted survival time (12 hours). Examination of gross liver morphology, along with histological analysis, reveals significant damage in the WT mice treated with GalN/LPS. Measurement of serum AST and ALT, a marker of hepatocellular injury which is often elevated due to activation of inflammatory pathways [434], shows that liver damage is also increased

dramatically in WT mice as compared to KO mice at the time of sacrifice. Caspase cleavage and activation is also strongly induced in WTs by the time of sacrifice, confirming the presence of massive apoptosis in WT but not in KO livers (Figure 16).

As mentioned previously, there is a perturbation in vitamin C biosynthesis in β-catenin KO mice [38], which could result in accumulation of D-glucuronate, a precursor of vitamin C [381]. D-galactosamine is known to reduce the hepatic content of UDP-glucose [259], which would decrease the amount of glucuronate, as well as inhibit the formation of de-novo glucuronate by depleting UDP-glucuronate [435]. Since β-catenin also plays a role in regulating glucose metabolism [436] and xenobiotic metabolism through regulation of various P450s [35, 94], there may therefore be altered metabolism of D-galactosamine, which may in turn impact its effect on transcriptional suppression prior to LPS administration. However, pretreatment of KO and control mice with actinomycin D, which is an independent class of transcriptional inhibitor, prior to LPS resulted in the recapitulation of the observations seen in GalN/LPS-treated KOs and controls (Table 4).

The transcription factor NF-κB plays a key role in both innate and adaptive immunity. It is known to positively regulate the transcription of anti-apoptotic genes such as c-IAPs, Trafs, Bcl-X_L, and c-FLIP [255], and is a common pro-survival factor. In addition, NF-κB plays a direct role in hepatocyte survival and regeneration [114, 243, 437], making it a likely candidate for imparting a phenotype to our KO model in response to TNF-α-mediated injury. Indeed, our results demonstrate that mRNA and protein expression of NF-κB subunits and downstream targets is increased in KO mice as compared to their WT counterparts at the time of sacrifice, and further examination reveals that p65 is activated basally in KOs as well (Figures 18-20). Although it was initially unclear why there was some inter-animal variability within the KO

genotype in response to TNF-α-mediated injury, the heterogeneity in the activation of NF-κB in the KOs may be due to factors that are yet to be determined. However, these results further strengthen the argument that the level of NF-κB expression determines an animal's fate after GalN/LPS. A similar phenotype is also seen in the Met-KO hepatocytes, which demonstrate hypersensitivity to Fas-induced apoptosis. These mice exhibit an intrinsic basal activation of NF-κB, which is most likely an adaptive response to both apoptosis and oxidative stress [438]. Interestingly, the c-Rel gene, a transcriptionally active member of the Rel/NF-kappaB family [439], is downregulated basally in our KO livers, perhaps to counter the increase in p65/RelA phosphorylation and activation.

As mentioned, an analysis of genes upregulated in KO mice in both resting and regenerating liver reveals several that are related to or induced by TNF-α stimulation **(**Table 2). Since release of TNF-α is one of the hallmarks of a classic inflammatory response, we investigated the presence of inflammatory markers in our KO livers and found a basal increase in expression of the TLR-4 receptor and CD45-positive cells, both indicative of an innate immune response (Figure 21). In addition, as discussed in the previous chapter, regucalcin, a critical component of anti-oxidant metabolism in hepatocytes through ascorbic acid biosynthesis and other mechanisms, is missing in KOs, which is also contributing to increased basal apoptosis. Therefore, we believe that levels of NF-κB are basally increased in order to counteract increased inflammation and increased oxidative stress, thus accounting for the protection seen in KO livers after challenge with GalN/LPS.

We analyzed gene array data from KO and WT mice and found a basal increase in anti-death genes such as Bcl-2 and Bcl-6 in our KOs, as well as decreased expression of caspases, including caspase-9, which has been linked to the mitochondrial death pathway [440] (Table 2).

This suggests that hepatocytes lacking β-catenin may overexpress anti-apoptotic genes and repress capase gene expression as a compensatory cytoprotective mechanism to counteract a basal apoptosis. Although our current studies have not yet elucidated the mechanism of increased apoptosis, indirect evidence suggests that it maybe due to activation of the Fas pathway. For example, we found an increase in Fas protein expression in the KOs at baseline and after GalN/LPS treatment. Additionally, Fas-activated serine threonine kinase was upregulated 6.9–fold in KO livers 40 hours after partial hepatectomy (PHx) in the KO, which corresponds to a time in which KOs are displaying suboptimal regeneration and a 2.5-fold increase in apoptosis [94].

Our laboratory has reported that β-catenin and the hepatocyte growth factor (HGF) receptor c-Met associate at the cell surface [49, 51]. Upon binding of HGF to c-Met, β-catenin becomes phosphorylated at tyrosine residues 654 and 670, dissociates from c-Met, and translocates to the nucleus, where it upregulates target genes [52, 53]. Other groups have shown that c-Met sequestration of the Fas receptor can prevent Fas-mediated apoptosis in hepatocytes [228], and that mice lacking c-Met were hypersensitive to Fas-induced apoptosis[229]. Intriguingly, expression of HGF mRNA is upregulated 9.27-fold in the KOs at baseline (Table 2), simultaneous with a dramatic reduction in the protein levels of Met receptor in KOs (Figure 21C). We have identified a novel interaction of Fas and β-catenin and based on existing knowledge of Fas-Met and Met-β-catenin complexes, it is likely that the three proteins interact to eventually impact Fas's availability to bind to its ligand. Lack of β-catenin may be inducing Fas-mediated apoptosis through lack of Fas-β-catenin interaction, decreased Met levels and enhanced free-Fas levels, which are further complemented by enhanced basal NF-κB activation. Based on this information, we hypothesize that in homeostasis, c-Met and β-catenin may be conferring

protection from Fas-mediated apoptosis, and that the absence of β-catenin would shift the balance toward a Fas-induced apoptotic fate. The role of β-catenin in Fas-mediated apoptosis, however, remains to be elucidated.

Our results suggest that although β-catenin is often thought to play a pro-survival role in liver biology, loss of β-catenin can paradoxically contribute to protection from apoptosis, likely through upregulation of anti-apoptotic machinery (Figure 24). The pivotal role of β-catenin in determining the mode of apoptosis in hepatocytes could be exploited for clinical therapies that enhance cell survival in liver diseases associated with massive cell death, such as alcoholic hepatitis, hepatic inflammation, viral hepatitis, cholestatic liver disease, and ischemia-reperfusion injury.

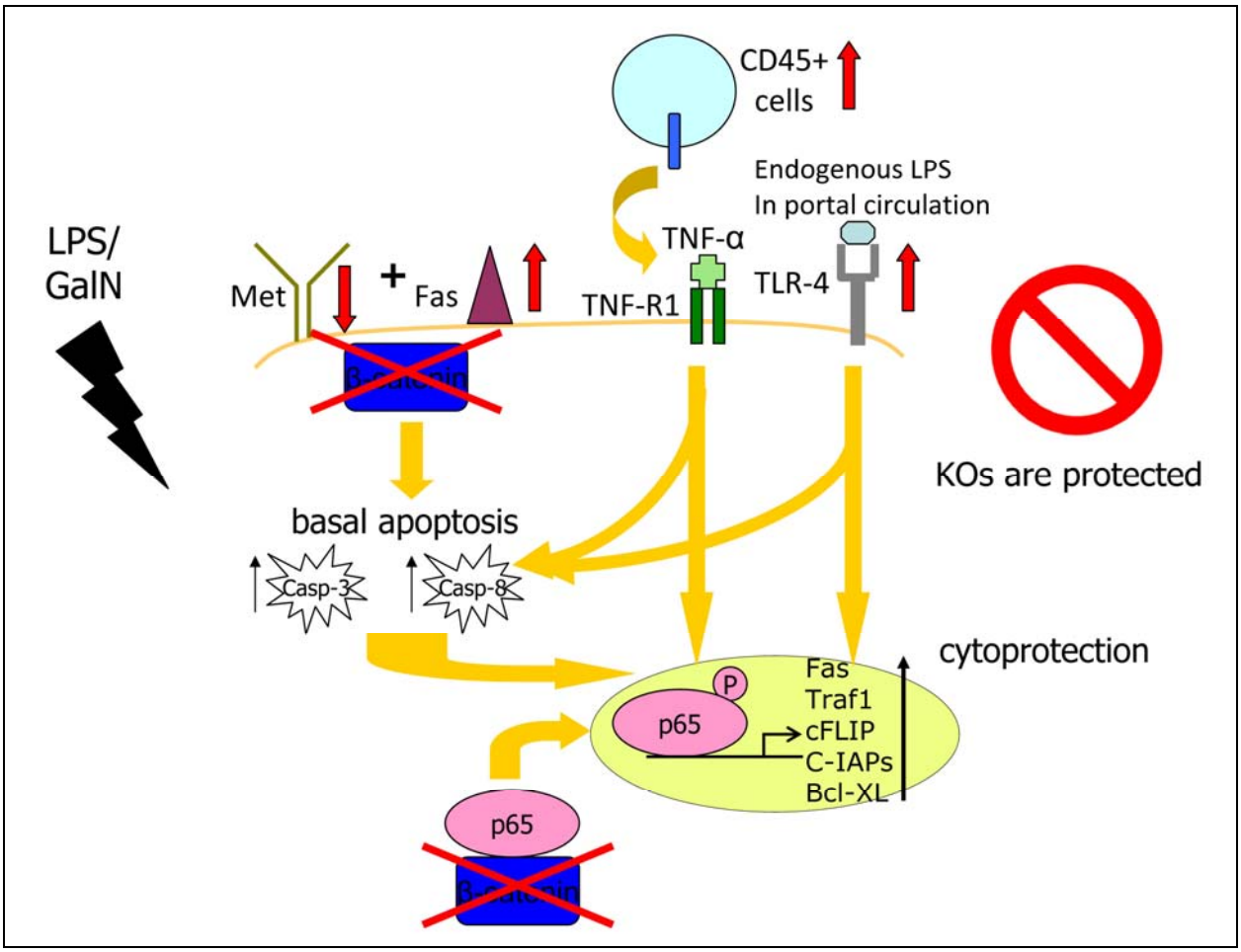

Figure 24: A proposed mechanism for resistance to TNF-α mediated apoptosis in β-catenin KO mice. Decreased Met and a simultaneous increase in Fas leads to a basal level of apoptosis, which can stimulate activation of NF-κB. A robust endogenous inflammatory response, indicated by an increase in CD45+ cells, can also activate NF-κB. CD45+ cells secrete TNF-α, which can bind to its receptor TNF-R1 and stimulate either apotosis or NF-κB activation. Basally increased TLF-4 in KOs would also respond to LPS normally present in portal circulation to induce basal NF-κB activation and apoptosis. Finally, the absence of β-catenin in KOs allows increased p65 translocation to the nucleus. In conclusion, levels of NF-κB in KOs are increased basally as well as after GalN clearance following LPS injection through several mechanisms, thus supporting the notion that these livers may be "primed" for protection against exogenous insult.

5.0 GENERAL DISCUSSION

5.1 BETA-CATENIN IN LIVER REGENERATION

The importance of β-catenin to liver regeneration was first described in the rat model [126]. Additional studies since then have highlighted the importance of β-catenin in regeneration in many species including zebrafish, mice, and humans [350, 351]. Decreased proliferation and liver/body weight ratio were noted as early as 24 hours in rats administered a β-catenin antisense oligonucleotide after 2/3 PHx [127]. β-catenin conditional knockout mice showed a delayed regeneration by 24 hours [94]. These results were independently corroborated by another laboratory that demonstrated a 12-24 hour delay in DNA synthesis and cell cycle progression in liver-specific β-catenin knockout mice [128]. In light of the above observations, it would be of translational significance to determine if activation of the Wnt/β-catenin signaling pathway would be beneficial to the process of regeneration. Indeed, our extensive data suggests not only that increased β-catenin enhances regeneration, but also that there is an opportunity to modulate Wnt/β-catenin signaling for the purpose of stimulating the regenerative process. Comparison of TG and WT hepatocyte proliferation in culture shows that thymidine incorporation (a measure of proliferation) and cell viability is increased in TG hepatocytes as compared to WT. The TG mice express increased cyclin-D1 protein, a key cell cycle regulator, several hours earlier than their WT counterparts after PHx. This in turn triggers upregulation of proliferation, as measured

by PCNA staining. Further, we provide evidence to suggest that the Met/β-catenin interaction at the cell surface is regulating β-catenin translocation to the nucleus contributing to enhanced proliferation. Finally, activation of β-catenin through Wnt-1 induction elicits similar effects to those seen in the TG mice, and confirms that β-catenin is an important intracellular regulator of liver regeneration.

Intriguingly, we did not detect any phenotype or abnormalities in metabolism, zonation, or proliferation in TG mice overexpressing S45D-β-catenin in our initial characterization. There was a modest increase in LW/BW ratio at 1 month which was transient and secondary to increased cyclin-D1 expression and proliferation. By 2 months, however, the TG livers had adapted to suppress excessive β-catenin by sequestering it at the hepatocyte membrane [441]. This unique means of turning "off" β-catenin signaling is under investigation and may have clinical implications in treatment of β-catenin-active tumors in the liver and elsewhere.

The role of constitutively active β-catenin in postnatal hepatic growth has been examined in sseveral mouse models. Increased cell proliferation leading to hyperplasia and hepatomegaly were described in a transgenic mouse that expresses an oncogenic form of β-catenin lacking the N-terminus, which is involved in protein stabilization [130]. Another report described enlarged livers shortly after adenoviral infection in a mouse strain carrying a mutant inducible form of β-catenin [131]. Two additional studies have also demonstrated the effect of β-catenin stabilization *albeit* indirectly through ablation of the APC gene, a known negative regulator of the pathway. In the first study, Apc-inactivated mice display both a clear increase in liver size and a high incidence of spontaneous hepatocellular cancer [442]. Another recent study has showed a regenerative advantage in APC-mutant mice livers after PHx [350]. While β-catenin activation was evident robustly in APC-mutant mice, it is unclear what impact loss of APC has

on other signaling pathways, as APC has recently been shown to affect cell processes such as epithelial integrity and DNA replication independent of β-catenin regulation [443, 444]. This is also relevant since APC loss is not evident in hepatocellular cancer in patients except in a case report [130-132, 445].

Intriguingly, no study to date has shown spontaneous HCC in TG mice overexpressing wild-type or constitutively active β-catenin [130-132]. For example, TG mice containing a deletion of exon-3 lacked spontaneous tumorigenesis; however, in the setting of simultaneous mutation of H-ras, this mutant form of β-catenin induced hepatic tumors [131] [446]. Akin to such observations, we did not detect spontaneous HCC in our TG mice. In light of observed membranous sequestration of excess β-catenin in TG mice, the phenotype, or lack thereof, is not surprising. Nonetheless, as in primary cultures or after PHx, diethylnitrosamine (DEN) exposure did lead to accelerated tumorigenesis in TG mice due to activation of the mutated β-catenin [441]. Thus, it appears that mutation of β-catenin alone is insufficient to cause spontaneous hepatic tumorigenesis and suggests that only after a "first hit", such as chemical induction or mutation of another oncogene, does mutant-β-catenin promote tumorigenesis.

While cyclin-D1 as a target of β-catenin signaling has been known for quite some time [25, 26], a direct link between β-catenin activation and expression of cyclin-D1 has been disputed [447] and is especially controversial in liver pathobiology [130, 442, 448]. It is of relevance to emphasize that that genetic targets of β-catenin signaling are not only tissue-dependent but also context–dependent. In the current study we clearly show a positive correlation of cyclin-D1 and β-catenin expression in many instances. Conditional disruption of β-catenin in the liver also led to a significant loss of expression of cyclin-D1 both during development and in adult liver [94, 96]. This deficit was more pronounced when a need for proliferation was created in the knockout

mice such as after PHx. In additional situations such as postnatal liver development, cyclin-D1 expression closely correlated with β-catenin expression [129]. It was interesting to observe that while cyclin-D1 appeared to be the central mechanism defective in regeneration in the absence of β-catenin, the TOPGAL reporter mice, as reported in the latter study, failed to elicit any sign of direct β-catenin activation following hepatectomy. The reason for this discrepancy remains unidentified and might be due to technical challenges for the use of TOPGAL mice to study β-catenin activation in adult tissues. It is conceivable that the site of integration for the transgene, which is obscure, may be subject to developmental regulation keeping it permissive in prenatal or early postnatal stages and rendering it non-permissive in adults. In any case, Wnt/β-catenin signaling in rats, mice and more recently in zebrafish is unquestionable [350]. Thus, while we are also cognizant of β-catenin-independent regulation of cyclin-D1, it is worth emphasizing that β-catenin is indeed one of the chief regulators of cyclin-D1 expression and in turn of cell proliferation in the liver and that such relationship would be especially evident during the events that require increase in cell proliferation. This observation also highlights the context-dependence of key genetic targets of Wnt/β-catenin signaling.

The Wnt/β-catenin pathway may be amenable to manipulation through exogenous activation, thus allowing it to be exploited for therapeutic purposes. Several studies have already addressed the effect of Wnt-1 stimulation on β-catenin activation and cell growth. For example, transfection of Wnt-1 plasmid in NIH3T3 cells causes translocation of β-catenin and nuclear association with LEF-1 [355]. Similarly, a decrease in GSK-3β activity and an increase in nuclear accumulation of β-catenin are seen in mammary epithelial cells transformed with Wnt-1 [50]. Further, Wnt-1 induces a growth response in cultured Rat-1 fibroblasts [356], which is partially mediated through activation of β-catenin [357]. In our study we demonstrate quite

clearly that Wnt-1 naked DNA delivered hydrodynamically leads to enhanced Wnt-1 expression, which then induces β-catenin activation along with an increase in cyclin-D1 expression and hepatocyte proliferation after partial hepatectomy. Thus our report for the first time provides evidence that exogenous manipulation of Wnt/β-catenin activation might be a novel way of inducing regeneration of the liver, when necessary. Clearly, this will be dependent on the availability of modulators of Wnt/β-catenin signaling pathway, which would need additional substantiation in preclinical models. While such modulators are not available currently, agents such as lithium chloride, which are know to inhibit GSK3β, a known negative regulator of β-catenin, might be relevant for further investigation in preclinical scenarios in liver regeneration [449].

This work also has the potential to enhance the effectiveness of current regenerative technologies in several ways. First, human hepatocytes genetically engineered with stable β-catenin may reduce the number of hepatocytes needed for cell transplant therapy and thus eliminate the need for serial transplants. Second, temporal suppression of the β-catenin pathway in transplant recipients may allow for more efficient cell engraftment by providing a growth advantage to donor cells. Additionally, inducing Wnt signaling after orthotopic liver transplant in the recipient may promote growth and function of the graft especially in small-for-size grafts. Finally, Wnt activation may also be of essence in promoting regeneration in acute liver failure. Thus, understanding the Wnt/β-catenin pathway may prove to be the key to successfully bridging liver biology, tissue engineering, and hepatic regenerative medicine.

5.2 BETA-CATENIN IN REGUCALCIN REGULATION, VITAMIN C SYNTHESIS, AND OXIDATIVE STRESS

The β-catenin signaling pathway is central to liver health and disease, and its activation is essential in many aspects of liver physiology including early development, regeneration, zonation, and oxidative stress [331]. Mutations in the β-catenin gene that render the protein aberrantly active have been implicated in various stages of liver tumorigenesis as well. Previous investigations of β-catenin knockout mice have validated downstream targets of β-catenin in the liver, such as glutamine synthetase [34]. Here, we report that regucalcin is a novel target of β-catenin in the liver, and examine its functional role in some of the canonical activities relevant to β-catenin such as regulating cell survival. In addition, we identify a unique role for β-catenin in regulating vitamin C biosynthesis and the redox state of a cell.

Initial experiments with β-catenin conditional knockout mice reveal the presence of regucalcin in wild-type mice and the absence of regucalcin in β-catenin knockout littermates after 3 months. The fact that a small amount of regucalcin still persisted in the KO animals at 1 month after birth can be attributed to the fact that the efficiency of recombination for Alb-Cre mice (the conversion from a floxed allele to a deleted allele) is only about 40% at birth and is not complete until the mice are 6 weeks old [391]. We examined the KO mice at 3 months of age, at which time regucalcin is absent in the livers of these mice. Similarly, β-catenin TG mice display elevated levels of regucalcin protein in the nucleus and cytosol of hepatocytes as compared to their WT counterparts. The strong correlation between regucalcin, GS, and nuclear β-catenin

after partial hepatectomy in the rat and in human hepatocellular carcinomas further supports the conclusion that β-catenin regulates regucalcin/SMP30 expression in murine, rat, and human livers.

Previous data on regucalcin in hepatocellular carcinomas has been somewhat sparse. Serum levels of regucalcin are markedly increased 30 days after administration of carbon tetrachloride, suggesting a role for regucalcin as a biomarker for chronic liver injury [450]. Decreased regucalcin expression was observed in CuZn superoxide dismutase deficient mice with fully-developed HCCs [451]. Another study showed that regucalcin RNA was downregulated in diethylnitrosamine (DEN)-generated mouse HCCs vs. regenerating (-10.7), quiescent (-12.6), and newborn liver (-4.4). In these samples, β-catenin, which was indirectly measured by the detection and measurement of β-catenin putative target genes, was not found to be deregulated [452]. Indeed, studies have shown that administration of DEN without phenobarbitol (PB) utilizes a non-Wnt pathway to induce HCC, whereas DEN plus PB- induced liver tumors display increased β-catenin mutations [453]. Another study of mouse liver carcinogenesis has shown that regucalcin was upregulated early in oxazepam-induced carcinogenesis, which is known to induce β-catenin gene mutations [365, 454]. This demonstrates correlation between β-catenin activation and regucalcin expression.

Analysis of the promoter region of mouse regucalcin revealed four putative TCF-4/LEF binding sites. These sites are between 750 and 150 bp upstream of the +1 site and are either very similar to or identical with consensus sequence sites for TCF-4 binding identified previously [19, 27, 455]. Using multiple deletion mutants isolated from the promoter region of the human regucalcin gene, we identified the specific site at which β–catenin transcriptionally regulates regucalcin expression [38]. The regucalcin promoter is also known to contain an AP-1 binding

site [170]. One recent study demonstrates that AP-1 and β-catenin can act in synergy to transcriptionally activate β-catenin target genes such as cyclin-D1 [456]. In previous studies, AP-1 has been shown to mediate regucalcin mRNA expression in hepatocytes by binding to the promoter region of the regucalcin gene and activating transcription [170]. The existence of AP-1 and TCF binding sites in the regucalcin promoter region may suggest a mechanism by which regucalcin can contribute to malignancy in HCCs secondary to β-catenin activation. An understanding of these mechanisms may also reveal potential exploitable therapeutic benefits.

To understand the biological implications of regucalcin especially in hepatoma cells, we utilized siRNA suppression of regucalcin. A significant increase in apoptosis in HepG2 cells occurred after regulacin suppression. This is in agreement with previous studies where regucalcin overexpression has been shown to suppress cell death [189]. Our results are also concordant with previous studies where mice containing a germline null mutation of SMP30 have been shown to be highly susceptible to death-receptor mediated apoptosis, thereby indicating that SMP30 plays a role in protection from apoptosis and contributes to cell survival [191, 192]. Previous work in our laboratory has also demonstrated that loss of β-catenin results in increased hepatocyte death as well [89, 94]. Thus, we suggest that loss of regucalcin in the KO mice may be one of the mechanisms by which basal apoptosis is increased, and becomes of relevance in cases of basal NF-κB activation.

Interestingly, inhibition of regucalcin in Hep3B and HepG2 tumor cells had little to no effect on proliferation, unlike what has been reported elsewhere [178, 373]. It has been previously suggested that regucalcin may have a role as an inhibitor of proliferation during liver regeneration in rats. These seemingly discordant observations may be a function of the multiple roles of molecules like regucalcin and β-catenin in cell proliferation, survival, and metabolism;

thus, the ultimate biological observations may really be an end result of tipping the balance between various responses and may be highly context-dependent.

We also show for the first time a physical association of β-catenin and regucalcin in HepG2 and Hep3B cells. Since β-catenin has been known to upregulate expression of Wnt pathway regulators such as Axin2 and Dkk1, we postulate that regucalcin may in fact be a negative regulator of β-catenin in the cytosol of the resting liver. If this is the case, transcriptional control of regucalcin by β-catenin would occur only when cytosolic and nuclear levels are excessive, thus limiting the duration of the signal. It might be that during regulated liver growth as in regeneration, regucalcin may function primarily as an activator of a feedback loop that negatively regulates β-catenin (and consequently cell proliferation) when intracellular levels of β-catenin reach a critical mass. However, in hepatocellular carcinomas, where the expression of β-catenin may be dysregulated due to mutations in the β-catenin gene, regucalcin (whose expression is amplified due to increased β-catenin) is no longer able to downregulate β-catenin and may in fact contribute to subsequent tumor formation through its secondary role as a promoter of tumor cell survival. In addition regucalcin has been shown to inhibit activity of phosphatases, especially tyrosine phosphatases. In HCC, where regucalcin expression is increased, there may be a greater inhibition of tyrosine phosphatase activity which would lead to increased tyrosine phosphorylation of β-catenin (due to the direct association between regucalcin and β-catenin), thus creating a positive feedback loop. Again, the negative and positive impact of regucalcin through diverse mechanisms may be dependent on cellular context and will require further careful investigation.

Both regucalcin and L-gulonolactone oxidase, another downstream target of β-catenin, are known to have an important role in vitamin C synthesis [193, 381]. We observed a significant

decrease in serum ascorbate levels in our KO mice, which have reduced expression levels of both genes. When cultured KO hepatocytes were supplemented with ascorbate, they showed a marked increase in cell survival, as measured by MTT uptake (data not shown). Ascorbate also rescued the cells from massive apoptosis which was present after 48 hours in culture. As we have previously noted, our β-catenin KO mouse show an increased basal level of hepatocyte apoptosis [94]. Reduction of apoptosis by ascorbate implicates lack of vitamin C synthesis in the KO phenotype. Other studies have also demonstrated a role for regucalcin as a suppressor of cell death [189, 191, 192]. Future studies in which β-catenin KO mice are supplemented with ascorbate would be needed to address whether vitamin C supplementation rescues the apoptotic phenotype observed in these mice.

Interestingly, we saw increased apoptosis in HepG2 cells and decreased survival in Hep3B cells treated with regucalcin siRNA, which was rescued by addition of ascorbate or N-acetyl cysteine (NAC) to the cultures. Both are known anti-oxidants that neutralize the effects of free radicals. Since humans and guinea pigs are unable to synthesize endogenous vitamin C due to mutations in the L-gulonolactone oxidase gene [381], the decreased cell survival after regucalcin knockdown seen in these human hepatoma cell lines may be a result of oxidative stress rather than the physiologic effects of vitamin C depletion. Other groups have also reported that lack of SMP30/regucalcin has been linked to an increase in oxidative stress [185, 186]. Further, overexpression of regucalcin leads to a reduction in reactive oxygen species in HepG2 cells [184]. We therefore propose that one mechanism for the increased apoptosis seen in our KO mice could be the increase in oxidative stress secondary to the loss of ascorbate. Indeed in our unpublished studies, we also observe a modest increase in basal lipid peroxidation, a marker of oxidative stress, in β-catenin conditional null mice. It is possible, therefore, that β-catenin

contributes to cell survival through the regulation of regucalcin, which may be an important modulator of redox state in hepatocytes.

5.3 BETA-CATENIN IN CELL SURVIVAL AND APOPTOSIS

Programmed cell death is a complex process regulated by several signaling pathways and containing a multitude of different checkpoints. Although many of these pathways and molecules have been well-described, the role of others has yet to be elucidated. β-catenin is a crucial component of the Wnt pathway that plays multiple roles in liver homeostasis through its regulation of proliferation, differentiation and regeneration. However, it is currently unknown whether this pathway plays any role in cell survival, and if so, what might be its function. Recent work in our laboratory has demonstrated enhanced apoptosis in β-catenin KO livers both basally and in the early stages of liver regeneration [94]. We also identified regucalcin as a β-catenin downstream target, which is implicated in modulating oxidative stress and suppressing cell death [38]. In fact, a higher incidence of apoptosis in both cultured hepatocytes from β-catenin KO mice and HepG2 cells treated with regucalcin siRNA was observed. Therefore, we hypothesized that β-catenin plays a pro-survival role in liver biology, and loss of β-catenin (and its downstream targets, including regucalcin) in hepatocytes can contribute to a basal increase in oxidative stress and apoptosis. Since several TNF-α related genes were upregulated basally in KO livers, we reasoned that the prevalence of basal apoptosis was occurring through activation of the TNF-α pathway, one of the major death-receptor mediated apoptotic pathways in liver. Indeed, there was a clear increase in basal inflammation in KO livers, as evidenced by the increase in CD45+ve cells, which are a prominent source of TNF-α. Intriguingly, however, we found that our β-catenin KO mice were largely protected from the effects of exogenous

GalN/LPS induced hepatotoxicity and apoptosis, while the WT animals died at the expected times and with predictable pathologic progression. All of the hallmarks of apoptotic death, which were present in the WTs – including caspase activation, elevated liver enzymes, TUNEL positivity, and disintegration of liver architecture – were absent or greatly diminished in the KO livers.

What is the mechanism of prolonged survival in these β-catenin KO mice? As mentioned previously, activation of the TNF-α pathway can stimulate two opposing pathways which result in either apoptosis (culminating in activation of caspase-8) or cell survival (culminating in activation of NF-κB) [403]. How this pathway is tipped to either direction remains an enigma. We do observe a basal increase in apoptosis in KOs but also demonstrate an increase in NF-κB pathway activation, and since the animals lack an overt phenotype, the balance clearly tips toward survival. Indeed, we found that levels of NF-κB and many of its downstream targets continue to be maintained in KO mice after insult. In addition, we found NF-κB to be basally active in KOs under unstimulated conditions, confirming that NF-κB is exerting a protective response in the KO animals.

A role for NF-κB in suppression of programmed cell death was first described in RelA (p65) knockout mice, which died at embryonic day 15 of massive hepatocyte apoptosis [457]. This effect is abolished in mice lacking TNF-R1 or TNF-α [458]. However, mice deficient in both RelA and TNF-R1 survive until shortly after birth, suggesting that RelA KO mice have an enhanced susceptibility to TNF-α, which is expressed in high amounts by hematopoietic progenitors [459]. Deletion of IKK2/β and NEMO, subunits of the IKK complex which activates NF-κB through phosphorylation of IκB, also result in lethality due to hepatocyte apoptosis during midgestation [460, 461]. Thus, proper activation and function of the NF-κB

pathway is critical to hepatocyte maturation and survival. Moreover, transfection of hepatocytes with a mutant IκBα superrepressor, which prevents NF-κB activation, sensitizes these cells to TNF-α apoptosis [243, 462, 463]. Further, conditional deletion of NEMO, the regulatory subunit of the IKK complex, in hepatocytes renders mice susceptible to even low doses of LPS [464], further highlighting the indispensible role of NF-κB in protection from TNF-α mediated apoptosis.

It has long been known that constitutive activation of NF-κB suppresses apoptosis in a variety of cancers [464]. However, very few experimental models have tested the effects of overexpression or constitutive activation of NF-κB on hepatocyte apoptosis. One study found that pretreatment with IL-1 protected mice from the effects of GalN/TNF-α apoptosis through activation of NF-κB [465]**.** To our knowledge, however, ours is the first to report on the effect of endogenous rather than inducible NF-κB on hepatocyte survival.

A recent report has described a mouse strain (Jcl-Imperial Cancer Research (ICR) mice) which is less sensitive than C57Bl/6 to GalN/TNF-α induced apoptosis [466]. Induction of NF-κB binding and activation after GalN/TNF-α treatment was similar in both ICR and C57Bl/6 mice, which the authors argue is evidence against an increase in NF-κB expression being the mechanism of protection from injury. Interestingly, however, the authors saw increased constitutive expression of a short form of c-FLIP, an inhibitor of caspase-8, in the ICR mice, which correlated with an increase in survival. As c-FLIP is a known target of NF-κB signaling, it is therefore plausible that basal activation of NF-κB, rather than binding or activation after injury, may account for the constitutive upregulation of of this anti-apoptotic protein. It remains to be seen if c-FLIP is also upregulated basally in our β-catenin KO mice, and if it contributes to the protection from TNF-α-mediated apoptosis seen in our model as well. Interestingly, these

ICR mice were also found to be more susceptible than C57Bl/6 mice to Jo-2 mediated apoptosis, which once again suggests an inverse relationship between resistance to TNF-α-mediated apoptosis (concurrent with NF-κB activation) and susceptibility to Fas-mediated apoptosis.

An important role for β-catenin in inhibiting NF-κB binding and transcriptional activity has recently been elucidated in cancer cells [266, 267]. Association of β-catenin with p65 prevents activation of NF-κB signaling and thus repression of NF-κB has been found in tumors with activated β-catenin. Furthermore, analysis of tumor tissues shows a strong inverse correlation between expression of β-catenin and Fas, a target gene of NF-κB [266], as well as between β-catenin and iNOS, another NF-κB-dependent gene [267]. It is still unclear whether the β-catenin and NF-κB interaction exists as part of a larger protein complex. Since it is known that GSK-3β can phosphorylate both β-catenin and NF-κB, it is plausible that the APC/Axin/GSK-3β/β-catenin complex is in close proximity to or in direct physical association with the β-catenin/NF-κB/IκK complex. If true, this association could potentiate the cross-regulation and signal integration of two distinct cell-survival pathways, thus positioning β-catenin as the effector molecule of both proliferation and apoptosis.

In recent years, several small molecule inhibitors of the Wnt/β-catenin pathway have been identified. Since aberrant Wnt pathway activation has been implicated in several types of cancers, these antagonists have been touted as the next generation of cancer therapeutics because of their anti-proliferative and pro-apoptotic effects [467-469]. Caution must be used, however, when prescribing such treatments, as they may have unintended consequences. As we have shown, inhibition of β-catenin does not automatically lead to apoptosis. Depending on the balance of NF-κB and Fas in the cell, as well as other secondary hepatic pathologies, cells treated with β-catenin antagonists may actually promote cell survival. Further pre-clinical work would

be necessary in order to determine the long-term effects of NF-κB activation in the context of β-catenin inhibition.

APPENDIX A

PUBLICATIONS

Peer-Reviewed Articles:

1. **Nejak-Bowen KN**, Thompson MD, Singh S, Bowen WC Jr, Dar MJ, Khillan J, Dai C, Monga SP. Accelerated liver regeneration and hepatocarcinogenesis in mice overexpressing serine-45 mutant beta-catenin. Hepatology. 2010 May;51(5):1603-13.

2. **Nejak-Bowen KN**, Zeng G, Tan X, Cieply B, Monga SP. Beta-catenin regulates vitamin C biosynthesis and cell survival in murine liver. J Biol Chem. 2009 Oct 9;284(41):28115-27. Epub 2009 Aug 18.

3. Paranjpe S, Bowen WC, Bell AW, **Nejak-Bowen K**, Luo JH, Michalopoulos GK. Cell cycle effects resulting from inhibition of hepatocyte growth factor and its receptor c-Met in regenerating rat livers by RNA interference. Hepatology. 2007 Jun;45(6):1471-7.

Reviews and Book Chapters:

1. Thompson M, **Nejak-Bowen K**, and Monga SPS. Crosstalk of the Wnt signaling pathway. Targeting the Wnt pathway in cancer. Springer. (Book chapter, in press).

2. **Nejak-Bowen K** and Monga SPS. Wnt/β-catenin signaling in hepatic organogenesis. Organogenesis. 2008 4(2): 92-99.

BIBLIOGRAPHY

1. Wodarz, A. and R. Nusse, *Mechanisms of Wnt signaling in development.* Annu Rev Cell Dev Biol, 1998. **14**: p. 59-88.
2. Cadigan, K.M. and R. Nusse, *Wnt signaling: a common theme in animal development.* Genes Dev, 1997. **11**(24): p. 3286-305.
3. Nusse, R., *Wnt signaling in disease and in development.* Cell Res, 2005. **15**(1): p. 28-32.
4. Logan, C.Y. and R. Nusse, *The Wnt signaling pathway in development and disease.* Annu Rev Cell Dev Biol, 2004. **20**: p. 781-810.
5. Peifer, M. and P. Polakis, *Wnt signaling in oncogenesis and embryogenesis--a look outside the nucleus.* Science, 2000. **287**(5458): p. 1606-9.
6. Willert, K., et al., *Wnt proteins are lipid-modified and can act as stem cell growth factors.* Nature, 2003. **423**(6938): p. 448-52.
7. Polakis, P., *Wnt signaling and cancer.* Genes Dev, 2000. **14**(15): p. 1837-51.
8. Paul, S. and A. Dey, *Wnt signaling and cancer development: therapeutic implication.* Neoplasma, 2008. **55**(3): p. 165-76.
9. Clevers, H., *Wnt/beta-catenin signaling in development and disease.* Cell, 2006. **127**(3): p. 469-80.
10. Wong, H.C., et al., *Direct binding of the PDZ domain of Dishevelled to a conserved internal sequence in the C-terminal region of Frizzled.* Mol Cell, 2003. **12**(5): p. 1251-60.
11. Cliffe, A., F. Hamada, and M. Bienz, *A role of Dishevelled in relocating Axin to the plasma membrane during wingless signaling.* Curr Biol, 2003. **13**(11): p. 960-6.
12. Zeng, X., et al., *Initiation of Wnt signaling: control of Wnt coreceptor Lrp6 phosphorylation/activation via frizzled, dishevelled and axin functions.* Development, 2008. **135**(2): p. 367-75.
13. Zeng, X., et al., *A dual-kinase mechanism for Wnt co-receptor phosphorylation and activation.* Nature, 2005. **438**(7069): p. 873-7.
14. Cadigan, K.M. and Y.I. Liu, *Wnt signaling: complexity at the surface.* J Cell Sci, 2006. **119**(Pt 3): p. 395-402.
15. Yokoya, F., et al., *beta-catenin can be transported into the nucleus in a Ran-unassisted manner.* Mol Biol Cell, 1999. **10**(4): p. 1119-31.
16. Huber, O., et al., *Nuclear localization of beta-catenin by interaction with transcription factor LEF-1.* Mech Dev, 1996. **59**(1): p. 3-10.

17. Boon, E.M., et al., *Wnt signaling regulates expression of the receptor tyrosine kinase met in colorectal cancer.* Cancer Res, 2002. **62**(18): p. 5126-8.
18. Rodilla, V., et al., *Jagged1 is the pathological link between Wnt and Notch pathways in colorectal cancer.* Proc Natl Acad Sci U S A, 2009. **106**(15): p. 6315-20.
19. Koh, T.J., et al., *Gastrin is a target of the beta-catenin/TCF-4 growth-signaling pathway in a model of intestinal polyposis.* J Clin Invest, 2000. **106**(4): p. 533-9.
20. Brabletz, T., et al., *beta-catenin regulates the expression of the matrix metalloproteinase-7 in human colorectal cancer.* Am J Pathol, 1999. **155**(4): p. 1033-8.
21. Zhang, T., et al., *Evidence that APC regulates survivin expression: a possible mechanism contributing to the stem cell origin of colon cancer.* Cancer Res, 2001. **61**(24): p. 8664-7.
22. Hendrix, N.D., et al., *Fibroblast growth factor 9 has oncogenic activity and is a downstream target of Wnt signaling in ovarian endometrioid adenocarcinomas.* Cancer Res, 2006. **66**(3): p. 1354-62.
23. Chamorro, M.N., et al., *FGF-20 and DKK1 are transcriptional targets of beta-catenin and FGF-20 is implicated in cancer and development.* Embo J, 2005. **24**(1): p. 73-84.
24. Shimokawa, T., et al., *Involvement of the FGF18 gene in colorectal carcinogenesis, as a novel downstream target of the beta-catenin/T-cell factor complex.* Cancer Res, 2003. **63**(19): p. 6116-20.
25. Shtutman, M., et al., *The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway.* Proc Natl Acad Sci U S A, 1999. **96**(10): p. 5522-7.
26. Tetsu, O. and F. McCormick, *Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells.* Nature, 1999. **398**(6726): p. 422-6.
27. He, T.C., et al., *Identification of c-MYC as a target of the APC pathway.* Science, 1998. **281**(5382): p. 1509-12.
28. Leung, J.Y., et al., *Activation of AXIN2 expression by beta-catenin-T cell factor. A feedback repressor pathway regulating Wnt signaling.* J Biol Chem, 2002. **277**(24): p. 21657-65.
29. Roose, J., et al., *Synergy between tumor suppressor APC and the beta-catenin-Tcf4 target Tcf1.* Science, 1999. **285**(5435): p. 1923-6.
30. Cadigan, K.M., et al., *Wingless repression of Drosophila frizzled 2 expression shapes the Wingless morphogen gradient in the wing.* Cell, 1998. **93**(5): p. 767-77.
31. Khan, Z., et al., *Analysis of endogenous LRP6 function reveals a novel feedback mechanism by which Wnt negatively regulates its receptor.* Mol Cell Biol, 2007. **27**(20): p. 7291-301.
32. Hovanes, K., et al., *Beta-catenin-sensitive isoforms of lymphoid enhancer factor-1 are selectively expressed in colon cancer.* Nat Genet, 2001. **28**(1): p. 53-7.
33. MacDonald, B.T., K. Tamai, and X. He, *Wnt/beta-catenin signaling: components, mechanisms, and diseases.* Dev Cell, 2009. **17**(1): p. 9-26.
34. Cadoret, A., et al., *New targets of beta-catenin signaling in the liver are involved in the glutamine metabolism.* Oncogene, 2002. **21**(54): p. 8293-301.
35. Loeppen, S., et al., *A beta-catenin-dependent pathway regulates expression of cytochrome P450 isoforms in mouse liver tumors.* Carcinogenesis, 2005. **26**(1): p. 239-48.
36. Giera, S., et al., *Wnt/beta-catenin signaling activates and determines hepatic zonal expression of glutathione S-transferases in mouse liver.* Toxicol Sci. **115**(1): p. 22-33.

37. Ovejero, C., et al., *Identification of the leukocyte cell-derived chemotaxin 2 as a direct target gene of beta-catenin in the liver.* Hepatology, 2004. **40**(1): p. 167-76.
38. Nejak-Bowen, K.N., et al., *Beta-catenin regulates vitamin C biosynthesis and cell survival in murine liver.* J Biol Chem, 2009. **284**(41): p. 28115-27.
39. Lilien, J. and J. Balsamo, *The regulation of cadherin-mediated adhesion by tyrosine phosphorylation/dephosphorylation of beta-catenin.* Curr Opin Cell Biol, 2005. **17**(5): p. 459-65.
40. Orsulic, S., et al., *E-cadherin binding prevents beta-catenin nuclear localization and beta-catenin/LEF-1-mediated transactivation.* J Cell Sci, 1999. **112 (Pt 8)**: p. 1237-45.
41. Aberle, H., et al., *Assembly of the cadherin-catenin complex in vitro with recombinant proteins.* J Cell Sci, 1994. **107 (Pt 12)**: p. 3655-63.
42. Roura, S., et al., *Regulation of E-cadherin/Catenin association by tyrosine phosphorylation.* J Biol Chem, 1999. **274**(51): p. 36734-40.
43. Piedra, J., et al., *Regulation of beta-catenin structure and activity by tyrosine phosphorylation.* J Biol Chem, 2001. **276**(23): p. 20436-43.
44. Theard, D., et al., *Cell polarity development and protein trafficking in hepatocytes lacking E-cadherin/beta-catenin-based adherens junctions.* Mol Biol Cell, 2007. **18**(6): p. 2313-21.
45. Tang, Y., et al., *Progenitor/stem cells give rise to liver cancer due to aberrant TGF-beta and IL-6 signaling.* Proc Natl Acad Sci U S A, 2008. **In press**.
46. Peinado, H., M. Quintanilla, and A. Cano, *Transforming growth factor beta-1 induces snail transcription factor in epithelial cell lines: mechanisms for epithelial mesenchymal transitions.* J Biol Chem, 2003. **278**(23): p. 21113-23.
47. Katuri, V., et al., *Critical interactions between TGF-beta signaling/ELF, and E-cadherin/beta-catenin mediated tumor suppression.* Oncogene, 2006. **25**(13): p. 1871-86.
48. Zarnegar, R., *Regulation of HGF and HGFR gene expression.* Exs, 1995. **74**: p. 33-49.
49. Monga, S.P., et al., *beta-Catenin regulation during matrigel-induced rat hepatocyte differentiation.* Cell Tissue Res, 2006. **323**(1): p. 71-9.
50. Papkoff, J. and M. Aikawa, *WNT-1 and HGF regulate GSK3 beta activity and beta-catenin signaling in mammary epithelial cells.* Biochem Biophys Res Commun, 1998. **247**(3): p. 851-8.
51. Monga, S.P., et al., *Hepatocyte growth factor induces Wnt-independent nuclear translocation of beta-catenin after Met-beta-catenin dissociation in hepatocytes.* Cancer Res, 2002. **62**(7): p. 2064-71.
52. Zeng, G., et al., *Tyrosine residues 654 and 670 in beta-catenin are crucial in regulation of Met-beta-catenin interactions.* Exp Cell Res, 2006. **312**(18): p. 3620-30.
53. Apte, U., et al., *Activation of Wnt/beta-catenin pathway during hepatocyte growth factor-induced hepatomegaly in mice.* Hepatology, 2006. **44**(4): p. 992-1002.
54. Ishibe, S., et al., *Cell confluence regulates hepatocyte growth factor-stimulated cell morphogenesis in a beta-catenin-dependent manner.* Mol Cell Biol, 2006. **26**(24): p. 9232-43.
55. Tward, A.D., et al., *Distinct pathways of genomic progression to benign and malignant tumors of the liver.* Proc Natl Acad Sci U S A, 2007. **104**(37): p. 14771-6.
56. Zaret, K.S., *Molecular genetics of early liver development.* Annu Rev Physiol, 1996. **58**: p. 231-51.

57. Zaret, K.S., *Hepatocyte differentiation: from the endoderm and beyond.* Curr Opin Genet Dev, 2001. **11**(5): p. 568-74.
58. Zaret, K.S., *Regulatory phases of early liver development: paradigms of organogenesis.* Nat Rev Genet, 2002. **3**(7): p. 499-512.
59. Spear, B.T., et al., *Transcriptional control in the mammalian liver: liver development, perinatal repression, and zonal gene regulation.* Cell Mol Life Sci, 2006. **63**(24): p. 2922-38.
60. Lee, C.S., et al., *The initiation of liver development is dependent on Foxa transcription factors.* Nature, 2005. **435**(7044): p. 944-7.
61. Jung, J., et al., *Initiation of mammalian liver development from endoderm by fibroblast growth factors.* Science, 1999. **284**(5422): p. 1998-2003.
62. Matsumoto, K., et al., *Liver organogenesis promoted by endothelial cells prior to vascular function.* Science, 2001. **294**(5542): p. 559-63.
63. Rossi, J.M., et al., *Distinct mesodermal signals, including BMPs from the septum transversum mesenchyme, are required in combination for hepatogenesis from the endoderm.* Genes Dev, 2001. **15**(15): p. 1998-2009.
64. Zhang, W., et al., *Regulation of Hex gene expression and initial stages of avian hepatogenesis by Bmp and Fgf signaling.* Dev Biol, 2004. **268**(2): p. 312-26.
65. Martinez Barbera, J.P., et al., *The homeobox gene Hex is required in definitive endodermal tissues for normal forebrain, liver and thyroid formation.* Development, 2000. **127**(11): p. 2433-45.
66. Bort, R., et al., *Hex homeobox gene controls the transition of the endoderm to a pseudostratified, cell emergent epithelium for liver bud development.* Dev Biol, 2006. **290**(1): p. 44-56.
67. Hunter, M.P., et al., *The homeobox gene Hhex is essential for proper hepatoblast differentiation and bile duct morphogenesis.* Dev Biol, 2007. **308**(2): p. 355-67.
68. Duncan, S.A., *Transcriptional regulation of liver development.* Dev Dyn, 2000. **219**(2): p. 131-42.
69. Schmidt, C., et al., *Scatter factor/hepatocyte growth factor is essential for liver development.* Nature, 1995. **373**(6516): p. 699-702.
70. Hentsch, B., et al., *Hlx homeo box gene is essential for an inductive tissue interaction that drives expansion of embryonic liver and gut.* Genes Dev, 1996. **10**(1): p. 70-9.
71. Sosa-Pineda, B., J.T. Wigle, and G. Oliver, *Hepatocyte migration during liver development requires Prox1.* Nat Genet, 2000. **25**(3): p. 254-5.
72. Zaret, K., *Early liver differentiation: genetic potentiation and multilevel growth control.* Curr Opin Genet Dev, 1998. **8**(5): p. 526-31.
73. Zaret, K.S., *Liver specification and early morphogenesis.* Mech Dev, 2000. **92**(1): p. 83-8.
74. Odom, D.T., et al., *Control of pancreas and liver gene expression by HNF transcription factors.* Science, 2004. **303**(5662): p. 1378-81.
75. Darlington, G.J., N. Wang, and R.W. Hanson, *C/EBP alpha: a critical regulator of genes governing integrative metabolic processes.* Curr Opin Genet Dev, 1995. **5**(5): p. 565-70.
76. Parviz, F., et al., *Hepatocyte nuclear factor 4alpha controls the development of a hepatic epithelium and liver morphogenesis.* Nat Genet, 2003. **34**(3): p. 292-6.
77. Zhao, R., et al., *GATA6 is essential for embryonic development of the liver but dispensable for early heart formation.* Mol Cell Biol, 2005. **25**(7): p. 2622-31.

78. Clotman, F., et al., *The onecut transcription factor HNF6 is required for normal development of the biliary tract.* Development, 2002. **129**(8): p. 1819-28.
79. Coffinier, C., et al., *Bile system morphogenesis defects and liver dysfunction upon targeted deletion of HNF1beta.* Development, 2002. **129**(8): p. 1829-38.
80. Lorent, K., et al., *Inhibition of Jagged-mediated Notch signaling disrupts zebrafish biliary development and generates multi-organ defects compatible with an Alagille syndrome phenocopy.* Development, 2004. **131**(22): p. 5753-66.
81. Zorn, A.M., K. Butler, and J.B. Gurdon, *Anterior endomesoderm specification in Xenopus by Wnt/beta-catenin and TGF-beta signalling pathways.* Dev Biol, 1999. **209**(2): p. 282-97.
82. Lemaigre, F. and K.S. Zaret, *Liver development update: new embryo models, cell lineage control, and morphogenesis.* Curr Opin Genet Dev, 2004. **14**(5): p. 582-90.
83. McLin, V.A., S.A. Rankin, and A.M. Zorn, *Repression of Wnt/beta-catenin signaling in the anterior endoderm is essential for liver and pancreas development.* Development, 2007. **134**(12): p. 2207-17.
84. Finley, K.R., J. Tennessen, and W. Shawlot, *The mouse secreted frizzled-related protein 5 gene is expressed in the anterior visceral endoderm and foregut endoderm during early post-implantation development.* Gene Expr Patterns, 2003. **3**(5): p. 681-4.
85. Pilcher, K.E. and P.A. Krieg, *Expression of the Wnt inhibitor, sFRP5, in the gut endoderm of Xenopus.* Gene Expr Patterns, 2002. **2**(3-4): p. 369-72.
86. Li, Y., et al., *Sfrp5 coordinates foregut specification and morphogenesis by antagonizing both canonical and noncanonical Wnt11 signaling.* Genes Dev, 2008. **22**(21): p. 3050-63.
87. Yoshida, Y., et al., *Calcineurin inhibitors block dorsal-side signaling that affect late-stage development of the heart, kidney, liver, gut and somitic tissue during Xenopus embryogenesis.* Dev Growth Differ, 2004. **46**(2): p. 139-52.
88. Ober, E.A., et al., *Mesodermal Wnt2b signalling positively regulates liver specification.* Nature, 2006. **442**(7103): p. 688-91.
89. Monga, S.P., et al., *Beta-catenin antisense studies in embryonic liver cultures: role in proliferation, apoptosis, and lineage specification.* Gastroenterology, 2003. **124**(1): p. 202-16.
90. Suksaweang, S., et al., *Morphogenesis of chicken liver: identification of localized growth zones and the role of beta-catenin/Wnt in size regulation.* Dev Biol, 2004. **266**(1): p. 109-22.
91. Micsenyi, A., et al., *Beta-catenin is temporally regulated during normal liver development.* Gastroenterology, 2004. **126**(4): p. 1134-46.
92. Wang, Q.M., et al., *[The role of beta-catenin in rat embryonic development and tumorigenesis].* Sichuan Da Xue Xue Bao Yi Xue Ban, 2006. **37**(6): p. 872-5.
93. Monga, S.P., et al., *Mouse fetal liver cells in artificial capillary beds in three-dimensional four-compartment bioreactors.* Am J Pathol, 2005. **167**(5): p. 1279-92.
94. Tan, X., et al., *Conditional deletion of beta-catenin reveals its role in liver growth and regeneration.* Gastroenterology, 2006. **131**(5): p. 1561-72.
95. Hussain, S.Z., et al., *Wnt impacts growth and differentiation in ex vivo liver development.* Exp Cell Res, 2004. **292**(1): p. 157-69.
96. Tan, X., et al., *Beta-catenin deletion in hepatoblasts disrupts hepatic morphogenesis and survival during mouse development.* Hepatology, 2008. **47**(5): p. 1667-79.

97. Eisenberg, L.M. and C.A. Eisenberg, *Evaluating the role of Wnt signal transduction in promoting the development of the heart.* ScientificWorldJournal, 2007. **7**: p. 161-76.
98. Tzahor, E., *Wnt/beta-catenin signaling and cardiogenesis: timing does matter.* Dev Cell, 2007. **13**(1): p. 10-3.
99. Lickert, H., et al., *Expression patterns of Wnt genes in mouse gut development.* Mech Dev, 2001. **105**(1-2): p. 181-4.
100. Ang, S.J., et al., *Spatial and temporal expression of Wnt and Dickkopf genes during murine lens development.* Gene Expr Patterns, 2004. **4**(3): p. 289-95.
101. Naito, A.T., et al., *Developmental stage-specific biphasic roles of Wnt/beta-catenin signaling in cardiomyogenesis and hematopoiesis.* Proc Natl Acad Sci U S A, 2006. **103**(52): p. 19812-7.
102. Kim, B.M., et al., *Phases of canonical Wnt signaling during the development of mouse intestinal epithelium.* Gastroenterology, 2007. **133**(2): p. 529-38.
103. Michalopoulos, G.K. and M.C. DeFrances, *Liver regeneration.* Science, 1997. **276**(5309): p. 60-6.
104. Higgins, G.M. and R.M. Anderson, *Experimental pathology of the liver, 1:Restoration of the liver of the white rat following partial surgical removal. .* Arch Pathol 1931. **12**: p. 186-202.
105. Michalopoulos, G.K., *Liver regeneration.* J Cell Physiol, 2007. **213**(2): p. 286-300.
106. Mars, W.M., et al., *Presence of urokinase in serum-free primary rat hepatocyte cultures and its role in activating hepatocyte growth factor.* Cancer Res, 1996. **56**(12): p. 2837-43.
107. Nakamura, T., et al., *Molecular cloning and expression of human hepatocyte growth factor.* Nature, 1989. **342**(6248): p. 440-3.
108. Stolz, D.B., et al., *Growth factor signal transduction immediately after two-thirds partial hepatectomy in the rat.* Cancer Res, 1999. **59**(16): p. 3954-60.
109. Delehedde, M., et al., *Hepatocyte growth factor/scatter factor stimulates migration of rat mammary fibroblasts through both mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt pathways.* Eur J Biochem, 2001. **268**(16): p. 4423-9.
110. Xiao, G.H., et al., *Anti-apoptotic signaling by hepatocyte growth factor/Met via the phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase pathways.* Proc Natl Acad Sci U S A, 2001. **98**(1): p. 247-52.
111. Pediaditakis, P., et al., *The processing and utilization of hepatocyte growth factor/scatter factor following partial hepatectomy in the rat.* Hepatology, 2001. **34**(4 Pt 1): p. 688-93.
112. Lindroos, P.M., R. Zarnegar, and G.K. Michalopoulos, *Hepatocyte growth factor (hepatopoietin A) rapidly increases in plasma before DNA synthesis and liver regeneration stimulated by partial hepatectomy and carbon tetrachloride administration.* Hepatology, 1991. **13**(4): p. 743-50.
113. Zarnegar, R., et al., *Expression of hepatocyte growth factor mRNA in regenerating rat liver after partial hepatectomy.* Biochem Biophys Res Commun, 1991. **177**(1): p. 559-65.
114. Taub, R., *Liver regeneration 4: transcriptional control of liver regeneration.* Faseb J, 1996. **10**(4): p. 413-27.
115. Su, A.I., et al., *Gene expression during the priming phase of liver regeneration after partial hepatectomy in mice.* Proc Natl Acad Sci U S A, 2002. **99**(17): p. 11181-6.
116. White, P., et al., *Identification of transcriptional networks during liver regeneration.* J Biol Chem, 2005. **280**(5): p. 3715-22.

117. Walker, P.R. and V.R. Potter, *Isozyme studies on adult, regenerating, precancerous and developing liver in relation to findings in hepatomas.* Adv Enzyme Regul, 1972. **10**: p. 339-64.
118. Rabes, H.M., *Kinetics of hepatocellular proliferation as a function of the microvascular structure and functional state of the liver.* Ciba Found Symp, 1977(55): p. 31-53.
119. Stocker, E. and W.D. Heine, *Regeneration of liver parenchyma under normal and pathological conditions.* Beitr Pathol, 1971. **144**(4): p. 400-8.
120. Stocker, E., H.K. Wullstein, and G. Brau, *[Capacity of regeneration in liver epithelia of juvenile, repeated partially hepatectomized rats. Autoradiographic studies after continous infusion of 3H-thymidine (author's transl)].* Virchows Arch B Cell Pathol, 1973. **14**(2): p. 93-103.
121. Grisham, J.W., *A morphologic study of deoxyribonucleic acid synthesis and cell proliferation in regenerating rat liver; autoradiography with thymidine-H3.* Cancer Res, 1962. **22**: p. 842-9.
122. Nelsen, C.J., et al., *Transient expression of cyclin D1 is sufficient to promote hepatocyte replication and liver growth in vivo.* Cancer Res, 2001. **61**(23): p. 8564-8.
123. Gonzalez, F.J., *Role of beta-catenin in the adult liver.* Hepatology, 2006. **43**(4): p. 650-3.
124. Benhamouche, S., et al., *Apc tumor suppressor gene is the "zonation-keeper" of mouse liver.* Dev Cell, 2006. **10**(6): p. 759-70.
125. Sekine, S., et al., *Liver-specific loss of beta-catenin blocks glutamine synthesis pathway activity and cytochrome p450 expression in mice.* Hepatology, 2006. **43**(4): p. 817-25.
126. Monga, S.P., et al., *Changes in WNT/beta-catenin pathway during regulated growth in rat liver regeneration.* Hepatology, 2001. **33**(5): p. 1098-109.
127. Sodhi, D., et al., *Morpholino oligonucleotide-triggered beta-catenin knockdown compromises normal liver regeneration.* J Hepatol, 2005. **43**(1): p. 132-41.
128. Sekine, S., et al., *Liver-specific loss of beta-catenin results in delayed hepatocyte proliferation after partial hepatectomy.* Hepatology, 2007. **45**(2): p. 361-8.
129. Apte, U., et al., *beta-Catenin is critical for early postnatal liver growth.* Am J Physiol Gastrointest Liver Physiol, 2007. **292**(6): p. G1578-85.
130. Cadoret, A., et al., *Hepatomegaly in transgenic mice expressing an oncogenic form of beta-catenin.* Cancer Res, 2001. **61**(8): p. 3245-9.
131. Harada, N., et al., *Lack of tumorigenesis in the mouse liver after adenovirus-mediated expression of a dominant stable mutant of beta-catenin.* Cancer Res, 2002. **62**(7): p. 1971-7.
132. Tan, X., et al., *Epidermal growth factor receptor: a novel target of the Wnt/beta-catenin pathway in liver.* Gastroenterology, 2005. **129**(1): p. 285-302.
133. Diesen, D.L. and P.C. Kuo, *Nitric oxide and redox regulation in the liver: Part I. General considerations and redox biology in hepatitis.* J Surg Res. **162**(1): p. 95-109.
134. Lander, H.M., *An essential role for free radicals and derived species in signal transduction.* Faseb J, 1997. **11**(2): p. 118-24.
135. Sies, H., *Oxidative stress: oxidants and antioxidants.* Exp Physiol, 1997. **82**(2): p. 291-5.
136. Kang, K.J., *Mechanism of hepatic ischemia/reperfusion injury and protection against reperfusion injury.* Transplant Proc, 2002. **34**(7): p. 2659-61.
137. Han, D., E. Williams, and E. Cadenas, *Mitochondrial respiratory chain-dependent generation of superoxide anion and its release into the intermembrane space.* Biochem J, 2001. **353**(Pt 2): p. 411-6.

138. Nohl, H., et al., *Are mitochondria a spontaneous and permanent source of reactive oxygen species?* Redox Rep, 2003. **8**(3): p. 135-41.
139. Sutton, H.C. and C.C. Winterbourn, *On the participation of higher oxidation states of iron and copper in Fenton reactions.* Free Radic Biol Med, 1989. **6**(1): p. 53-60.
140. Kessova, I.G., et al., *Alcohol-induced liver injury in mice lacking Cu, Zn-superoxide dismutase.* Hepatology, 2003. **38**(5): p. 1136-45.
141. Koop, D.R. and D.J. Tierney, *Multiple mechanisms in the regulation of ethanol-inducible cytochrome P450IIE1.* Bioessays, 1990. **12**(9): p. 429-35.
142. Qiu, Y., L.Z. Benet, and A.L. Burlingame, *Identification of the hepatic protein targets of reactive metabolites of acetaminophen in vivo in mice using two-dimensional gel electrophoresis and mass spectrometry.* J Biol Chem, 1998. **273**(28): p. 17940-53.
143. Gong, G., et al., *Human hepatitis C virus NS5A protein alters intracellular calcium levels, induces oxidative stress, and activates STAT-3 and NF-kappa B.* Proc Natl Acad Sci U S A, 2001. **98**(17): p. 9599-604.
144. Okuda, M., et al., *Mitochondrial injury, oxidative stress, and antioxidant gene expression are induced by hepatitis C virus core protein.* Gastroenterology, 2002. **122**(2): p. 366-75.
145. Zamara, E., et al., *4-Hydroxynonenal as a selective pro-fibrogenic stimulus for activated human hepatic stellate cells.* J Hepatol, 2004. **40**(1): p. 60-8.
146. McCord, J.M., *Oxygen-derived free radicals in postischemic tissue injury.* N Engl J Med, 1985. **312**(3): p. 159-63.
147. Harbrecht, B.G., et al., *Nitric oxide synthesis serves to reduce hepatic damage during acute murine endotoxemia.* Crit Care Med, 1992. **20**(11): p. 1568-74.
148. Kamiya, K., et al., *The cytoprotective role of lipopolysaccharide-induced nitric oxide against liver damage during early phase of endotoxemia in rats.* Shock, 2000. **14**(2): p. 229-33.
149. Jaeschke, H., *Reactive oxygen and mechanisms of inflammatory liver injury.* J Gastroenterol Hepatol, 2000. **15**(7): p. 718-24.
150. Hug, H., et al., *Reactive oxygen intermediates are involved in the induction of CD95 ligand mRNA expression by cytostatic drugs in hepatoma cells.* J Biol Chem, 1997. **272**(45): p. 28191-3.
151. Poli, G., J.C. Cutrin, and F. Biasi, *Lipid peroxidation in the reperfusion injury of the liver.* Free Radic Res, 1998. **28**(6): p. 547-51.
152. Jaeschke, H., et al., *Glutathione peroxidase-deficient mice are more susceptible to neutrophil-mediated hepatic parenchymal cell injury during endotoxemia: importance of an intracellular oxidant stress.* Hepatology, 1999. **29**(2): p. 443-50.
153. Bellezzo, J.M., et al., *Modulation of lipopolysaccharide-mediated activation in rat Kupffer cells by antioxidants.* J Lab Clin Med, 1998. **131**(1): p. 36-44.
154. Adamson, G.M. and R.E. Billings, *Tumor necrosis factor induced oxidative stress in isolated mouse hepatocytes.* Arch Biochem Biophys, 1992. **294**(1): p. 223-9.
155. Hampton, M.B. and S. Orrenius, *Dual regulation of caspase activity by hydrogen peroxide: implications for apoptosis.* FEBS Lett, 1997. **414**(3): p. 552-6.
156. Lemasters, J.J., et al., *The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy.* Biochim Biophys Acta, 1998. **1366**(1-2): p. 177-96.

157. Sanchez, A., et al., *Apoptosis induced by transforming growth factor-beta in fetal hepatocyte primary cultures: involvement of reactive oxygen intermediates.* J Biol Chem, 1996. **271**(13): p. 7416-22.
158. Glantzounis, G.K., et al., *The contemporary role of antioxidant therapy in attenuating liver ischemia-reperfusion injury: a review.* Liver Transpl, 2005. **11**(9): p. 1031-47.
159. Liu, P., et al., *Beneficial effects of extracellular glutathione against endotoxin-induced liver injury during ischemia and reperfusion.* Circ Shock, 1994. **43**(2): p. 64-70.
160. Wefers, H. and H. Sies, *Oxidation of glutathione by the superoxide radical to the disulfide and the sulfonate yielding singlet oxygen.* Eur J Biochem, 1983. **137**(1-2): p. 29-36.
161. Chance, B., H. Sies, and A. Boveris, *Hydroperoxide metabolism in mammalian organs.* Physiol Rev, 1979. **59**(3): p. 527-605.
162. Zelko, I.N., T.J. Mariani, and R.J. Folz, *Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression.* Free Radic Biol Med, 2002. **33**(3): p. 337-49.
163. Yamaguchi, M., *Role of regucalcin in maintaining cell homeostasis and function (review).* Int J Mol Med, 2005. **15**(3): p. 371-89.
164. Takahasi, H. and M. Yamaguchi, *Enhancement of plasma membrane (Ca(2+)-Mg2+)-ATPase activity in regenerating rat liver: involvement of endogenous activating protein regucalcin.* Mol Cell Biochem, 1996. **162**(2): p. 133-8.
165. Fujita, T., *Senescence marker protein-30 (SMP30): structure and biological function.* Biochem Biophys Res Commun, 1999. **254**(1): p. 1-4.
166. Yamaguchi, M., *Mitochondrial uptake of 45Ca2+ bound to calcium-binding protein isolated from rat liver cytosol.* Chem Pharm Bull (Tokyo), 1985. **33**(8): p. 3390-4.
167. Takahashi, H. and M. Yamaguchi, *Role of regucalcin as an activator of Ca(2+)-ATPase activity in rat liver microsomes.* J Cell Biochem, 1999. **74**(4): p. 663-9.
168. Yamaguchi, M., *Effect of calcium-binding protein regucalcin on Ca2+ transport system in rat liver nuclei: stimulation of Ca2+ release.* Mol Cell Biochem, 1992. **113**(1): p. 63-70.
169. Tsurusaki, Y. and M. Yamaguchi, *Role of endogenous regucalcin in the regulation of Ca(2+)-ATPase activity in rat liver nuclei.* J Cell Biochem, 2000. **78**(4): p. 541-9.
170. Murata, T. and M. Yamaguchi, *Ca2+ administration stimulates the binding of AP-1 factor to the 5'-flanking region of the rat gene for the Ca2+-binding protein regucalcin.* Biochem J, 1998. **329 (Pt 1)**: p. 157-63.
171. Shimokawa, N. and M. Yamaguchi, *Calcium administration stimulates the expression of calcium-binding protein regucalcin mRNA in rat liver.* FEBS Lett, 1992. **305**(2): p. 151-4.
172. Tsurusaki, Y. and M. Yamaguchi, *Role of endogenous regucalcin in transgenic rats: suppression of protein tyrosine phosphatase and ribonucleic acid synthesis activities in liver nucleus.* Int J Mol Med, 2003. **12**(2): p. 207-11.
173. Yamaguchi, M. and M. Isogai, *Tissue concentration of calcium-binding protein regucalcin in rats by enzyme-linked immunoadsorbent assay.* Mol Cell Biochem, 1993. **122**(1): p. 65-8.
174. Yamaguchi, M. and H. Yoshida, *Regulatory effect of calcium-binding protein isolated from rat liver cytosol on activation of fructose 1,6-diphosphatase by Ca2+-calmodulin.* Chem Pharm Bull (Tokyo), 1985. **33**(10): p. 4489-93.

175. Yamaguchi, M. and H. Tai, *Inhibitory effect of calcium-binding protein regucalcin on Ca2+/calmodulin-dependent cyclic nucleotide phosphodiesterase activity in rat liver cytosol.* Mol Cell Biochem, 1991. **106**(1): p. 25-30.
176. Yamaguchi, M., *The role of regucalcin in nuclear regulation of regenerating liver.* Biochem Biophys Res Commun, 2000. **276**(1): p. 1-6.
177. Yamaguchi, M. and Y. Kanayama, *Enhanced expression of calcium-binding protein regucalcin mRNA in regenerating rat liver.* J Cell Biochem, 1995. **57**(2): p. 185-90.
178. Yamaguchi, M. and Y. Kanayama, *Calcium-binding protein regucalcin inhibits deoxyribonucleic acid synthesis in the nuclei of regenerating rat liver.* Mol Cell Biochem, 1996. **162**(2): p. 121-6.
179. Yamaguchi, M. and S. Ueoka, *Inhibitory effect of calcium-binding protein regucalcin on ribonucleic acid synthesis in isolated rat liver nuclei.* Mol Cell Biochem, 1997. **173**(1-2): p. 169-75.
180. Tsurusaki, Y. and M. Yamaguchi, *Suppressive effect of endogenous regucalcin on the enhancement of protein synthesis and aminoacyl-tRNA synthetase activity in regenerating rat liver.* Int J Mol Med, 2000. **6**(3): p. 295-9.
181. Tsurusaki, Y. and M. Yamaguchi, *Overexpression of regucalcin modulates tumor-related gene expression in cloned rat hepatoma H4-II-E cells.* J Cell Biochem, 2003. **90**(3): p. 619-26.
182. Fukaya, Y. and M. Yamaguchi, *Regucalcin increases superoxide dismutase activity in rat liver cytosol.* Biol Pharm Bull, 2004. **27**(9): p. 1444-6.
183. Yamaguchi, M., H. Takahashi, and Y. Tsurusaki, *Suppressive role of endogenous regucalcin in the enhancement of nitric oxide synthase activity in liver cytosol of normal and regucalcin transgenic rats.* J Cell Biochem, 2003. **88**(6): p. 1226-34.
184. Handa, S., N. Maruyama, and A. Ishigami, *Over-expression of Senescence Marker Protein-30 decreases reactive oxygen species in human hepatic carcinoma Hep G2 cells.* Biol Pharm Bull, 2009. **32**(10): p. 1645-8.
185. Sato, T., et al., *Senescence marker protein-30 protects mice lungs from oxidative stress, aging, and smoking.* Am J Respir Crit Care Med, 2006. **174**(5): p. 530-7.
186. Son, T.G., et al., *SMP30 deficiency causes increased oxidative stress in brain.* Mech Ageing Dev, 2006. **127**(5): p. 451-7.
187. Kondo, Y., et al., *Vitamin C depletion increases superoxide generation in brains of SMP30/GNL knockout mice.* Biochem Biophys Res Commun, 2008. **377**(1): p. 291-6.
188. Yamaguchi, M. and T. Sakurai, *Inhibitory effect of calcium-binding protein regucalcin on Ca2(+)-activated DNA fragmentation in rat liver nuclei.* FEBS Lett, 1991. **279**(2): p. 281-4.
189. Izumi, T. and M. Yamaguchi, *Overexpression of regucalcin suppresses cell death in cloned rat hepatoma H4-II-E cells induced by tumor necrosis factor-alpha or thapsigargin.* J Cell Biochem, 2004. **92**(2): p. 296-306.
190. Izumi, T. and M. Yamaguchi, *Overexpression of regucalcin suppresses cell death and apoptosis in cloned rat hepatoma H4-II-E cells induced by lipopolysaccharide, PD 98059, dibucaine, or Bay K 8644.* J Cell Biochem, 2004. **93**(3): p. 598-608.
191. Matsuyama, S., et al., *Senescence marker protein-30 regulates Akt activity and contributes to cell survival in Hep G2 cells.* Biochem Biophys Res Commun, 2004. **321**(2): p. 386-90.

192. Ishigami, A., et al., *Senescence marker protein-30 knockout mouse liver is highly susceptible to tumor necrosis factor-alpha- and Fas-mediated apoptosis.* Am J Pathol, 2002. **161**(4): p. 1273-81.
193. Kondo, Y., et al., *Senescence marker protein 30 functions as gluconolactonase in L-ascorbic acid biosynthesis, and its knockout mice are prone to scurvy.* Proc Natl Acad Sci U S A, 2006. **103**(15): p. 5723-8.
194. Ding, W.X. and X.M. Yin, *Dissection of the multiple mechanisms of TNF-alpha-induced apoptosis in liver injury.* J Cell Mol Med, 2004. **8**(4): p. 445-54.
195. Yoon, J.H. and G.J. Gores, *Death receptor-mediated apoptosis and the liver.* J Hepatol, 2002. **37**(3): p. 400-10.
196. Akazawa, Y. and G.J. Gores, *Death receptor-mediated liver injury.* Semin Liver Dis, 2007. **27**(4): p. 327-38.
197. Nagata, S., *Apoptosis by death factor.* Cell, 1997. **88**(3): p. 355-65.
198. Yin, X.M. and W.X. Ding, *Death receptor activation-induced hepatocyte apoptosis and liver injury.* Curr Mol Med, 2003. **3**(6): p. 491-508.
199. Galle, P.R. and P.H. Krammer, *CD95-induced apoptosis in human liver disease.* Semin Liver Dis, 1998. **18**(2): p. 141-51.
200. Canbay, A., et al., *Fas enhances fibrogenesis in the bile duct ligated mouse: a link between apoptosis and fibrosis.* Gastroenterology, 2002. **123**(4): p. 1323-30.
201. Faouzi, S., et al., *Anti-Fas induces hepatic chemokines and promotes inflammation by an NF-kappa B-independent, caspase-3-dependent pathway.* J Biol Chem, 2001. **276**(52): p. 49077-82.
202. Galle, P.R., et al., *Involvement of the CD95 (APO-1/Fas) receptor and ligand in liver damage.* J Exp Med, 1995. **182**(5): p. 1223-30.
203. Ribeiro, P.S., et al., *Hepatocyte apoptosis, expression of death receptors, and activation of NF-kappaB in the liver of nonalcoholic and alcoholic steatohepatitis patients.* Am J Gastroenterol, 2004. **99**(9): p. 1708-17.
204. Rudiger, H.A. and P.A. Clavien, *Tumor necrosis factor alpha, but not Fas, mediates hepatocellular apoptosis in the murine ischemic liver.* Gastroenterology, 2002. **122**(1): p. 202-10.
205. Muto, Y., et al., *Enhanced tumour necrosis factor and interleukin-1 in fulminant hepatic failure.* Lancet, 1988. **2**(8602): p. 72-4.
206. McClain, C.J. and D.A. Cohen, *Increased tumor necrosis factor production by monocytes in alcoholic hepatitis.* Hepatology, 1989. **9**(3): p. 349-51.
207. Grove, J., et al., *Association of a tumor necrosis factor promoter polymorphism with susceptibility to alcoholic steatohepatitis.* Hepatology, 1997. **26**(1): p. 143-6.
208. Berke, G., *The CTL's kiss of death.* Cell, 1995. **81**(1): p. 9-12.
209. Ogasawara, J., et al., *Lethal effect of the anti-Fas antibody in mice.* Nature, 1993. **364**(6440): p. 806-9.
210. Peter, M.E. and P.H. Krammer, *The CD95(APO-1/Fas) DISC and beyond.* Cell Death Differ, 2003. **10**(1): p. 26-35.
211. Chinnaiyan, A.M., et al., *FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis.* Cell, 1995. **81**(4): p. 505-12.
212. Ashkenazi, A. and V.M. Dixit, *Death receptors: signaling and modulation.* Science, 1998. **281**(5381): p. 1305-8.

213. Boldin, M.P., et al., *A novel protein that interacts with the death domain of Fas/APO1 contains a sequence motif related to the death domain.* J Biol Chem, 1995. **270**(14): p. 7795-8.
214. Thorburn, A., *Death receptor-induced cell killing.* Cell Signal, 2004. **16**(2): p. 139-44.
215. Thornberry, N.A. and Y. Lazebnik, *Caspases: enemies within.* Science, 1998. **281**(5381): p. 1312-6.
216. Scaffidi, C., et al., *Two CD95 (APO-1/Fas) signaling pathways.* Embo J, 1998. **17**(6): p. 1675-87.
217. Luo, X., et al., *Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors.* Cell, 1998. **94**(4): p. 481-90.
218. Yin, X.M., et al., *Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis.* Nature, 1999. **400**(6747): p. 886-91.
219. de la Coste, A., et al., *Differential protective effects of Bcl-xL and Bcl-2 on apoptotic liver injury in transgenic mice.* Am J Physiol, 1999. **277**(3 Pt 1): p. G702-8.
220. Lacronique, V., et al., *Bcl-2 protects from lethal hepatic apoptosis induced by an anti-Fas antibody in mice.* Nat Med, 1996. **2**(1): p. 80-6.
221. Rodriguez, I., et al., *A bcl-2 transgene expressed in hepatocytes protects mice from fulminant liver destruction but not from rapid death induced by anti-Fas antibody injection.* J Exp Med, 1996. **183**(3): p. 1031-6.
222. Scaffidi, C., et al., *Differential modulation of apoptosis sensitivity in CD95 type I and type II cells.* J Biol Chem, 1999. **274**(32): p. 22532-8.
223. Tschopp, J., M. Irmler, and M. Thome, *Inhibition of fas death signals by FLIPs.* Curr Opin Immunol, 1998. **10**(5): p. 552-8.
224. Irmler, M., et al., *Inhibition of death receptor signals by cellular FLIP.* Nature, 1997. **388**(6638): p. 190-5.
225. Scaffidi, C., et al., *The role of c-FLIP in modulation of CD95-induced apoptosis.* J Biol Chem, 1999. **274**(3): p. 1541-8.
226. Deveraux, Q.L., et al., *X-linked IAP is a direct inhibitor of cell-death proteases.* Nature, 1997. **388**(6639): p. 300-4.
227. Daemen, M.A., et al., *Functional protection by acute phase proteins alpha(1)-acid glycoprotein and alpha(1)-antitrypsin against ischemia/reperfusion injury by preventing apoptosis and inflammation.* Circulation, 2000. **102**(12): p. 1420-6.
228. Zou, C., et al., *Lack of Fas antagonism by Met in human fatty liver disease.* Nat Med, 2007. **13**(9): p. 1078-85.
229. Huh, C.G., et al., *Hepatocyte growth factor/c-met signaling pathway is required for efficient liver regeneration and repair.* Proc Natl Acad Sci U S A, 2004. **101**(13): p. 4477-82.
230. Zhao, Y., et al., *Promotion of Fas-mediated apoptosis in Type II cells by high doses of hepatocyte growth factor bypasses the mitochondrial requirement.* J Cell Physiol, 2007. **213**(2): p. 556-63.
231. Smyth, L.A. and H.J. Brady, *cMet and Fas receptor interaction inhibits death-inducing signaling complex formation in endothelial cells.* Hypertension, 2005. **46**(1): p. 100-6.
232. Schwabe, R.F. and D.A. Brenner, *Mechanisms of Liver Injury. I. TNF-alpha-induced liver injury: role of IKK, JNK, and ROS pathways.* Am J Physiol Gastrointest Liver Physiol, 2006. **290**(4): p. G583-9.

233. Chen, G. and D.V. Goeddel, *TNF-R1 signaling: a beautiful pathway.* Science, 2002. **296**(5573): p. 1634-5.
234. Hsu, H., et al., *TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways.* Cell, 1996. **84**(2): p. 299-308.
235. Hsu, H., J. Xiong, and D.V. Goeddel, *The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation.* Cell, 1995. **81**(4): p. 495-504.
236. Micheau, O. and J. Tschopp, *Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes.* Cell, 2003. **114**(2): p. 181-90.
237. Wei, M.C., et al., *Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death.* Science, 2001. **292**(5517): p. 727-30.
238. Chen, X., et al., *Bid-independent mitochondrial activation in tumor necrosis factor alpha-induced apoptosis and liver injury.* Mol Cell Biol, 2007. **27**(2): p. 541-53.
239. Harper, N., et al., *Fas-associated death domain protein and caspase-8 are not recruited to the tumor necrosis factor receptor 1 signaling complex during tumor necrosis factor-induced apoptosis.* J Biol Chem, 2003. **278**(28): p. 25534-41.
240. Stennicke, H.R., et al., *Pro-caspase-3 is a major physiologic target of caspase-8.* J Biol Chem, 1998. **273**(42): p. 27084-90.
241. Chen, L.F. and W.C. Greene, *Shaping the nuclear action of NF-kappaB.* Nat Rev Mol Cell Biol, 2004. **5**(5): p. 392-401.
242. Wajant, H., K. Pfizenmaier, and P. Scheurich, *Tumor necrosis factor signaling.* Cell Death Differ, 2003. **10**(1): p. 45-65.
243. Bellas, R.E., et al., *Inhibition of NF-kappa B activity induces apoptosis in murine hepatocytes.* Am J Pathol, 1997. **151**(4): p. 891-6.
244. Chen, S., et al., *Suppression of tumor necrosis factor-mediated apoptosis by nuclear factor kappaB-independent bone morphogenetic protein/Smad signaling.* J Biol Chem, 2001. **276**(42): p. 39259-63.
245. Plumpe, J., et al., *NF-kappaB determines between apoptosis and proliferation in hepatocytes during liver regeneration.* Am J Physiol Gastrointest Liver Physiol, 2000. **278**(1): p. G173-83.
246. Tacke, F., T. Luedde, and C. Trautwein, *Inflammatory pathways in liver homeostasis and liver injury.* Clin Rev Allergy Immunol, 2009. **36**(1): p. 4-12.
247. Devin, A., et al., *The distinct roles of TRAF2 and RIP in IKK activation by TNF-R1: TRAF2 recruits IKK to TNF-R1 while RIP mediates IKK activation.* Immunity, 2000. **12**(4): p. 419-29.
248. Perkins, N.D., *The Rel/NF-kappa B family: friend and foe.* Trends Biochem Sci, 2000. **25**(9): p. 434-40.
249. Zhong, H., et al., *The transcriptional activity of NF-kappaB is regulated by the IkappaB-associated PKAc subunit through a cyclic AMP-independent mechanism.* Cell, 1997. **89**(3): p. 413-24.
250. Wang, D., et al., *Tumor necrosis factor alpha-induced phosphorylation of RelA/p65 on Ser529 is controlled by casein kinase II.* J Biol Chem, 2000. **275**(42): p. 32592-7.
251. Leitges, M., et al., *Targeted disruption of the zetaPKC gene results in the impairment of the NF-kappaB pathway.* Mol Cell, 2001. **8**(4): p. 771-80.
252. Hoeflich, K.P., et al., *Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation.* Nature, 2000. **406**(6791): p. 86-90.

253. Wang, C.Y., et al., *NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation.* Science, 1998. **281**(5383): p. 1680-3.
254. Karin, M. and A. Lin, *NF-kappaB at the crossroads of life and death.* Nat Immunol, 2002. **3**(3): p. 221-7.
255. Ghosh, S. and M. Karin, *Missing pieces in the NF-kappaB puzzle.* Cell, 2002. **109 Suppl**: p. S81-96.
256. Lehmann, V., M.A. Freudenberg, and C. Galanos, *Lethal toxicity of lipopolysaccharide and tumor necrosis factor in normal and D-galactosamine-treated mice.* J Exp Med, 1987. **165**(3): p. 657-63.
257. Shiratori, Y., et al., *Role of endotoxin-responsive macrophages in hepatic injury.* Hepatology, 1990. **11**(2): p. 183-92.
258. Morikawa, A., et al., *Apoptotic cell death in the response of D-galactosamine-sensitized mice to lipopolysaccharide as an experimental endotoxic shock model.* Infect Immun, 1996. **64**(3): p. 734-8.
259. Decker, K. and D. Keppler, *Galactosamine hepatitis: key role of the nucleotide deficiency period in the pathogenesis of cell injury and cell death.* Rev Physiol Biochem Pharmacol, 1974(71): p. 77-106.
260. Galanos, C., M.A. Freudenberg, and W. Reutter, *Galactosamine-induced sensitization to the lethal effects of endotoxin.* Proc Natl Acad Sci U S A, 1979. **76**(11): p. 5939-43.
261. Leist, M., et al., *Murine hepatocyte apoptosis induced in vitro and in vivo by TNF-alpha requires transcriptional arrest.* J Immunol, 1994. **153**(4): p. 1778-88.
262. Shou, J., et al., *Human Dkk-1, a gene encoding a Wnt antagonist, responds to DNA damage and its overexpression sensitizes brain tumor cells to apoptosis following alkylation damage of DNA.* Oncogene, 2002. **21**(6): p. 878-89.
263. Chen, S., et al., *Wnt-1 signaling inhibits apoptosis by activating beta-catenin/T cell factor-mediated transcription.* J Cell Biol, 2001. **152**(1): p. 87-96.
264. Myung, S.J., et al., *Wnt signaling enhances the activation and survival of human hepatic stellate cells.* FEBS Lett, 2007. **581**(16): p. 2954-8.
265. Wang, Z., et al., *Beta-catenin promotes survival of renal epithelial cells by inhibiting Bax.* J Am Soc Nephrol, 2009. **20**(9): p. 1919-28.
266. Deng, J., et al., *beta-catenin interacts with and inhibits NF-kappa B in human colon and breast cancer.* Cancer Cell, 2002. **2**(4): p. 323-34.
267. Du, Q., et al., *Wnt/beta-catenin signaling regulates cytokine-induced human inducible nitric oxide synthase expression by inhibiting nuclear factor-kappaB activation in cancer cells.* Cancer Res, 2009. **69**(9): p. 3764-71.
268. Strom, S.C., J.R. Chowdhury, and I.J. Fox, *Hepatocyte transplantation for the treatment of human disease.* Semin Liver Dis, 1999. **19**(1): p. 39-48.
269. Grossman, M., et al., *Successful ex vivo gene therapy directed to liver in a patient with familial hypercholesterolaemia.* Nat Genet, 1994. **6**(4): p. 335-41.
270. Mito, M., M. Kusano, and Y. Kawaura, *Hepatocyte transplantation in man.* Transplant Proc, 1992. **24**(6): p. 3052-3.
271. Kusano, M. and M. Mito, *Observations on the fine structure of long-survived isolated hepatocytes inoculated into rat spleen.* Gastroenterology, 1982. **82**(4): p. 616-28.
272. Mito, M., et al., *Morphology and function of isolated hepatocytes transplanted into rat spleen.* Transplantation, 1979. **28**(6): p. 499-505.

273. Sokal, E.M., *Liver transplantation for inborn errors of liver metabolism.* J Inherit Metab Dis, 2006. **29**(2-3): p. 426-30.
274. Fox, I.J., et al., *Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation.* N Engl J Med, 1998. **338**(20): p. 1422-6.
275. Strom, S.C., et al., *Hepatocyte transplantation as a bridge to orthotopic liver transplantation in terminal liver failure.* Transplantation, 1997. **63**(4): p. 559-69.
276. Dhawan, A., et al., *Hepatocyte transplantation for inherited factor VII deficiency.* Transplantation, 2004. **78**(12): p. 1812-4.
277. Stephenne, X., et al., *Cryopreserved liver cell transplantation controls ornithine transcarbamylase deficient patient while awaiting liver transplantation.* Am J Transplant, 2005. **5**(8): p. 2058-61.
278. Muraca, M., et al., *Hepatocyte transplantation as a treatment for glycogen storage disease type 1a.* Lancet, 2002. **359**(9303): p. 317-8.
279. Habibullah, C.M., et al., *Human fetal hepatocyte transplantation in patients with fulminant hepatic failure.* Transplantation, 1994. **58**(8): p. 951-2.
280. Bilir, B.M., et al., *Hepatocyte transplantation in acute liver failure.* Liver Transpl, 2000. **6**(1): p. 32-40.
281. Soriano, H.E., et al., *Hepatocellular transplantation (HCT) in children with fulminant liver failure(FLF).* . Hepatology 1997. **26**: p. 239A(443).
282. Asahina, K., K. Teramoto, and H. Teraoka, *Embryonic stem cells: hepatic differentiation and regenerative medicine for the treatment of liver disease.* Curr Stem Cell Res Ther, 2006. **1**(2): p. 139-56.
283. Lysy, P.A., et al., *Stem cells for liver tissue repair: current knowledge and perspectives.* World J Gastroenterol, 2008. **14**(6): p. 864-75.
284. Sharma, A.D., et al., *The role of stem cells in physiology, pathophysiology, and therapy of the liver.* Stem Cell Rev, 2006. **2**(1): p. 51-8.
285. Si-Tayeb, K., et al., *Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells.* Hepatology. **51**(1): p. 297-305.
286. Shiraki, N., et al., *Differentiation of mouse and human embryonic stem cells into hepatic lineages.* Genes Cells, 2008.
287. Drobinskaya, I., et al., *Scalable Selection of Hepatocyte- and Hepatocyte Precursor-like Cells from Culture of Differentiating Transgenically Modified Murine ES Cells.* Stem Cells, 2008.
288. Jochheim, A., et al., *Quantitative gene expression profiling reveals a fetal hepatic phenotype of murine ES-derived hepatocytes.* Int J Dev Biol, 2004. **48**(1): p. 23-9.
289. Chinzei, R., et al., *Embryoid-body cells derived from a mouse embryonic stem cell line show differentiation into functional hepatocytes.* Hepatology, 2002. **36**(1): p. 22-9.
290. Yamada, T., et al., *In vitro differentiation of embryonic stem cells into hepatocyte-like cells identified by cellular uptake of indocyanine green.* Stem Cells, 2002. **20**(2): p. 146-54.
291. Tsutsui, M., et al., *Characterization of cytochrome P450 expression in murine embryonic stem cell-derived hepatic tissue system.* Drug Metab Dispos, 2006. **34**(4): p. 696-701.
292. Soto-Gutierrez, A., et al., *Reversal of mouse hepatic failure using an implanted liver-assist device containing ES cell-derived hepatocytes.* Nat Biotechnol, 2006. **24**(11): p. 1412-9.

293. Sharma, A.D., et al., *Human cord blood stem cells generate human cytokeratin 18-negative hepatocyte-like cells in injured mouse liver.* Am J Pathol, 2005. **167**(2): p. 555-64.
294. Lagasse, E., et al., *Purified hematopoietic stem cells can differentiate into hepatocytes in vivo.* Nat Med, 2000. **6**(11): p. 1229-34.
295. Ishikawa, F., et al., *Transplanted human cord blood cells give rise to hepatocytes in engrafted mice.* Ann N Y Acad Sci, 2003. **996**: p. 174-85.
296. Sandhu, J.S., et al., *Stem cell properties and repopulation of the rat liver by fetal liver epithelial progenitor cells.* Am J Pathol, 2001. **159**(4): p. 1323-34.
297. Wang, X., et al., *The origin and liver repopulating capacity of murine oval cells.* Proc Natl Acad Sci U S A, 2003. **100 Suppl 1**: p. 11881-8.
298. Monga, S.P., et al., *Expansion of hepatic and hematopoietic stem cells utilizing mouse embryonic liver explants.* Cell Transplant, 2001. **10**(1): p. 81-9.
299. Oertel, M., et al., *Repopulation of rat liver by fetal hepatoblasts and adult hepatocytes transduced ex vivo with lentiviral vectors.* Hepatology, 2003. **37**(5): p. 994-1005.
300. Sharma, A.D., et al., *Murine embryonic stem cell-derived hepatic progenitor cells engraft in recipient livers with limited capacity of liver tissue formation.* Cell Transplant, 2008. **17**(3): p. 313-23.
301. Teramoto, K., et al., *Teratoma formation and hepatocyte differentiation in mouse liver transplanted with mouse embryonic stem cell-derived embryoid bodies.* Transplant Proc, 2005. **37**(1): p. 285-6.
302. Kobayashi, N., et al., *Partial hepatectomy and subsequent radiation facilitates engraftment of mouse embryonic stem cells in the liver.* Transplant Proc, 2004. **36**(8): p. 2352-4.
303. Schumacher, I.K., et al., *Transplantation of conditionally immortalized hepatocytes to treat hepatic encephalopathy.* Hepatology, 1996. **24**(2): p. 337-43.
304. Baba, Y., K.P. Garrett, and P.W. Kincade, *Constitutively active beta-catenin confers multilineage differentiation potential on lymphoid and myeloid progenitors.* Immunity, 2005. **23**(6): p. 599-609.
305. Baba, Y., et al., *Constitutively active beta-catenin promotes expansion of multipotent hematopoietic progenitors in culture.* J Immunol, 2006. **177**(4): p. 2294-303.
306. Simon, M., et al., *Constitutive activation of the Wnt/beta-catenin signalling pathway in acute myeloid leukaemia.* Oncogene, 2005. **24**(14): p. 2410-20.
307. Reya, T., et al., *A role for Wnt signalling in self-renewal of haematopoietic stem cells.* Nature, 2003. **423**(6938): p. 409-14.
308. Natori, S., et al., *Hepatocyte apoptosis is a pathologic feature of human alcoholic hepatitis.* J Hepatol, 2001. **34**(2): p. 248-53.
309. Kurose, I., et al., *Oxidative stress-mediated apoptosis of hepatocytes exposed to acute ethanol intoxication.* Hepatology, 1997. **25**(2): p. 368-78.
310. Zhou, Z., X. Sun, and Y.J. Kang, *Ethanol-induced apoptosis in mouse liver: Fas- and cytochrome c-mediated caspase-3 activation pathway.* Am J Pathol, 2001. **159**(1): p. 329-38.
311. Feldstein, A.E., et al., *Hepatocyte apoptosis and fas expression are prominent features of human nonalcoholic steatohepatitis.* Gastroenterology, 2003. **125**(2): p. 437-43.
312. Faubion, W.A., et al., *Toxic bile salts induce rodent hepatocyte apoptosis via direct activation of Fas.* J Clin Invest, 1999. **103**(1): p. 137-45.

313. Strand, S., et al., *Hepatic failure and liver cell damage in acute Wilson's disease involve CD95 (APO-1/Fas) mediated apoptosis.* Nat Med, 1998. **4**(5): p. 588-93.
314. Kountouras, J., C. Zavos, and D. Chatzopoulos, *Apoptosis in hepatitis C.* J Viral Hepat, 2003. **10**(5): p. 335-42.
315. Mochizuki, K., et al., *Fas antigen expression in liver tissues of patients with chronic hepatitis B.* J Hepatol, 1996. **24**(1): p. 1-7.
316. Hiramatsu, N., et al., *Immunohistochemical detection of Fas antigen in liver tissue of patients with chronic hepatitis C.* Hepatology, 1994. **19**(6): p. 1354-9.
317. Ramalho, R.M., et al., *Apoptosis and Bcl-2 expression in the livers of patients with steatohepatitis.* Eur J Gastroenterol Hepatol, 2006. **18**(1): p. 21-9.
318. Bird, G.L., et al., *Increased plasma tumor necrosis factor in severe alcoholic hepatitis.* Ann Intern Med, 1990. **112**(12): p. 917-20.
319. Colell, A., et al., *Selective glutathione depletion of mitochondria by ethanol sensitizes hepatocytes to tumor necrosis factor.* Gastroenterology, 1998. **115**(6): p. 1541-51.
320. Marinos, G., et al., *Tumor necrosis factor receptors in patients with chronic hepatitis B virus infection.* Gastroenterology, 1995. **108**(5): p. 1453-63.
321. Kallinowski, B., et al., *Induction of tumour necrosis factor (TNF) receptor type p55 and p75 in patients with chronic hepatitis C virus (HCV) infection.* Clin Exp Immunol, 1998. **111**(2): p. 269-77.
322. Fang, J.W., et al., *Activation of the tumor necrosis factor-alpha system in the liver in chronic hepatitis B virus infection.* Am J Gastroenterol, 1996. **91**(4): p. 748-53.
323. Tokushige, K., et al., *Significance of soluble TNF receptor-I in acute-type fulminant hepatitis.* Am J Gastroenterol, 2000. **95**(8): p. 2040-6.
324. Malhi, H., M.E. Guicciardi, and G.J. Gores, *Hepatocyte death: a clear and present danger.* Physiol Rev. **90**(3): p. 1165-94.
325. Spahr, L., et al., *Combination of steroids with infliximab or placebo in severe alcoholic hepatitis: a randomized controlled pilot study.* J Hepatol, 2002. **37**(4): p. 448-55.
326. Masuoka, H.C., M.E. Guicciardi, and G.J. Gores, *Caspase inhibitors for the treatment of hepatitis C.* Clin Liver Dis, 2009. **13**(3): p. 467-75.
327. Pockros, P.J., et al., *Oral IDN-6556, an antiapoptotic caspase inhibitor, may lower aminotransferase activity in patients with chronic hepatitis C.* Hepatology, 2007. **46**(2): p. 324-9.
328. Valentino, K.L., et al., *First clinical trial of a novel caspase inhibitor: anti-apoptotic caspase inhibitor, IDN-6556, improves liver enzymes.* Int J Clin Pharmacol Ther, 2003. **41**(10): p. 441-9.
329. Kumar, D. and R.K. Tandon, *Use of ursodeoxycholic acid in liver diseases.* J Gastroenterol Hepatol, 2001. **16**(1): p. 3-14.
330. Rodrigues, C.M. and C.J. Steer, *The therapeutic effects of ursodeoxycholic acid as an anti-apoptotic agent.* Expert Opin Investig Drugs, 2001. **10**(7): p. 1243-53.
331. Thompson, M.D. and S.P. Monga, *WNT/beta-catenin signaling in liver health and disease.* Hepatology, 2007. **45**(5): p. 1298-305.
332. Burke, Z.D., et al., *Liver zonation occurs through a beta-catenin-dependent, c-Myc-independent mechanism.* Gastroenterology, 2009. **136**(7): p. 2316-2324 e1-3.
333. Aberle, H., et al., *beta-catenin is a target for the ubiquitin-proteasome pathway.* Embo J, 1997. **16**(13): p. 3797-804.

334. Yokoyama, H.O., et al., *Regeneration of mouse liver after partial hepatectomy.* Cancer Res, 1953. **13**(1): p. 80-5.
335. Kaimori, A., et al., *Transforming growth factor-beta1 induces an epithelial-to-mesenchymal transition state in mouse hepatocytes in vitro.* J Biol Chem, 2007. **282**(30): p. 22089-101.
336. Seglen, P.O., *Preparation of isolated rat liver cells.* Methods Cell Biol, 1976. **13**: p. 29-83.
337. Block, G.D., et al., *Population expansion, clonal growth, and specific differentiation patterns in primary cultures of hepatocytes induced by HGF/SF, EGF and TGF alpha in a chemically defined (HGM) medium.* J Cell Biol, 1996. **132**(6): p. 1133-49.
338. Yang, J., et al., *Sustained expression of naked plasmid DNA encoding hepatocyte growth factor in mice promotes liver and overall body growth.* Hepatology, 2001. **33**(4): p. 848-59.
339. Cieply, B., et al., *Unique phenotype of hepatocellular cancers with exon-3 mutations in beta-catenin gene.* Hepatology, 2008.
340. Miyazaki, M., et al., *Phenobarbital suppresses growth and accelerates restoration of differentiation markers of primary culture rat hepatocytes in the chemically defined hepatocyte growth medium containing hepatocyte growth factor and epidermal growth factor.* Exp Cell Res, 1998. **241**(2): p. 445-57.
341. Pediaditakis, P., et al., *Differential mitogenic effects of single chain hepatocyte growth factor (HGF)/scatter factor and HGF/NK1 following cleavage by factor Xa.* J Biol Chem, 2002. **277**(16): p. 14109-15.
342. Hansen, M.B., S.E. Nielsen, and K. Berg, *Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill.* J Immunol Methods, 1989. **119**(2): p. 203-10.
343. Fausto, N., *Liver regeneration.* J Hepatol, 2000. **32**(1 Suppl): p. 19-31.
344. Ku, N.O., et al., *Keratin binding to 14-3-3 proteins modulates keratin filaments and hepatocyte mitotic progression.* Proc Natl Acad Sci U S A, 2002. **99**(7): p. 4373-8.
345. Boulikas, T., *Control of DNA replication by protein phosphorylation.* Anticancer Res, 1994. **14**(6B): p. 2465-72.
346. Wang, X., et al., *Increased levels of forkhead box M1B transcription factor in transgenic mouse hepatocytes prevent age-related proliferation defects in regenerating liver.* Proc Natl Acad Sci U S A, 2001. **98**(20): p. 11468-73.
347. Legoix, P., et al., *Beta-catenin mutations in hepatocellular carcinoma correlate with a low rate of loss of heterozygosity.* Oncogene, 1999. **18**(27): p. 4044-6.
348. Yan, H.X., et al., *Protein-tyrosine phosphatase PCP-2 inhibits beta-catenin signaling and increases E-cadherin-dependent cell adhesion.* J Biol Chem, 2006. **281**(22): p. 15423-33.
349. Bonvini, P., et al., *Geldanamycin abrogates ErbB2 association with proteasome-resistant beta-catenin in melanoma cells, increases beta-catenin-E-cadherin association, and decreases beta-catenin-sensitive transcription.* Cancer Res, 2001. **61**(4): p. 1671-7.
350. Goessling, W., et al., *APC mutant zebrafish uncover a changing temporal requirement for wnt signaling in liver development.* Dev Biol, 2008. **320**(1): p. 161-74.
351. Apte, U., et al., *Beta-catenin activation promotes liver regeneration after acetaminophen-induced injury.* Am J Pathol, 2009. **175**(3): p. 1056-65.

352. Albrecht, J.H. and L.K. Hansen, *Cyclin D1 promotes mitogen-independent cell cycle progression in hepatocytes.* Cell Growth Differ, 1999. **10**(6): p. 397-404.
353. Bioulac-Sage, P., et al., *Hepatocellular adenoma: what is new in 2008.* Hepatol Int, 2008. **2**(3): p. 316-21.
354. Apte, U., et al., *Wnt/beta-catenin signaling mediates oval cell response in rodents.* Hepatology, 2007.
355. Hsu, S.C., J. Galceran, and R. Grosschedl, *Modulation of transcriptional regulation by LEF-1 in response to Wnt-1 signaling and association with beta-catenin.* Mol Cell Biol, 1998. **18**(8): p. 4807-18.
356. Young, C.S., et al., *Wnt-1 induces growth, cytosolic beta-catenin, and Tcf/Lef transcriptional activation in Rat-1 fibroblasts.* Mol Cell Biol, 1998. **18**(5): p. 2474-85.
357. Young, C.S., T.N. Masckauchan, and J. Kitajewski, *Beta-catenin/Tcf activation partially mimics the transforming activity of Wnt-1 in Rat-1 fibroblasts.* Differentiation, 2003. **71**(8): p. 477-85.
358. Haegel, H., et al., *Lack of beta-catenin affects mouse development at gastrulation.* Development, 1995. **121**(11): p. 3529-37.
359. Hugh, T.J., et al., *beta-catenin expression in primary and metastatic colorectal carcinoma.* Int J Cancer, 1999. **82**(4): p. 504-11.
360. Jaiswal, A.S., C.H. Kennedy, and S. Narayan, *A correlation of APC and c-myc mRNA levels in lung cancer cell lines.* Oncol Rep, 1999. **6**(6): p. 1253-6.
361. Rimm, D.L., et al., *Frequent nuclear/cytoplasmic localization of beta-catenin without exon 3 mutations in malignant melanoma.* Am J Pathol, 1999. **154**(2): p. 325-9.
362. Smalley, M.J. and T.C. Dale, *Wnt signaling and mammary tumorigenesis.* J Mammary Gland Biol Neoplasia, 2001. **6**(1): p. 37-52.
363. Morin, P.J., *beta-catenin signaling and cancer.* Bioessays, 1999. **21**(12): p. 1021-30.
364. Tien, L.T., et al., *Expression of beta-catenin in hepatocellular carcinoma.* World J Gastroenterol, 2005. **11**(16): p. 2398-401.
365. Devereux, T.R., et al., *Mutation of beta-catenin is an early event in chemically induced mouse hepatocellular carcinogenesis.* Oncogene, 1999. **18**(33): p. 4726-33.
366. de La Coste, A., et al., *Somatic mutations of the beta-catenin gene are frequent in mouse and human hepatocellular carcinomas.* Proc Natl Acad Sci U S A, 1998. **95**(15): p. 8847-51.
367. Wei, Y., et al., *Activation of beta-catenin in epithelial and mesenchymal hepatoblastomas.* Oncogene, 2000. **19**(4): p. 498-504.
368. Fujita, T., et al., *Hepatic and renal expression of senescence marker protein-30 and its biological significance.* J Gastroenterol Hepatol, 1998. **13 Suppl**: p. S124-31.
369. Yamaguchi, M. and S. Ueoka, *Expression of calcium-binding protein regucalcin mRNA in fetal rat liver is stimulated by calcium administration.* Mol Cell Biochem, 1998. **178**(1-2): p. 283-7.
370. Shinya, N. and M. Yamaguchi, *Stimulatory effect of calcium administration on regucalcin mRNA expression is attenuated in the kidney cortex of rats ingested with saline.* Mol Cell Biochem, 1998. **178**(1-2): p. 275-81.
371. Murata, T. and M. Yamaguchi, *Molecular cloning of the cDNA coding for regucalcin and its mRNA expression in mouse liver: the expression is stimulated by calcium administration.* Mol Cell Biochem, 1997. **173**(1-2): p. 127-33.

372. Tsurusaki, Y. and M. Yamaguchi, *Role of regucalcin in liver nuclear function: binding of regucalcin to nuclear protein or DNA and modulation of tumor-related gene expression.* Int J Mol Med, 2004. **14**(2): p. 277-81.
373. Tsurusaki, Y. and M. Yamaguchi, *Suppressive role of endogenous regucalcin in the enhancement of deoxyribonucleic acid synthesis activity in the nucleus of regenerating rat liver.* J Cell Biochem, 2002. **85**(3): p. 516-22.
374. Tsurusaki, Y. and M. Yamaguchi, *Role of endogenous regucalcin in nuclear regulation of regenerating rat liver: suppression of the enhanced ribonucleic acid synthesis activity.* J Cell Biochem, 2002. **87**(4): p. 450-7.
375. Izumi, T., Y. Tsurusaki, and M. Yamaguchi, *Suppressive effect of endogenous regucalcin on nitric oxide synthase activity in cloned rat hepatoma H4-II-E cells overexpressing regucalcin.* J Cell Biochem, 2003. **89**(4): p. 800-7.
376. Fukaya, Y. and M. Yamaguchi, *Characterization of protein tyrosine phosphatase activity in rat liver microsomes: suppressive effect of endogenous regucalcin in transgenic rats.* Int J Mol Med, 2004. **14**(3): p. 427-32.
377. Inagaki, S., H. Misawa, and M. Yamaguchi, *Role of endogenous regucalcin in protein tyrosine phosphatase regulation in the cloned rat hepatoma cells (H4-II-E).* Mol Cell Biochem, 2000. **213**(1-2): p. 43-50.
378. Makino, R. and M. Yamaguchi, *Expression of calcium-binding protein regucalcin mRNA in hepatoma cells.* Mol Cell Biochem, 1996. **155**(1): p. 85-90.
379. Murata, T., N. Shinya, and M. Yamaguchi, *Expression of calcium-binding protein regucalcin mRNA in the cloned human hepatoma cells (HepG2): stimulation by insulin.* Mol Cell Biochem, 1997. **175**(1-2): p. 163-8.
380. Misawa, H. and M. Yamaguchi, *Transcript heterogeneity of the human gene for Ca2+-binding protein regucalcin.* Int J Mol Med, 2000. **5**(3): p. 283-7.
381. Linster, C.L. and E. Van Schaftingen, *Vitamin C. Biosynthesis, recycling and degradation in mammals.* Febs J, 2007. **274**(1): p. 1-22.
382. Majer, B.J., et al., *Genotoxic effects of dietary and lifestyle related carcinogens in human derived hepatoma (HepG2, Hep3B) cells.* Mutat Res, 2004. **551**(1-2): p. 153-66.
383. Zeng, G., et al., *Wnt'er in liver: expression of Wnt and frizzled genes in mouse.* Hepatology, 2007. **45**(1): p. 195-204.
384. Kohler, C., et al., *Expression of Notch-1 and its ligand Jagged-1 in rat liver during liver regeneration.* Hepatology, 2004. **39**(4): p. 1056-65.
385. Fleige, S., et al., *Comparison of relative mRNA quantification models and the impact of RNA integrity in quantitative real-time RT-PCR.* Biotechnol Lett, 2006. **28**(19): p. 1601-13.
386. Zeng, G., et al., *Aberrant Wnt/beta-catenin signaling in pancreatic adenocarcinoma.* Neoplasia, 2006. **8**(4): p. 279-89.
387. Michalopoulos, G.K., et al., *Histological organization in hepatocyte organoid cultures.* Am J Pathol, 2001. **159**(5): p. 1877-87.
388. Bell, A.W. and G.K. Michalopoulos, *Phenobarbital regulates nuclear expression of HNF-4alpha in mouse and rat hepatocytes independent of CAR and PXR.* Hepatology, 2006. **44**(1): p. 186-94.
389. Barroso, M.P., et al., *Ascorbate and alpha-tocopherol prevent apoptosis induced by serum removal independent of Bcl-2.* Arch Biochem Biophys, 1997. **343**(2): p. 243-8.

390. Bajt, M.L., et al., *Acetaminophen-induced oxidant stress and cell injury in cultured mouse hepatocytes: protection by N-acetyl cysteine.* Toxicol Sci, 2004. **80**(2): p. 343-9.
391. Postic, C. and M.A. Magnuson, *DNA excision in liver by an albumin-Cre transgene occurs progressively with age.* Genesis, 2000. **26**(2): p. 149-50.
392. Nejak-Bowen, K. and S.P. Monga, *Wnt/beta-catenin signaling in hepatic organogenesis.* Organogenesis, 2008. **4**(2).
393. Hanada, S., et al., *The genetic background modulates susceptibility to mouse liver Mallory-Denk body formation and liver injury.* Hepatology, 2008. **48**(3): p. 943-52.
394. Jho, E.H., et al., *Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway.* Mol Cell Biol, 2002. **22**(4): p. 1172-83.
395. Niida, A., et al., *DKK1, a negative regulator of Wnt signaling, is a target of the beta-catenin/TCF pathway.* Oncogene, 2004. **23**(52): p. 8520-6.
396. Behari, J., et al., *Liver-specific beta-catenin knockout mice exhibit defective bile acid and cholesterol homeostasis and increased susceptibility to diet-induced steatohepatitis.* Am J Pathol. **176**(2): p. 744-53.
397. Zhang, X.F., et al., *Conditional beta-catenin loss in mice promotes chemical hepatocarcinogenesis: role of oxidative stress and platelet-derived growth factor receptor alpha/phosphoinositide 3-kinase signaling.* Hepatology. **52**(3): p. 954-65.
398. Galanos, C., et al., *Hypersensitivity to endotoxin and mechanisms of host-response.* Prog Clin Biol Res, 1988. **272**: p. 295-308.
399. Leist, M., et al., *The 55-kD tumor necrosis factor receptor and CD95 independently signal murine hepatocyte apoptosis and subsequent liver failure.* Mol Med, 1996. **2**(1): p. 109-24.
400. Guicciardi, M.E. and G.J. Gores, *Life and death by death receptors.* Faseb J, 2009. **23**(6): p. 1625-37.
401. Van Antwerp, D.J., et al., *Suppression of TNF-alpha-induced apoptosis by NF-kappaB.* Science, 1996. **274**(5288): p. 787-9.
402. Beg, A.A. and D. Baltimore, *An essential role for NF-kappaB in preventing TNF-alpha-induced cell death.* Science, 1996. **274**(5288): p. 782-4.
403. Wallach, D., et al., *Tumor necrosis factor receptor and Fas signaling mechanisms.* Annu Rev Immunol, 1999. **17**: p. 331-67.
404. Kyriakis, J.M., *Life-or-death decisions.* Nature, 2001. **414**(6861): p. 265-6.
405. Zhao, Y., et al., *Activation of pro-death Bcl-2 family proteins and mitochondria apoptosis pathway in tumor necrosis factor-alpha-induced liver injury.* J Biol Chem, 2001. **276**(29): p. 27432-40.
406. Akahori, M., et al., *Nitric oxide ameliorates actinomycin D/endotoxin-induced apoptotic liver failure in mice.* J Surg Res, 1999. **85**(2): p. 286-93.
407. Prince, J.M., et al., *Toll-like receptor-4 signaling mediates hepatic injury and systemic inflammation in hemorrhagic shock.* J Am Coll Surg, 2006. **202**(3): p. 407-17.
408. Stock, P., et al., *Platelet-derived growth factor receptor-alpha: a novel therapeutic target in human hepatocellular cancer.* Mol Cancer Ther, 2007. **6**(7): p. 1932-41.
409. Pritchard, M.T., et al., *Early growth response-1 contributes to galactosamine/lipopolysaccharide-induced acute liver injury in mice.* Am J Physiol Gastrointest Liver Physiol, 2007. **293**(6): p. G1124-33.

410. Feng, B., et al., *Dynamic metabonomic analysis of BALB/c mice with different outcomes after D-galactosamine/lipopolysaccharide-induced fulminant hepatic failure.* Liver Transpl, 2008. **14**(11): p. 1620-31.
411. Josephs, M.D., et al., *Lipopolysaccharide and D-galactosamine-induced hepatic injury is mediated by TNF-alpha and not by Fas ligand.* Am J Physiol Regul Integr Comp Physiol, 2000. **278**(5): p. R1196-201.
412. Bradham, C.A., et al., *Mechanisms of hepatic toxicity. I. TNF-induced liver injury.* Am J Physiol, 1998. **275**(3 Pt 1): p. G387-92.
413. Chang, L., et al., *The E3 ubiquitin ligase itch couples JNK activation to TNFalpha-induced cell death by inducing c-FLIP(L) turnover.* Cell, 2006. **124**(3): p. 601-13.
414. Baeuerle, P.A. and T. Henkel, *Function and activation of NF-kappa B in the immune system.* Annu Rev Immunol, 1994. **12**: p. 141-79.
415. Blackwell, T.S., et al., *Multiorgan nuclear factor kappa B activation in a transgenic mouse model of systemic inflammation.* Am J Respir Crit Care Med, 2000. **162**(3 Pt 1): p. 1095-101.
416. Sakurai, H., et al., *IkappaB kinases phosphorylate NF-kappaB p65 subunit on serine 536 in the transactivation domain.* J Biol Chem, 1999. **274**(43): p. 30353-6.
417. Schwabe, R.F. and D.A. Brenner, *Role of glycogen synthase kinase-3 in TNF-alpha-induced NF-kappaB activation and apoptosis in hepatocytes.* Am J Physiol Gastrointest Liver Physiol, 2002. **283**(1): p. G204-11.
418. Chan, H., D.P. Bartos, and L.B. Owen-Schaub, *Activation-dependent transcriptional regulation of the human Fas promoter requires NF-kappaB p50-p65 recruitment.* Mol Cell Biol, 1999. **19**(3): p. 2098-108.
419. Wegenka, U.M., et al., *The interleukin-6-activated acute-phase response factor is antigenically and functionally related to members of the signal transducer and activator of transcription (STAT) family.* Mol Cell Biol, 1994. **14**(5): p. 3186-96.
420. Medzhitov, R., P. Preston-Hurlburt, and C.A. Janeway, Jr., *A human homologue of the Drosophila Toll protein signals activation of adaptive immunity.* Nature, 1997. **388**(6640): p. 394-7.
421. Janeway, C.A., Jr. and R. Medzhitov, *Innate immune recognition.* Annu Rev Immunol, 2002. **20**: p. 197-216.
422. Klein, I., et al., *Kupffer cell heterogeneity: functional properties of bone marrow derived and sessile hepatic macrophages.* Blood, 2007. **110**(12): p. 4077-85.
423. Jung, D.Y., et al., *TLR4, but not TLR2, signals autoregulatory apoptosis of cultured microglia: a critical role of IFN-beta as a decision maker.* J Immunol, 2005. **174**(10): p. 6467-76.
424. Wang, X., et al., *A mechanism of cell survival: sequestration of Fas by the HGF receptor Met.* Mol Cell, 2002. **9**(2): p. 411-21.
425. Michalopoulos, G.K., et al., *HGF-, EGF-, and dexamethasone-induced gene expression patterns during formation of tissue in hepatic organoid cultures.* Gene Expr, 2003. **11**(2): p. 55-75.
426. Li, G., et al., *Cyclooxygenase-2 prevents fas-induced liver injury through up-regulation of epidermal growth factor receptor.* Hepatology, 2009. **50**(3): p. 834-43.
427. Grimm, S., et al., *Bcl-2 down-regulates the activity of transcription factor NF-kappaB induced upon apoptosis.* J Cell Biol, 1996. **134**(1): p. 13-23.

428. Velasco, M., et al., *Rapid Up-regulation of IkappaBbeta and abrogation of NF-kappaB activity in peritoneal macrophages stimulated with lipopolysaccharide.* J Biol Chem, 1997. **272**(37): p. 23025-30.
429. Tapalaga, D., G. Tiegs, and S. Angermuller, *NFkappaB and caspase-3 activity in apoptotic hepatocytes of galactosamine-sensitized mice treated with TNFalpha.* J Histochem Cytochem, 2002. **50**(12): p. 1599-609.
430. Son, Y.H., et al., *Roles of MAPK and NF-kappaB in interleukin-6 induction by lipopolysaccharide in vascular smooth muscle cells.* J Cardiovasc Pharmacol, 2008. **51**(1): p. 71-7.
431. Steinhusen, U., et al., *Apoptosis-induced cleavage of beta-catenin by caspase-3 results in proteolytic fragments with reduced transactivation potential.* J Biol Chem, 2000. **275**(21): p. 16345-53.
432. Van de Craen, M., et al., *Proteolytic cleavage of beta-catenin by caspases: an in vitro analysis.* FEBS Lett, 1999. **458**(2): p. 167-70.
433. Tiegs, G., M. Wolter, and A. Wendel, *Tumor necrosis factor is a terminal mediator in galactosamine/endotoxin-induced hepatitis in mice.* Biochem Pharmacol, 1989. **38**(4): p. 627-31.
434. Clark, J.M., F.L. Brancati, and A.M. Diehl, *The prevalence and etiology of elevated aminotransferase levels in the United States.* Am J Gastroenterol, 2003. **98**(5): p. 960-7.
435. Linster, C.L. and E. Van Schaftingen, *Rapid stimulation of free glucuronate formation by non-glucuronidable xenobiotics in isolated rat hepatocytes.* J Biol Chem, 2003. **278**(38): p. 36328-33.
436. Chafey, P., et al., *Proteomic analysis of beta-catenin activation in mouse liver by DIGE analysis identifies glucose metabolism as a new target of the Wnt pathway.* Proteomics, 2009. **9**(15): p. 3889-900.
437. Bellas, R.E. and G.E. Sonenshein, *Nuclear factor kappaB cooperates with c-Myc in promoting murine hepatocyte survival in a manner independent of p53 tumor suppressor function.* Cell Growth Differ, 1999. **10**(5): p. 287-94.
438. Gomez-Quiroz, L.E., et al., *Hepatocyte-specific c-Met deletion disrupts redox homeostasis and sensitizes to Fas-mediated apoptosis.* J Biol Chem, 2008.
439. Bernard, D., et al., *The c-Rel transcription factor can both induce and inhibit apoptosis in the same cells via the upregulation of MnSOD.* Oncogene, 2002. **21**(28): p. 4392-402.
440. Hakem, R., et al., *Differential requirement for caspase 9 in apoptotic pathways in vivo.* Cell, 1998. **94**(3): p. 339-52.
441. Nejak-Bowen, K.N., et al., *Accelerated liver regeneration and hepatocarcinogenesis in mice overexpressing serine-45 mutant beta-catenin.* Hepatology. **51**(5): p. 1603-13.
442. Colnot, S., et al., *Liver-targeted disruption of Apc in mice activates beta-catenin signaling and leads to hepatocellular carcinomas.* Proc Natl Acad Sci U S A, 2004. **101**(49): p. 17216-21.
443. Qian, J., et al., *The APC tumor suppressor inhibits DNA replication by directly binding to DNA via its carboxyl terminus.* Gastroenterology, 2008. **135**(1): p. 152-62.
444. Prosperi, J.R., et al., *The APC tumor suppressor is required for epithelial integrity in the mouse mammary gland.* J Cell Physiol, 2009. **220**(2): p. 319-31.
445. Su, L.K., et al., *Biallelic inactivation of the APC gene is associated with hepatocellular carcinoma in familial adenomatous polyposis coli.* Cancer, 2001. **92**(2): p. 332-9.

446. Harada, N., et al., *Hepatocarcinogenesis in mice with beta-catenin and Ha-ras gene mutations.* Cancer Res, 2004. **64**(1): p. 48-54.
447. Sansom, O.J., et al., *Cyclin D1 is not an immediate target of beta-catenin following Apc loss in the intestine.* J Biol Chem, 2005. **280**(31): p. 28463-7.
448. Prange, W., et al., *Beta-catenin accumulation in the progression of human hepatocarcinogenesis correlates with loss of E-cadherin and accumulation of p53, but not with expression of conventional WNT-1 target genes.* J Pathol, 2003. **201**(2): p. 250-9.
449. Manisastry, S.M., M. Han, and K.K. Linask, *Early temporal-specific responses and differential sensitivity to lithium and Wnt-3A exposure during heart development.* Dev Dyn, 2006. **235**(8): p. 2160-74.
450. Yamaguchi, M., et al., *Potential role of regucalcin as a specific biochemical marker of chronic liver injury with carbon tetrachloride administration in rats.* Mol Cell Biochem, 2002. **241**(1-2): p. 61-7.
451. Elchuri, S., et al., *Identification of biomarkers associated with the development of hepatocellular carcinoma in CuZn superoxide dismutase deficient mice.* Proteomics, 2007. **7**(12): p. 2121-9.
452. Graveel, C.R., et al., *Expression profiling and identification of novel genes in hepatocellular carcinomas.* Oncogene, 2001. **20**(21): p. 2704-12.
453. Calvisi, D.F., et al., *Activation of beta-catenin provides proliferative and invasive advantages in c-myc/TGF-alpha hepatocarcinogenesis promoted by phenobarbital.* Carcinogenesis, 2004. **25**(6): p. 901-8.
454. Iida, M., et al., *Changes in global gene and protein expression during early mouse liver carcinogenesis induced by non-genotoxic model carcinogens oxazepam and Wyeth-14,643.* Carcinogenesis, 2003. **24**(4): p. 757-70.
455. Dihlmann, S., et al., *Regulation of AKT1 expression by beta-catenin/Tcf/Lef signaling in colorectal cancer cells.* Carcinogenesis, 2005. **26**(9): p. 1503-12.
456. Toualbi, K., et al., *Physical and functional cooperation between AP-1 and beta-catenin for the regulation of TCF-dependent genes.* Oncogene, 2006.
457. Beg, A.A., et al., *Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B.* Nature, 1995. **376**(6536): p. 167-70.
458. Doi, T.S., et al., *Absence of tumor necrosis factor rescues RelA-deficient mice from embryonic lethality.* Proc Natl Acad Sci U S A, 1999. **96**(6): p. 2994-9.
459. Rosenfeld, M.E., et al., *Prevention of hepatic apoptosis and embryonic lethality in RelA/TNFR-1 double knockout mice.* Am J Pathol, 2000. **156**(3): p. 997-1007.
460. Li, Q., et al., *Severe liver degeneration in mice lacking the IkappaB kinase 2 gene.* Science, 1999. **284**(5412): p. 321-5.
461. Rudolph, D., et al., *Severe liver degeneration and lack of NF-kappaB activation in NEMO/IKKgamma-deficient mice.* Genes Dev, 2000. **14**(7): p. 854-62.
462. Xu, Y., et al., *NF-kappaB inactivation converts a hepatocyte cell line TNF-alpha response from proliferation to apoptosis.* Am J Physiol, 1998. **275**(4 Pt 1): p. C1058-66.
463. Wullaert, A., et al., *Hepatic tumor necrosis factor signaling and nuclear factor-kappaB: effects on liver homeostasis and beyond.* Endocr Rev, 2007. **28**(4): p. 365-86.
464. Luedde, T., et al., *Deletion of IKK2 in hepatocytes does not sensitize these cells to TNF-induced apoptosis but protects from ischemia/reperfusion injury.* J Clin Invest, 2005. **115**(4): p. 849-59.

465. Libert, C., et al., *Involvement of the liver, but not of IL-6, in IL-1-induced desensitization to the lethal effects of tumor necrosis factor.* J Immunol, 1991. **146**(8): p. 2625-32.
466. Takai, S., et al., *Intrinsic resistance to TNF-alpha-induced hepatocyte apoptosis in ICR mice correlates with expression of a short form of c-FLIP.* Lab Invest, 2007. **87**(6): p. 572-81.
467. Handeli, S. and J.A. Simon, *A small-molecule inhibitor of Tcf/beta-catenin signaling down-regulates PPARgamma and PPARdelta activities.* Mol Cancer Ther, 2008. **7**(3): p. 521-9.
468. Lepourcelet, M., et al., *Small-molecule antagonists of the oncogenic Tcf/beta-catenin protein complex.* Cancer Cell, 2004. **5**(1): p. 91-102.
469. Emami, K.H., et al., *A small molecule inhibitor of beta-catenin/CREB-binding protein transcription [corrected].* Proc Natl Acad Sci U S A, 2004. **101**(34): p. 12682-7.