Role and regulation of p65/β-catenin association during liver injury and regeneration: a ‘complex’ relationship

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ABSTRACT

An important role for β-catenin in regulating p65 (a subunit of NF-κB) during acute liver injury has recently been elucidated through use of conditional β-catenin knockout mice, which show protection from apoptosis through increased activation of p65. Thus, we hypothesized that the p65/β-catenin complex may play a role in regulating processes such as cell proliferation during liver regeneration. We show through in vitro and in vivo studies that the p65/β-catenin complex is regulated through the TNF-α pathway, and not through Wnt signaling. However, this complex is unchanged after partial hepatectomy (PH), despite increased p65 and β-catenin nuclear translocation as well as cyclin D1 activation. We demonstrate through both in vitro silencing experiments and chromatin immunoprecipitation after PH that β-catenin, and not p65, regulates cyclin D1 expression. Conversely, using reporter mice we show p65 is activated exclusively in the non-parenchymal (NPC) compartment during liver regeneration. Furthermore, stimulation of macrophages by TNF-α induces activation of NF-κB and subsequent secretion of Wnts essential for β-catenin activation in hepatocytes. Thus, we show that β-catenin and p65 are activated in separate cellular compartments during liver regeneration, with p65 activity in NPCs contributing to the activation of hepatocyte β-catenin, cyclin D1 expression, and subsequent proliferation.
INTRODUCTION

Fulminant hepatic failure (FHF) can be initiated by a variety of etiologies, including viruses and toxins. Orthotopic liver transplantation is currently the most effective and widely used method for treating FHF, which has a mortality rate in excess of 80% without intervention. However, the relatively high risk of the surgery, combined with a requirement for lifelong immunosuppression and a shortage of healthy donor organs, has prompted clinicians to seek out promising alternatives. Thus, understanding the signaling pathways involved in liver regeneration is important in order to identify new therapies for treating acute liver injury and FHF.

The most common method of inducing liver regeneration experimentally is surgical removal of three of five liver lobes from the rodent, referred to as partial hepatectomy (PH). A key cell-cycle associated gene critical for initiation of cell proliferation is cyclin D1, which is expressed as early as 6 hours after PH. β-catenin is a key driver of cyclin D1 expression and cell proliferation. We have previously shown that direct overexpression of β-catenin enhances liver growth and LR through cyclin D1 regulation. Conversely, hepatocyte-specific β-catenin KO displayed suboptimal LR, the result of a 2-fold decrease in the number of proliferating cells, which corresponds with deficient expression of cyclin D1.

NF-κB is a group of dimeric transcription factors that initiate a wide variety of cellular programs in response to inflammation and injury, and is robustly activated by TNF-α. Recently, NF-κB has been recognized as a key apoptosis inhibitor by inducing transcription of genes that suppress caspase activation. Interestingly, NF-κB is also activated very early during liver regeneration, translocating to the nucleus to bind DNA within one hour after PH. However, the location, function, and importance of NF-κB activation in different liver cell populations have long been a source of debate. Some
publications have shown through inhibition or binding studies that NF-κB activation primarily occurs in hepatocytes and is essential for initiating cell proliferation\textsuperscript{11-14}. Other studies, however, demonstrated that activation of NF-κB occurs primarily in Kupffer cells, not hepatocytes, during regeneration.\textsuperscript{15,16,17} Thus, although NF-κB is activated during liver regeneration, the ambiguity of the above findings suggest that its overall contribution to regeneration has yet to be definitively determined\textsuperscript{18}.

The NF-κB subunit p65 has also been shown to physically associate with β-catenin in the context of cancer, resulting in inhibition of p65 transactivation and target gene expression\textsuperscript{19,20}. Our laboratory has recently demonstrated that liver-specific β-catenin knockout (KO1) mice unexpectedly exhibit prolonged survival and reduced injury to TNF-α induced apoptosis. We elucidated this to be due to the existence of the p65/β-catenin complex in the cytosol of hepatocytes, which undergoes dynamic regulation during the process of liver injury and its absence in the KO1 led to earlier and protracted NF-κB activation\textsuperscript{21}. We believe that these findings warrant further investigation into the dynamics of β-catenin and NF-κB in other injury/repair models such as liver regeneration after PH. Furthermore, since both β-catenin and NF-κB/p65 play essential roles in liver regeneration, we hypothesized that one or both pathways may be activating cyclin D1 expression during the proliferative stage, and that the p65/β-catenin complex could be a contributory source of β-catenin and/or p65 during regeneration.

We first examined the p65/β-catenin complex in the context of acute liver injury to determine the upstream regulation of dissociation. We found that TNF-α, and not Wnt signaling, is required for complex dissociation, as mice lacking Wnt signaling in hepatocytes are susceptible to injury, in contrast to KO1, which are protected. Contrary to our hypothesis, however, this complex does not change after PH and thus is not responsible for either p65 or β-catenin activation during liver regeneration. We show
through both in vitro silencing experiments and chromatin immunoprecipitation of livers after PH that β-catenin, and not p65, regulates cyclin D1 expression. To clarify the role of NF-κB during regeneration, we used a reporter mouse that demonstrated activation of this signaling pathway in the non-parenchymal cells (NPCs) of the liver, rather than in hepatocytes. Finally, stimulation of macrophages with TNF-α was sufficient to induce expression of Wnts required for activation of β-catenin and subsequent proliferation in hepatocytes. We conclude that while β-catenin and NF-κB activation are occurring in distinct cell populations during liver regeneration, these signaling pathways are intricately interconnected and cross-regulate one another during the regenerative process.
MATERIALS AND METHODS

Mice, induction of acute liver injury, and partial hepatectomy

Liver-specific β-catenin KO (KO1) mice and liver-specific LRP5/6 KO (KO2) mice were generated as previously described 6, 22. Induction of TNF-α-mediated liver injury by intra-peritoneal administration of 700mg/kg body weight (BW) D-galactosamine, followed 30 minutes later by 50μg/kg BW lipopolysaccharide, was performed as described in our previous publication 21. Partial hepatectomy was performed on WT and KO1 mice as described elsewhere 5. Mice were sacrificed at various time points after PH or at the first signs of morbidity for GalN/LPS. Blood samples were collected from the orbital sinus at the time of sacrifice. Serum biochemical measurements for alanine aminotransferase (ALT) were performed by the University of Pittsburgh Medical Center Clinical Chemistry laboratory. For GalN/LPS treatment, n=3 WT and n=4 LRP5/6 KO were analyzed. For each time point after PH, at least n=2 samples were pooled and analyzed.

The NF-κB-lacZ transgenic mice, which harbor a lacZ reporter gene under the control of an NF-κB-responsive promoter (kappaB-lacZ) were obtained from Xue-Ru Wu of NYU 23, 24. The transgenic mice were identified by polymerase chain reaction (PCR) analysis using tail genomic DNA digestion and the following primers: lacZ fwd: 5’-GCA TCG AGC TGG GTA ATA AGC CTT GGC AAT-3’; lacZ rev: 5’-GAC ACC AGA CCA ACT GGT AAT GGT AGC GAC-3’.

Protein extraction, Western blotting, and immunoprecipitation

Whole-cell lysates from mouse livers were prepared by homogenizing in RIPA with Halt inhibitor cocktail (Pierce, Rockford, IL) as previously described 21. Nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit as per the manufacturer’s instructions (Thermo Fisher Scientific, Rockford,
IL). For immunoprecipitation (IP) studies, 1000μg of liver lysate, or 1000μg of lysates from Hep3B cells or Raw264 cells, were precleared with rabbit, mouse, or goat IgG together with Protein A/G agarose for 30 minutes at 4°C. After centrifugation, the supernatants were incubated with p65 antibody (Santa Cruz Biotechnology; sc-109 or sc-8008) overnight at 4°C; alternatively, supernatants were incubated with β-catenin conjugated agarose beads (Santa Cruz Biotechnology; sc-1496) for 2.5 hours at room temperature. The next day, samples immunoprecipitated with p65 antibodies were incubated with Protein A/G agarose for 1 hour at 4°C. In all conditions, pellets were collected, washed in PBS containing inhibitors, and resuspended in loading buffer.

Eluate from immunoprecipitated complexes, liver lysates, or cell lysates were subjected to electrophoresis and Western blotting as described previously 21. Antibodies used in this study were as follows: NF-κB p65 (A clone; 1:200), NF-κB p65 (F-6 clone; 1:500), glutamine synthetase (1:200), Axin2/conductin (1:200), β-catenin (H-102 clone; 1:200), and GAPDH (1:800; Santa Cruz Biotechnology, Santa Cruz, CA); β-catenin (1:500; BD Biosciences, San Jose, CA); cyclin D1 (1:200; Neomarkers, Fremont, CA); and β-actin (1:5000, Chemicon). Secondary antibodies were horseradish peroxidase-conjugated goat anti-mouse (1:35,000) and donkey anti-rabbit (1:35,000) (Millipore, Temecula, CA). Representative Western blots from n≥2 experiments are shown.

**RNA, real-time PCR, and NF-κB cDNA array**

RNA was extracted from WT livers after PH at various time points using Trizol (Invitrogen, Carlsbad, CA). RNA was DNase-treated and equal microgram amounts of RNA from each sample (n=2 per time point) were used to make cDNA samples with the SuperScript III First-Strand Synthesis kit (Invitrogen). Quantitative expression levels of cyclin D1 were determined by real-time PCR using SYBR green and RT² qPCR primers.
for mouse Ccnd1 (Qiagen, Valencia, CA). Reverse-transcribed samples were amplified in parallel on an ABI StepOnePlus instrument (Applied Biosystems). Each sample was run in duplicate. Expression levels of cyclin D1 were normalized relative to expression of cyclophilin in each sample. Gene expression was calculated by using the $2^{-\Delta\Delta Ct}$ method, which was derived from average Ct and expressed as fold change or percent expression of control.

NF-κB target gene expression was measured in WT and KO livers (n=3 pooled samples per genotype) 12 hours after PH using the 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°C 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 luminescence. The data were normalized to control (WT) values and then plotted on a bar graph.

**Histology and Immunohistochemistry**

Liver tissues fixed in 10% formalin and embedded in paraffin were sectioned 4 µm in thickness and assessed for histological changes by hematoxylin and eosin (H&E) staining. Apoptotic nuclei were detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining using the ApopTag Peroxidase kit (EMD Millipore, Billerica, MA).

Immunohistochemistry (IHC) for PCNA on paraffin-embedded sections of mouse livers was performed as previously described 21. Briefly, after antigen retrieval in 1% ZnSO$_4$ and treatment for endogenous peroxidase, sections were stained with anti-PCNA
at a 1:4000 dilution (Dako, Denmark) for 1 hour, then incubated with biotinylated horse anti-mouse secondary (Vector Laboratories, Inc., Burlingame, CA), developed using the ABC/DAB kit (Vector Laboratories), and counterstained with Shandon’s hematoxylin. The average number of positive cells per liver lobe (n≥4) from n≥3 animals for each time point was plotted in Excel.

For β-catenin IHC, sections were subjected to antigen retrieval in Tris-EDTA buffer, pH9, in a pressure cooker for 20 minutes and then treated for endogenous peroxidases. After blocking, slides were incubated with anti-β-catenin antibody specific for the carboxy terminus (Cell Signaling Technology, Danvers, MA; catalog #9587; 1:100) for 1 hour at room temperature. Slides were incubated with biotinylated anti-rabbit secondary antibody (Vector Laboratories), then developed and counterstained as described above.

**Determination of β-galactosidase activity in tissue sections**

Tissue from freshly harvested liver was fixed in 4% paraformaldehyde at 4°C for 2 hours, followed by 15% sucrose (diluted in PBS) at 4°C overnight. The next day, tissues were transferred to 30% sucrose in PBS and incubated at 4°C overnight. Tissues were then embedded in OCT, sectioned at 4 µm thickness, and dried overnight at 37°C. Sections were stained with a β-galactosidase Staining Kit (Mirus Bio, Madison, WI). Briefly, after washing in PBS, tissues were incubated in Cell Staining Working Solution and incubated in a humidified chamber in the dark at 37°C overnight. The next day, tissues were repeatedly washed in PBS, counterstained in eosin for 30 seconds, and dehydrated before coverslipping. Representative images from n≥2 animals per time point are shown.
**ChIP assay**

Freshly harvested liver (T0 and 6H PH; n=2 each) was finely minced on ice and added to PBS containing 1% formaldehyde, 0.1M PMSF, 0.5M EDTA, and Halt inhibitor cocktail. Tissue was cross-linked for 15 minutes on a rotator at room temperature, followed by quenching with 0.125 M glycine and rinsing with cold PBS. Tissue was then collected and homogenized in cold PBS plus additives with a Wheaton overhead homogenizer. After pelleting, cells were lysed in 5Mm PIPES (pH 8.0), 85mM KCl, and 0.5% NP-40 using a Dounce homogenizer and incubated on ice for 15 minutes to release nuclei. Nuclei were resuspended in 50 mM Tris, pH 8.1, 10 mM EDTA, 1%SDS, and inhibitors at 5X the cell volume and incubated on ice for 20 minutes, then sonicated with a Bioruptor at 10 minute intervals until chromatin fragments are 200-500bp in length. Then 25μl of the chromatin was removed and saved as input. Chromatin aliquots were diluted in IP dilution buffer and precleared with Protein G-Sepharose beads (GE Healthcare, Pittsburgh, PA) for 3 hours at 4 °C. Supernatants were incubated overnight at 4°C with 10μg of either TCF4 antibody (C9B9 clone; Cell Signaling) or p65 antibody (A clone, ChIP formulation; Santa Cruz). Antibody-chromatin complexes were recovered by incubation with Protein G-Sepharose for 3 hours at 4°C and then centrifuged. Additional IP buffer was added to each sample, and then samples were loaded onto a ChIP filtration column (CHIP-IT High Sensitivity Kit; Active Motif, Carlsbad, CA) and gravity filtered, followed by washing and elution by centrifugation. Samples were de-crosslinked by incubation with Proteinase K at 55°C for 30 min then followed by 80°C for 2 hours, then purified with the MiniElute kit (Qiagen).

The resulting DNA fragments and input controls were subjected to real-time PCR as described above using the following primer sets: cyclin D1 TCF4 binding site: 5'- CCG GCT TTG ATC TCT GCT TA -3' and 5'- CGC GGA GTC TGT AGC TCT CT -3'; cyclin
D1 p65 binding site: 5'-CAA CAG TAA CGT CAC ACG T -3' and 5'-CAC AGG AGC TGG TGT TCC AT -3'. ChIP q-PCR data was normalized to percent input for each sample, and then normalized to the T0 remnant liver corresponding to the experimental sample (6H post-PH) to determine fold change.

**Cell culture, transfection, and luciferase reporter assays**

Hep3B human hepatoma cell lines were obtained from ATCC, Manassas, VA. Hep3Bs were transfected and measured for luciferase activity as previously described. Briefly, Hep3Bs grown in Eagle's Minimal Essential Medium (American Type Culture Collection, Manassas, VA) with 10% FBS (Atlanta Biologicals, Lawrenceville, GA) were seeded onto 6-well plates and transiently transfected with siRNA combined with reporters as indicated. Human β-catenin (CTNNB1) siRNA, human p65 siRNA, or negative control siRNA (Ambion, Inc., Austin, TX) was used at a final concentration of 50 nM in the presence of Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA), as per the manufacturer's instructions. Simultaneously, either 0.2μg of TOPflash reporter plasmid (which measures β-catenin/TCF-dependent transcriptional activation; Upstate Biotechnology), 0.2μg of p65 reporter plasmid, or 0.2μg of cyclin D1 reporter plasmid was co-transfected with 0.2 μg of *Renilla* plasmid, also in the presence of Lipofectamine 2000. The cells were harvested 48 hours after transfection for protein extraction and luciferase assay. Luciferase assays were performed using the Dual Luciferase Assay System kit, in accordance with the manufacturer's protocols (Promega, Madison, WI). Relative luciferase activity (in arbitrary units) was reported as fold induction after normalization to *Renilla* for transfection efficiency.

For TNF-α treatment, Hep3B cells were transfected with *Renilla* and either p65 reporter plasmid or cyclin D1 reporter plasmid as described above. After 5 hours, fresh
media containing either vehicle control or TNF-α (R&D) at a final concentration of 25ng/ml was added to the cells. The media was changed 24 hours after transfection and fresh TNF-α or vehicle control was added to the wells. Cells were harvested 48 hours after transfection for luciferase assays, which were performed as described above. Finally, Hep3B cells were treated with 50 ng/ml TNF-α in 100mM dishes and harvested at various time points after treatment for protein extraction and immunoprecipitation as described above.

A Hep3B cell line expressing a constitutively active form of β-catenin, which is mutated at serine 33 to tyrosine (S33Y), was recently described. These stably transfected cells were then transiently transfected with control, p65, or β-catenin siRNAs as well as cyclin D1 and Renilla reporters at a concentration of 0.2 μg/ml each. Cells were harvested for luciferase assay 48 hours after transfection as described above.

AML12 cells were obtained from ATCC and maintained in Dulbecco Eagle’s minimal essential medium (DMEM) / Ham’s F-12 (1:1) mixed media supplemented with 10% FBS, 5 μg/ml Insulin, 5 μg/ml Transferrin, 5 μg/ml Sodium Selenite (Roche), and 100nM dexamethasone. Cells were transfected with either mouse β-catenin siRNA, mouse p65 siRNA, or negative control siRNA (Ambion, Inc., Austin, TX), concomitant with transfection of cyclin D1 and Renilla reporters, and harvested for luciferase assay as described above.

**Cell Growth Assay**

A [³H]thymidine uptake assay was utilized to measure cell proliferation. Briefly, Hep3B cells transfected with control, p65, or β-catenin siRNA as described above were dosed with 2.5 μCi/ml of [³H]thymidine (Perkin Elmer, Waltham, MA) for 48 hours before harvesting. The counts/min were determined with a Beckman scintillation counter.
RAW-Blue TNF-α treatment and NF-κB reporter assay

RAW-Blue cells (Invivogen, San Diego, CA), derived from RAW264.7 macrophages and containing a secreted embryonic alkaline phosphatase (SEAP) reporter construct inducible by NF-κB and AP-1, were cultured as previously described. Briefly, RAW-Blue cells (1 × 10^5 cells/well) were cultured in DMEM supplemented with 10% (v/v) FBS (HyClone, Logan, UT) and zeocin (Invivogen, 200 μg/ml) and stimulated by different doses of TNF-α for 18 hours. RAW-Blue cells were then harvested and supernatant added to QUANTI-blue alkaline phosphatase substrate (Invivogen), followed by incubation at 37°C for 1–3 h. Absorbance was measured at 620 nm in a BMG Polarstar microplate reader.

To analyze expression of Wnt2 gene expression after TNF-α stimulation, RAW-Blue macrophages were first grown to 40-50% confluency and serum starved overnight. The following morning, 0.1ng/ml of TNF-α was added. Treated and untreated wells were pooled (2-3 wells) and an RNA kit (Qiagen) was used to extract RNA. After DNase treatment, samples were converted to cDNA (Invitrogen) and qPCR was used to identify levels of Wnt2. Primers used are as follows: GAPDH forward: 5′- AAC TTT GGC ATT GTG GAA GG -3′; GAPDH reverse: 5′- ACA CAT TGG GGG TAG GAA CA -3′; Wnt2 forward: 5′- TCT GTC TAT CTT GGG CAT TCT G -3′; Wnt2 reverse: 5′- TTC CTT CGC TAT GTG ATG TTT C -3′; Experiment was repeated 3 times and data shown is from one representative experiment.

Statistics

Data are presented as mean ± SD as indicated. All statistics were performed using Prism 6 software (GraphPad) or Excel 2011 for Mac OS X. Comparisons between
various groups were performed by Student’s t-test. Values of $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$ are considered significant throughout the study.

**Study approval**

All animal experiments were performed under the guidelines of the National Institutes of Health and the Institutional Animal Use and Care Committee at the University of Pittsburgh. The studies performed in the current report were approved by the Institutional Animal Use and Care Committee at the University of Pittsburgh (protocol #14013027 and #14084364).
RESULTS

Wnt signaling does not regulate the p65/β-catenin complex after injury.

Previously, we demonstrated that the p65/β-catenin complex changes dynamically over time in response to acute liver injury \(^2\). Further, absence of β-catenin in liver-specific knockout mice (KO1) allowed for protection against LPS/TNF\(\alpha\) injury. However, the upstream mechanisms regulating the association of this complex, especially the role of β-catenin upstream effector Wnt, are unclear. Indeed, β-catenin transcriptional activation of a subset of target genes is ongoing during TNF-α induced injury despite cleavage of β-catenin itself, which is likely due to caspase activity (Supplemental Figure 1) \(^2\). Thus, to address if active Wnt signaling could be inhibiting p65 activity by maintaining the presence of the p65-β-catenin complex, we generated hepatocyte-specific double LRP5/6 mice (KO2) by interbreeding albumin-cre and LRP5/6-double floxed mice. In these mice, canonical Wnt signaling is disrupted but β-catenin is still present in the cell. Thus, formation of the p65/β-catenin complex should be unaltered in these livers. Indeed, unlike KO1, p65 and β-catenin associate strongly in KO2 as well as WT livers at baseline (Figure 1A). KO2 and WT mice were then injected with galactosamine (GalN) followed by lipopolysaccharide (LPS), which activates the TNF-α pathway. Unlike in KO1, which are protected from injury \(^2\), there was no difference in time to morbidity between WT and KO mice, with both groups succumbing to injury around 8 hours (8H) after GalN/LPS (Figure 1B). Histologically, KO2 livers were indistinguishable from WT livers, with characteristic centrilobular congestion, hepatocellular degeneration, and necrosis; abundant TUNEL-positive apoptotic cells were also apparent in both genotypes (Figure 1C). Measurement of serum alanine aminotransferase (ALT) also showed insignificant differences between WT and KO2 (Figure 1D). Additionally, p65 and β-catenin are still strongly associated in both WT and
KO2 livers at the time of morbidity, implying inability of NF-κB to activate pro-survival target genes (Figure 1E). Thus, maintenance of the p65/β-catenin complex in the cytoplasm during TNF-α injury is due to the presence of β-catenin, irrespective of upstream signaling.

**p65/β-catenin complex dissociation is induced by TNF-α signaling and correlates with increased activation of DNA synthesis.**

As we have previously shown, disruption of p65/β-catenin association occurs within hours after LPS treatment. To confirm that this is directly caused by TNF-α activation of p65, we stimulated Hep3B cells with TNF-α and monitored dissociation of the complex. Figure 2A shows that β-catenin and p65 dissociate in Hep3Bs 3 hours after treatment with TNF-α. Interestingly, the kinetics of complex dissociation are similar in WT mice treated with GalN/LPS, with dissociation of the complex at 3-4H after induction of apoptosis coinciding with peak p65 protein expression (Figure 2B and Supplemental Figure 1). Both in vitro and in vivo, the complex reforms at around 5 hours after treatment, although the total amount of complex is lesser due to increased liver injury causing decreased expression of p65 and β-catenin. Thus, TNF-α signaling, and not Wnt signaling, is responsible for the dynamic changes seen in the p65/β-catenin complex during liver injury.

To determine whether this association corresponds to cellular functions other than hepatocyte survival, we examined WT liver sections for PCNA, a marker of cells in S-phase of the cell cycle. Interestingly, hepatocytes enter the cell cycle in response to injury 3H after GalN/LPS. However, D-galactosamine suppresses transcription, which is necessary for cell cycle progression. Thus, although the hepatocyte may enter S-phase, cell cycle progression is likely arrested at this stage, which contributes to and
exacerbates the injury seen in WT. Nonetheless, p65/β-catenin dissociation correlates with increased activation of the cell cycle response despite failed proliferation (Figure 2C). On the other hand, the re-association of p65 and β-catenin at 4H correlates with decreased number of hepatocytes in S-phase and progression of liver injury. These data suggest that p65/β-catenin dissociation is required for proper activation of pro-survival pathways and may be directly or indirectly contributing to induction of cell cycle machinery during acute liver injury.

β-catenin is an important component of a proper regenerative response, as it regulates hepatocyte proliferation through activation of cyclin D1 expression. Indeed, KO1 mice show a delayed regenerative response after partial hepatectomy, which coincided with decreased cyclin D1 expression. In the GalN/LPS model, proliferation is absent in KO1 mice until after the transcriptional block caused by GalN is lifted (data not shown). Therefore, we hypothesized that the p65/β-catenin complex is mutually inhibitory to both components (β-catenin as part of Wnt signaling and p65 as a component of the NF-kB pathway), and that dissociation of this complex may induce β-catenin activation. To test this, we transfected Hep3B cells with TOPflash reporter (a measure for β-catenin/TCF4 transcriptional activity) and either control, p65, or β-catenin siRNA. Indeed, knocking down p65 expression in Hep3B cells increases β-catenin activity (Figure 2D). Thus, p65 inhibits β-catenin activity in much the same way as β-catenin inhibits p65 activity.

**Lack of notable changes in the p65/β-catenin complex during liver regeneration despite nuclear translocation of β-catenin and p65 and increased cyclin D1 expression.**
We and others have described the consequences of p65/β-catenin association in liver injury \(^{19-21}\); however, the status and function of this complex in the early stages of liver regeneration is unknown. This is relevant because the complex could be a contributory source of β-catenin that would augment cell proliferation during regeneration. We utilized partial hepatectomy (PH) to examine any changes in the p65/β-catenin association, as well as activation status of both p65 and β-catenin and target gene expression. We found unremarkable changes in the status of this complex during the first 24 hours after PH (Figure 3A). Intriguingly however, both p65 and β-catenin translocate to the nucleus within 3 hours after PH (Figure 3B). Concomitantly, small but significant increases are seen in both cyclin D1 mRNA and protein expression at 1 hour after PH, corresponding to β-catenin nuclear translocation; cyclin D1 expression again increases at 12 hours, which is the period immediately prior to peak DNA synthesis \(^{29,30}\) (Figure 3C and 3D). Thus, dissociation of the p65/β-catenin complex does not appear to be contributing to the expression of target genes essential for regeneration such as cyclin D1.

**β-catenin, and not p65 or TNF-α, regulates cyclin D1 expression and proliferation.**

Despite lack of any changes in the p65/β-catenin complex, the nuclear translocation of β-catenin and p65 after PH along with the concomitant increase in cyclin D1 expression was intriguing. Since both the human and mouse cyclin D1 promoter contain highly conserved consensus sequences for both TCF/LEF and NF-κB \(^{31-33}\), we next wanted to determine whether β-catenin, p65, or a synergistic co-activation of the two proteins was responsible for cyclin D1 upregulation during cell proliferation. To reduce the confounding effects of cross-talk from multiple cell types, we decided to analyze effect of loss of β-catenin and p65 on cyclin D1 in vitro using Hep3B cells. After
confirming that p65 siRNA decreases p65 reporter activity in this system, while β-catenin siRNA increases p65 activity (Figure 4A), we next looked at how inhibiting β-catenin or p65 affected cyclin D1 expression and cell proliferation in vitro. Figure 4B shows that cyclin D1 activity is decreased in the absence of β-catenin but not in the absence of p65. A similar decrease in cyclin D1 activity in the absence of β-catenin but not in the absence of p65 was observed in the mouse immortalized hepatocyte cell line AML12 (data not shown). Inhibiting β-catenin also results in suppressed cell proliferation, while loss of p65 had no effect on proliferation (Figure 4C). Finally, we transfected cells with plasmid expressing constitutively active S33Y-mutated β-catenin, which stimulates hepatocyte cell growth, in order to study the effect of p65 or β-catenin loss on actively proliferating cells. Despite the overabundance of active β-catenin in these cells, siRNA against β-catenin effectively suppresses cyclin D1 activity (Figure 4D). We have previously shown that this mutated form of β-catenin significantly represses p65 reporter activity; thus S33Y-expressing Hep3B cells transfected with p65 siRNA have maximally suppressed p65 due to both decreased p65 activity and decreased p65 expression. Despite this, there is still no reduction in cyclin D1 activity in p65 siRNA-treated, actively proliferating Hep3Bs, and reporter activity remains comparable to that of control siRNA (Figure 4D).

Since TNF-α is a critical component in the early phase of liver regeneration after partial hepatectomy, we wanted to directly address if it could be regulating proliferation pathways in hepatocytes. As expected, addition of 25ng/ml TNF-α to Hep3B cells for 48 hours increased p65 expression over 3-fold (Figure 4E). However, cyclin D1 activity remained completely unchanged after TNF-α administration (Figure 4F).

Taken together, we conclude that β-catenin is required for cyclin D1 activity and subsequent proliferation, while TNF-α/p65 is dispensable.
Cyclin D1 expression during liver regeneration is β-catenin/TCF4 dependent.

To verify these findings in vivo in a model of liver regeneration, we performed ChIP assay on livers isolated from WT animals at baseline and 6 hours after PH. We chose this time point because binding of TCF4 or p65 to DNA should precede induction of cyclin D1 expression. We analyzed TCF4 occupancy on a confirmed binding site in the cyclin D1 promoter, and found an approximately two-fold higher recruitment of TCF4 after PH compared to baseline (Figure 5A). Since this primer set also encompasses the NF-κB sequence, we next designed primers that exclude the TCF4 binding site and contain only the NF-κB site, in order to more precisely determine the contribution of these two transcription factors in activation of cyclin D1. Using the NF-κB-specific primers, we determined that occupancy of p65 on the NF-κB binding site did not change after PH and remained at baseline levels (Figure 5B). Thus, as in vitro, β-catenin but not p65 drives cyclin D1 activity in response to a regenerative stimulus.

Since p65 was not affecting hepatocyte proliferation during regeneration despite increased nuclear translocation, we next wanted to investigate the role of p65/NF-κB signaling after PH by analysis of target gene expression. Additionally, we compared WT to KO1 at 12 hours after PH to determine if suppression of β-catenin results in activation of an altered p65 gene signature. As expected, in the absence of β-catenin, cyclin D1 gene expression is decreased in KO1. However, expression of cytokines, growth factors, and tissue remodelers (such as IL1A, TNFA, FasL, VCAM1, VEGFC, and MMP1) are increased in KO1 compared to WT (Figure 5C). These results suggest that p65 target genes are differentially regulated in WT and β-catenin KO 12H after PH, favoring a more inflammatory, pro-apoptotic profile in the absence of β-catenin.
**p65 is activated in the non-parenchymal cell compartment during liver regeneration, while β-catenin activation is occurring primarily in hepatocytes.**

Since β-catenin nuclear translocation after PH is occurring in hepatocytes to induce cyclin D1 expression, we next investigate the source of p65 nuclear translocation that was evident in whole cell lysates after PH despite lack of any changes in the p65/β-catenin complex (Figure 3A, 3B). To investigate the cell compartment in which NF-κB is activated during liver regeneration, we utilized NF-κB lacZ transgenic mice, which exhibit β-galactosidase (β-gal) activity in the presence of transcriptionally active NF-κB 23, 24 (Figure 6A), and subjected these mice to PH. NF-κB activity was notably induced 6 hours after PH, as indicated by the increased number of β-gal-positive cells in the liver lobules (Figure 6B). However, these β-gal-positive cells were exclusively localized to the NPCs both before and immediately after PH. We did not detect NF-κB activity in hepatocytes at any time point after PH. On the other hand, IHC shows that β-catenin is predominantly localized to the hepatocyte membrane and undergoes activation primarily in this cell type after PH, as demonstrated by strong cytoplasmic and nuclear localization at 3 hours and 6 hours, respectively (Figure 6C). Thus, although the kinetics of NF-κB and β-catenin activation are similar during liver regeneration, they are occurring in different cell compartments.

**TNF-α activates p65 and stimulates Wnt production in macrophages.**

Because NF-κB is activated in NPCs during liver regeneration, we next compared the expression of both β-catenin and p65 in Hep3B cells to Raw264 cells, a macrophage cell line, by Western blot. Interestingly, although Hep3Bs have an abundance of both β-catenin and p65 compared to Raw264 cells, the ratio of β-catenin to p65 differs significantly: in Hep3B cells, there is more β-catenin than p65, while in
Raw264 cells, the amount of p65 is greater than the amount of β-catenin (Figure 7A). Furthermore, the p65/β-catenin complex is present albeit at low levels in macrophages as well (Figure 7A). Based on input and IP studies, most β-catenin in macrophages appears to be associated with p65 while only a small fraction of β-catenin is associated with p65 in Hep3B cells (Figure 7A, 7B).

Since TNF-α is both released by and acts upon macrophages, we evaluated if TNF-α impacts macrophage NF-κB activity. TNF-α strongly activated p65 activation in a dose-dependent manner in RAW-Blue cells, a macrophage cell line containing an inducible NF-κB reporter (Figure 7C)\(^{27}\). Since resident liver macrophages (Kupffer cells) are important in altering cytokine and growth factor expression in the liver after PH \(^{17, 35}\), we examined if TNF-α could indirectly impact hepatocyte proliferation through induction of Wnt gene expression in macrophages. Previously, we have reported macrophages to be a source of Wnts after PH. Indeed, incubation of RAW-Blue cells with 0.1ng/ml (100pg/ml) of TNF-α, which represents the physiological concentration of this cytokine in the blood during liver regeneration \(^{36, 37}\), led to a several-fold increase in Wnt2, one of the Wnts implicated in metabolic zonation and regeneration \(^{38}\), at both 6 hours and 24 hours after treatment (Figure 7D).

Taken together these data suggest that during early liver regeneration, NF-κB is activated in NPCs such as macrophages in a TNF-α-dependent manner to then induce expression of mitogens that have a direct effect on hepatocyte proliferation.
DISCUSSION

In the current study, we describe the complex interaction and regulation of β-catenin and p65 in both liver injury and regeneration. As we have published previously, the p65/β-catenin complex is a critical regulator of cell survival during liver injury such as that induced by GalN/LPS. Dissociation of this complex in hepatocytes is mediated by release of TNF-α from Kupffer cells in response to LPS, facilitating nuclear translocation of p65 (Figure 8A). The absence of β-catenin augments this response, resulting in early, robust and protracted activation of p65. Interestingly, in liver regeneration, we have demonstrated that the p65/β-catenin complex is not contributing to the regenerative process. Instead, increased TNF-α levels as a result of PH leads to activation of NF-κB in Kupffer cells and subsequent production of cytokines and growth factors including Wnts, which in turn induce proliferation in hepatocytes (Figure 8B). Thus, although this complex does not play a direct role in activation of either p65 or β-catenin in hepatocytes after PH, the activation of these pathways in distinct cell types is nonetheless essential for liver regeneration.

In our earlier publication, we showed that ablation of β-catenin renders hepatocytes more resistant to apoptosis due to lack of the NK-κB-inhibitory p65/β-catenin complex. However, the mechanisms underlying the association of this complex at homeostasis and the regulation of this complex during hepatocyte survival were not understood. In this manuscript, we utilize mice lacking Wnt-driven β-catenin signaling but retaining intact β-catenin (LRP5/6 KO; KO2). We find that in response to GalN/LPS treatment, KO2 mice do not phenocopy β-catenin KO1 mice in terms of protection from TNF-α induced apoptosis, but instead show similar kinetics of injury as WT animals. If Wnt signaling were contributing to dissociation of this complex, we would expect to see earlier mortality in LRP5/6 KO mice. Conversely, if Wnt signaling, and not β-catenin
itself, were responsible for protection, we would expect the LRP5/6 KO to be resistant to injury, similar to KO1. Neither of these two scenarios occurred in the LRP5/6 KO. We previously also described lack of p65 reporter activity in the presence of ICG001, which blocks β-catenin’s downstream transcriptional activity. The LRP5/6 KO mice mirror this in vitro state, as β-catenin protein is intact in hepatocytes despite lack of Wnt signaling. Thus, we validate that physical association of β-catenin and p65 regulates survival or apoptosis, while also showing that Wnt signaling does not regulate this complex during acute liver injury. Instead, p65/β-catenin complex dissociation is induced by TNF-α signaling.

Analysis of the kinetics of p65/β-catenin dissociation in the presence of TNF-α revealed an important finding: that dissociation of this complex occurs at a predictable rate, regardless of the source of TNF-α. In TNF-α-treated Hep3B cells as well as in the GalN/LPS liver injury model, we observed a decrease in p65/β-catenin association 3H after treatment, and in both cases this dissociation appears to be crucial for optimal activation of downstream pathways. However, this dissociation is absent after PH, and the p65/β-catenin complex is intact. In fact, the extent of dissociation in response to TNF-α may be dose-dependent. In mouse models of endotoxic shock, such as LPS-induced acute liver injury, serum TNF-α levels range from 3-25 ng/ml within 90 minutes of treatment. On the other hand, some groups have been unable to detect the presence of serum TNF-α at any time after PH, while others have reported serum TNF-α in pg/ml amounts within 1 hour after PH. We hypothesize that pathological levels of TNF-α, such as that found in LPS-induced liver injury, have a more profound and prolonged effect on the p65/β-catenin complex than the physiological TNF-α released during liver regeneration.
Despite the increased nuclear translocation of both β-catenin and p65, we observed a lack of NF-κB activation in hepatocytes after PH. Indeed, our in vivo studies support the hypothesis that activation of NF-κB occurs primarily in NPCs after PH. Yang et al. also demonstrated through use of a transgenic mouse expressing enhanced green fluorescent protein under the transcriptional control of NF-κB that activation primarily occurs in Kupffer cells rather than hepatocytes. We believe that decreased levels of p65/β-catenin complex in macrophages may also be contributing to NF-κB activation in these cells. WB demonstrates that macrophages have a lower ratio of β-catenin to p65 than do hepatocytes, which contain notably higher levels of β-catenin. This disparity would result in more free p65 in macrophages, thus creating a lowered activation threshold. The presence of physiological levels of TNF-α, which is insufficient to cause complex dissociation in hepatocytes, may nonetheless induce p65 activation in a cell type with less inhibitory β-catenin. Therefore, we believe that the increased nuclear translocation of both β-catenin and p65 as observed in whole cell lysates after PH is occurring in different cell types, with β-catenin activation occurring primarily in hepatocytes, and NF-κB activation occurring in macrophages.

Another interesting finding from our studies is that addition of TNF-α to RawBLUE cells results in increased expression of Wnt2 gene expression. Indeed, recent reports have demonstrated that Kupffer cells contribute Wnts that are necessary for β-catenin activation during regeneration. We postulate that the physiological increase in TNF-α after PH stimulates release of Wnt2 from macrophages, which then acts in a paracrine manner on neighboring hepatocytes to induce β-catenin activation and subsequent proliferation. Further studies will be needed to validate these findings in vivo.
Cyclin D1, a critical driver of cell cycle progression and proliferation, has previously been reported to be a downstream target of both the NF-κB and the β-catenin signaling pathways. Because of similarities in activation/translocation kinetics after PH, we wondered if NF-κB may be a potential β-catenin binding partner during liver regeneration. In support of this hypothesis, previous studies have shown that direct association of p50 and β-catenin on the promoter of C-reactive protein is required for transcription. Interestingly, β-catenin also occupies an NF-κB binding site on the cyclin D1 promoter in combination with p65, and overexpression of p65 represses β-catenin-induced cyclin D1 expression through protein-protein interactions. Additionally, there was no significant increase in β-catenin-driven TCF-4 activation after PH in TOPGal reporter mice despite increased cyclin D1 expression and proliferation, suggesting that other transcription factors that may potentially partner with β-catenin to induce specific target gene expression. In order to test whether β-catenin and NF-κB synergistically regulate proliferation through activation of cyclin D1 gene expression, we performed in vitro studies silencing either β-catenin or p65 and examined proliferation and cyclin D1 reporter activity. We found that only loss of β-catenin negatively affected cyclin D1 activation and proliferation, which were sustained in the absence of p65. Furthermore, transfection with a dominant-negative TCF4 plasmid also decreased both proliferation and cyclin D1 reporter activity (data not shown), demonstrating that β-catenin/TCF4 association is critical for this process. ChIP assay confirmed this observation in vivo, showing increased binding of TCF4 but not p65 to the cyclin D1 promoter 6 hours after PH. We conclude that β-catenin is necessary and sufficient for activation of cyclin D1 expression.

Although the role of the p65/β-catenin complex in acute liver injury has been described, the physiological significance of this interaction in hepatocytes during liver
regeneration, if any, is still unclear. However, the increase in cytokines and apoptotic factors observed in β-catenin KO livers after PH may provide some clues. Many of these same genes were also upregulated in KO1 livers after GalN/LPS, which in that model facilitated prolonged survival. However, it is important to note that in the GalN/LPS model, the requirement for survival outweighs the proliferative drive, whereas in the PH model these roles are reversed. Thus, in the context of liver regeneration, our recent data indicate that β-catenin may divert p65 away from activation of survival/inflammation pathways after PH in favor of promoting proliferation. However, when β-catenin is absent from hepatocytes, as in the case of KO1, this inhibition is lifted, allowing aberrant p65 signaling to occur. In support of this hypothesis, we have previously shown that apoptosis increases in KO1 mice after PH at all stages, while liver regeneration is delayed. Thus, the role of this complex in liver regeneration may be to prevent over-activation of p65, which could lead to a pro-inflammatory or pro-apoptotic phenotype and thus hinder proliferation, regeneration, and repair.

Our current observations lead us to conclude that β-catenin may be an important component of balancing the context-dependent pro-proliferation versus pro-survival functions of NF-κB. The pivotal role of β-catenin in determining or altering the cellular programming of hepatocytes could thus be exploited for clinical therapies that enhance cell survival and/or proliferation in FHF as well as other liver diseases associated with massive cell death.
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REFERENCES

FIGURE LEGENDS

Figure 1: LPR5/6 KO mice are susceptible to GalN/LPS induced liver injury. (A) IP shows that p65 and β-catenin associate strongly in both WT and LRP5/6 KO (KO2) livers at baseline. (B) Kaplan-Meier analysis of WT and KO2 mice shows no difference in survival after GalN/LPS (n≥3). (C) H&E (top panels) shows massive inflammation, necrosis, and hemorrhage in both WT and KO2; TUNEL staining (bottom panels) shows significant apoptosis in both WT and KO2 as well. (D) Serum ALT levels are equivalent in WT and KO2 mice 6H after GalN/LPS treatment (n≥3). (E) IP shows maintenance of p65/β-catenin association in both WT and KO2 after GalN/LPS-induced injury.

Figure 2: Dissociation of the inhibitory p65/β-catenin complex after TNF-α coincides with initiation of DNA replication during acute liver injury and increased β-catenin activation in vitro. (A) IP shows that p65/β-catenin association is lost 3 hours after TNF-α treatment in Hep3B cells, but rebounds at 6 hours. (B) p65/β-catenin association also decreases 3 hours after GalN/LPS treatment. (C) Quantification of PCNA staining shows a strong induction of cell cycle initiation 3 hours after GalN/LPS treatment in WT livers, which is virtually absent at later time points. * p<0.05. (D) Inhibition of p65 increases β-catenin/TCF4 reporter activity in Hep3B cells.** p<0.01.

Figure 3: Despite increases in both p65 and β-catenin nuclear translocation, as well as increased cyclin D1 expression, the p65/β-catenin complex is unchanged during early liver regeneration. (A) IP shows insignificant changes in p65/β-catenin association after PH. (B) β-catenin translocates to the nucleus 1 hour after PH, while p65 increases in the nucleus 3H after PH, as assessed by WB of nuclear lysates. (C) Real-time PCR for cyclin D1 mRNA shows significant increases in cyclin D1 expression at
both 1 hour and 12 hours after PH. *p<0.05; ** p<0.01. (D) WB shows a significant increase in cyclin D1 protein 12 hours after PH.

**Figure 4: Cyclin D1 activity and proliferation in vitro requires β-catenin but not TNF-α/p65.** (A) p65 siRNA decreases p65 reporter activity, while β-catenin siRNA increases p65 activity. (B) β-catenin siRNA significantly decreases cyclin D1 reporter activity in Hep3B cells, while p65 siRNA has no effect. (C) β-catenin siRNA significantly decreases proliferation of Hep3B cells, as assessed by thymidine incorporation assay. (D) In actively proliferating cells expressing mutant S33Y β-catenin, siRNA against β-catenin, but not p65, decreases cyclin D1 reporter activity. *p<0.05; ** p<0.01. (E) TNF-α induces p65 reporter activity in Hep3B cells. ** p<0.01. (F) Addition of TNF-α to Hep3B cells does not change cyclin D1 reporter activity.

**Figure 5: β-catenin/TCF4 regulates cyclin D1 expression in a p65-independent manner after PH, while in the absence of β-catenin, p65 target gene expression increases.** (A) ChIP using TCF4 antibody shows an increase in cyclin D1 promoter occupancy 6 hours after PH compared to baseline. (B) ChIP using p65 antibody demonstrates no difference in cyclin D1 promoter occupancy after PH. (C) cDNA analysis of NF-κB targets after PH shows that KO1 have a several-fold increase in several cytokine and apoptosis-related genes compared to WT.

**Figure 6: NF-κB activation after PH occurs in NPCs such as macrophages, while β-catenin activation occurs in hepatocytes.** (A) Schematic of the NF-κB LacZ reporter mouse, which has 3 functional NF-KB sites upstream of a nuclear localization sequence and LacZ. (B) NF-κB activity is confined to NPCs before and immediately after PH, as
assessed by β-galactosidase staining of histology sections. (C) β-catenin IHC demonstrates activation of β-catenin (as determined by translocation into the cytoplasm and nucleus) predominantly in the hepatocytes at 3 and 6 hours after PH. For B and C, n≥2 mice per time point.

**Figure 7: Macrophages, which have a higher ratio of p65 to β-catenin than hepatocytes, produce Wnts after TNF-α stimulation.** (A) WB of β-catenin and p65 shows that Hep3B cells have more β-catenin than p65, compared to Raw264 cells, which have more p65 than β-catenin. (B) IP for β-catenin shows presence of the p65/β-catenin complex in macrophages as well as Hep3Bs, despite low abundance of β-catenin. (C) RAWBlue macrophages treated with TNF-α show a dose-dependent increase in NF-κB activity. ** p<0.01. (D) RAW-Blue cells showed increased Wnt2 mRNA expression 6 hours and 24 hours after incubation with a physiological dose of TNF-α. * p<0.05.

**Figure 8: Schematic for the role of β-catenin and NF-κB/p65 activation in liver injury and regeneration.** (A) In acute liver injury, administration of LPS activates NF-κB in Kupffer cells, resulting in production of TNF-α, which in turn acts on neighboring hepatocytes to dissociate the p65/β-catenin complex and induce nuclear translocation of p65. (B) During liver regeneration, cytokines such as TNF-α activate NF-κB in Kupffer cells, resulting in expression of genes encoding growth factors as well as Wnt proteins; these factors are then released and act on neighboring hepatocytes in a paracrine manner to induce proliferation through transcription of cyclin D1.
Supplemental Figure 1: Expression of β-catenin and p65 change over time in WT livers after GalN/LPS. (A) WB shows cleavage of β-catenin occurs 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 GS and Axin2 remains unchanged over time, while others such as cyclin D1 decrease with increasing β-catenin degradation. Expression of p65 also decreases in WTs after GalN/LPS treatment, and is absent by 6 hours.
Figure 1
Figure 2

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**C**

PCNA+ cells after GalN/LPS

**D**

TOPflash reporter activity

- Control siRNA
- p65 siRNA
- Beta-catenin siRNA

Figure 3

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**B**

NUCLEAR LYSATES

- β-catenin
- p65
- actin

**C**

Cyclin D1 mRNA expression after PH

**D**

Cyclin D1

actin
Figure 4
Figure 5
Figure 6
Figure 7

A. Hep3B Raw264
   - β-catenin
   - p65
   - GAPDH

B. β-catenin IP
   - Hep3B Raw264
   - IP: β-catenin
   - WB: p65
   - IP: β-catenin
   - WB: β-catenin

C. RAWBlue TNF-α activation
   - Control
   - TNF-α

D. Wnt2 mRNA expression
   - 6hr NT
   - 6hr TNF-α
   - 24hr NT
   - 24hr TNF-α

Fold change from no treatment

* p < 0.05
** p < 0.01
Figure 8
Supplemental Figure 1