ROLE OF DNA DAMAGE RESPONSE IN LIVER REGENERATION AFTER APAP OVERDOSE INDUCED ALF

BY

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ABSTRACT

Acetaminophen (APAP) overdose is a leading cause of acute liver failure (ALF) with limited treatment options. The mechanisms of APAP-induced liver injury include formation of a reactive metabolite N-acetyl-p-quinoneimine (NAPQI) and it's covalent binding to protein, oxidative stress, mitochondrial damage and subsequent nuclear DNA damage leading to necrosis. The replacement of necrotic cells and restoration of liver function occurs through liver regeneration. Although several studies have shown that liver regeneration plays a crucial role in the final outcome of APAP-induced ALF patients, the mechanisms are not entirely known. DNA damage can activate the DNA damage response (DDR) – a signaling cascade that senses the damage and co-ordinates cell cycle progression with DNA repair and other cellular processes. Although APAP toxicity involves severe nuclear DNA damage, role of DDR in regulation of liver regeneration after APAP induced acute liver injury ALI has not been investigated. My **hypothesis** is that the DDR and p53- a DDR effector protein which induces cell cycle arrest, are activated after APAP overdose and connect liver injury response to initiation of liver regeneration.

We studied DDR using incremental dose model with two different doses of APAP in mice [300 mg/kg (APAP300)-a regenerative dose and 600 mg/kg i.p. (APAP600)-a non-regenerative dose]. We began by analyzing microarray data obtained from the incremental dose model using the Ingenuity Pathway Analysis program. This analysis revealed significant differences in DNA damage, replication and checkpoint related pathways in regenerative and non-regenerative doses of APAP. We found that APAP

overdose causes DNA Double Strand Break (DSB) in both groups of animals and it is sustained in mice treated with APAP600 relative to mice treated with APAP300. We also observed a subsequent increase in DNA repair proteins in APAP300 treated mice but not in APAP600 treated mice. The DNA repair pathway was significantly suppressed and p53 activation was significantly higher in mice treated with APAP600 as compared to APAP300. These data illustrate that delayed DSB repair response occurs after APAP overdose leading to prolonged growth arrest and may be a crucial mechanism involved in inhibition of liver regeneration.

Next, we investigated roles of p53 in detail using WT and p53KO mice (C57BL/6J background) following APAP300 treatment. Remarkably, deletion of p53 resulted in a 3-fold higher liver injury when compared to WT mice implying a protective role for p53 in injury progression. Deletion of p53 did not affect APAP bioactivation however it delayed clearance of APAP protein adducts from liver. Intriguingly, despite higher injury p53KO mice recovered similarly as the WT mice due to faster liver regeneration. Global transcriptomic and molecular analysis revealed several mechanisms triggering higher progression of liver injury yet faster regeneration in p53KO animals after APAP300 treatment. Impaired metabolic homeostasis, GSH transsulfuration and reduced expression of mitochondrial complexes in p53KO mice resulted in higher liver injury. However, increased inflammatory signaling and proliferative signaling through AKT, ERK and mTOR pathways improved recovery in p53KO mice despite very high injury. These studies show that p53 plays a pleotropic role after APAP overdose where it prevents progression of liver injury by maintaining mitochondrial and metabolic

homeostasis and also regulates initiation of liver regeneration through inflammatory and proliferative signaling.

Overall, my studies comprehensively investigated roles of DDR and p53 in liver injury and regeneration after APAP overdose. These studies have uncovered novel mechanisms that connect cellular injury to initiation of liver regeneration after APAP-induced ALF, which will be useful for future therapeutic development.

ACKNOWLEDGEMENTS

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TABLE OF CONTENTS

CHAPTER I: INTRODUCTION

1.1 ACUTE LIVER FAILURE (ALF)

Acute Liver Failure (ALF) is a clinical syndrome characterized by rapid onset of severe hepatocyte injury and associated with coagulopathy and encephalopathy in a patient without preexisting liver disease (Lee 1993, Hoofnagle, Carithers et al. 1995). The signs or symptoms associated with ALF are hepatic dysfunction, abnormal liver biochemical values, coagulopathy and encephalopathy, culminating in multiorgan failure (Lee 2012). ALF is the result of severe liver cell injury from a variety of different etiologies, such as neoplastic infiltration, acute Budd–Chiari syndrome, heatstroke, mushroom poisoning, Wilson's disease, ischemic hepatocellular injury, viral infections, drug induced liver injury and idiosyncratic drug reactions (Bernal and Wendon 2013). In developing countries, viral infections (hepatitis A, B, and E) and drug induced liver injury (especially that of anti-tubercular drugs) are main causes of ALF. However, in the Western world, overdose of the commonly used analgesic and antipyretic agent acetaminophen (APAP) is the major cause of ALF accounting for nearly 50% of all ALF cases in the US, 70% in the UK, 40% in Sweden and 15% in Germany (Ellis 1998, Ostapowicz, Fontana et al. 2002, Bernal 2003, Larson, Polson et al. 2005, Wei, Bergquist et al. 2007, Bernal and Wendon 2013).

1.2 ACETAMINOPHEN TOXICITY

Acetaminophen (APAP) is the widely used analgesic and antipyretic drug (Kaufman, Kelly et al. 2002). It is an active ingredient in more than 600 over the counter as well as prescription medications indicated for pain, fever, cold, flu, and allergies. It is

considered safe at recommended doses however overdose of APAP causes severe liver injury, which can develop into ALF. Annually over 78,000 emergency room visits, 33,000 hospitalizations and around 500 deaths have been reported due to APAP overdose in the USA (Budnitz, Lovegrove et al. 2011, Manthripragada, Zhou et al. 2011). A daily dose of 4g is the maximum recommended dose with margin of safety (Watkins, Kaplowitz et al. 2006). However, intake of APAP exceeding the limit of 4g has been observed in intentional and unintentional overdose due to concomitant use of multiple medications containing APAP leading to Acute Liver Injury (ALI). Likewise, combination medicines containing opiates with APAP also increase risk of liver injury (Budnitz, Pollock et al. 2006).

At therapeutic doses, majority of APAP is metabolized in the liver by UDP-glucuronosyl transferase (UGT) and Sulfotransferase (SULT) accounting for 50-70% of APAP-glucuronide and 25-35% APAP-sulfate metabolites excreted in urine. About 2% of ingested APAP is excreted unchanged in urine (Fig.1.2.1). Only 5-15% of APAP is metabolized by Cytochrome P450 enzymes to reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI) that reacts with cellular glutathione (GSH) spontaneously or in presence of glutathione S-transferase (Miner and Kissinger 1979). APAP-GSH as cysteine conjugate is excreted in bile and then in urine (Cummings, King et al. 1967, McGill and Jaeschke 2013).

After APAP overdose excess NAPQI is formed, which far exceeds the amount of cellular GSH and extra free NAPQI then reacts with protein sulfhydryl groups to form protein adducts (Jaeschke, McGill et al. 2012). Binding of NAPQI to mitochondrial

proteins compromises mitochondrial respiration, stops ATP production and generates oxidative (ROS) as well as nitrosative (RNS) stress in mitochondria leading to initiation of the liver cell injury (Jaeschke, Knight et al. 2003, Jaeschke, McGill et al. 2012). These initiating events require further amplification and propagation to cause cell death, which is caused by a cascade of kinase-mediated signaling. The most prominent of these is activation of c-Jun N-terminal kinase (JNK), and Rip1/3 kinase (Hanawa, Shinohara et al. 2008, Ramachandran, McGill et al. 2013). Mitochondrial oxidative stress causes activation of mitogen activated protein kinase (MAPK) and apoptosis signal regulating kinase 1 (ASK1) which in turn activates JNK (Nakagawa, Maeda et al. 2008). Activated JNK translocates to mitochondria and further enhances mitochondrial ROS generation. Mitochondrial ROS causes mitochondrial DNA damage, BAX translocation to mitochondria, opening of mitochondrial permeability transition pore (MPT) resulting in collapse of membrane potential and decreased ATP production. These events result in mitochondrial swelling and rupture with release of endonuclease G and AIF. Both these proteins translocate to nucleus and cause massive nuclear DNA fragmentation and eventually hepatocyte necrosis (Kon, Kim et al. 2004, Jaeschke and Bajt 2006, Latchoumycandane, Goh et al. 2007, Han, Dara et al. 2013, Du, Xie et al. 2015).

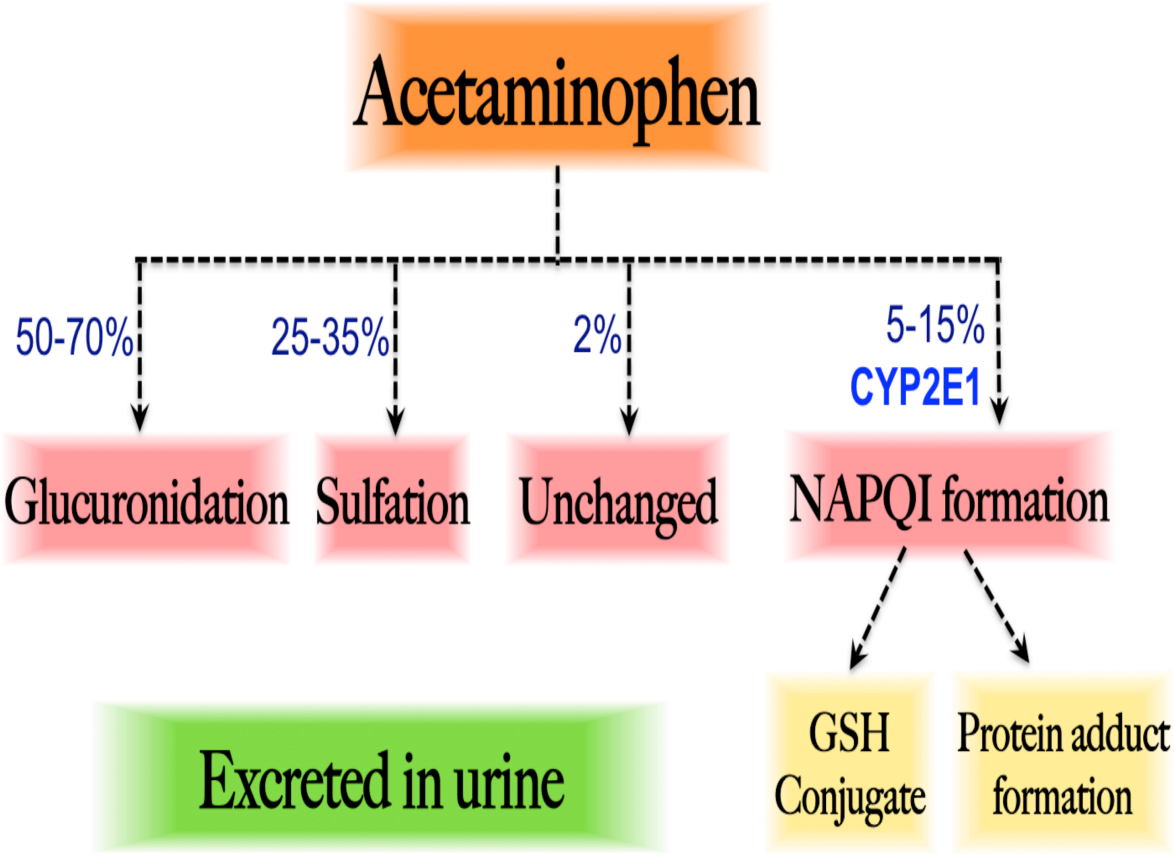


Fig. 1.2.1 Schematic of APAP metabolism. Most of ingested APAP is metabolized by glucuronidation and sulfation. Very small fraction of APAP is bioactivated by Cytochrome p450 2E1 enzyme (CYP2E1) to N-acetyl-p-benzoquinone imine (NAPQI). NAPQI can be detoxified by glutathione (GSH) or form protein adducts. All conjugated metabolites and some unchanged APAP are excreted in urine.

During hepatocyte necrosis due to APAP overdose, various damage- associated molecular patterns (DAMPs) such as nuclear DNA fragments and HMGB1 are released. These DAMPs activate Kupffer cells (KCs) through TLR receptors leading to increased cytokine and chemokine production (Jaeschke, Williams et al. 2012). Pro-inflammatory cytokines and chemokines such as IL1β, TNFα, and MCP1 activate and recruit neutrophils and monocytes to the liver (Williams, Farhood et al. 2010). Monocyte-derived M2 macrophages get recruited to the liver and secrete anti-inflammatory cytokines such as IL10. These activated phagocytes can facilitate removal of dead cells from liver and initiate wound healing process (Bourdi, Masubuchi et al. 2002, Woolbright and Jaeschke 2017). Several studies have indicated role of inflammatory mediators such as vascular endothelial growth factor (VEGF), IL6, and TNFα in promoting liver regeneration after an APAP induced liver injury as well (Chiu, Gardner et al. 2003, James, Lamps et al. 2003, Donahower, McCullough et al. 2006, Kato, Ito et al. 2011).

1.3 LIVER REGENERATION – A PLAUSIBLE THERAPY FOR ACETAMINOPHEN TOXICITY

Therapeutic intervention for ALF is a challenge because of its rapid progression, few treatment options and frequent bad outcomes. APAP overdose is a major cause of ALF in the Western world however, therapeutic options for APAP-induced ALF are extremely limited. At present, N-acetylcysteine is the only antidote for APAP and shows maximum efficacy when given within 8-10 h of overdose (Larson 2007, Fontana 2008, Lee, Larson et al. 2009). NAC is a precursor of GSH and facilitates GSH synthesis

thereby scavenge NAPQI and prevents toxicity at early stage. At later stages, NAC treatment is less effective. However, it still improves oxidative stress by facilitating GSH synthesis and, in mitochondria, supports mitochondrial energy metabolism (Smilkstein, Knapp et al. 1988, Larson 2007, Saito, Zwingmann et al. 2010). Another treatment option is liver transplantation for patients who progress to ALF. However, only 25-30% ALF patients get liver transplants due to lack of donor organs (Lee 2012). Currently, around 14,000 patients are on liver transplant waitlist. Moreover, life-long immunosuppression further complicates patients' post-transplant existence.

Several studies have underlined the importance of enhanced liver regeneration following toxic chemical induced liver injury including APAP in improving final outcome in rodents and humans (Mehendale 2005, Jaeschke and Bajt 2006, Apte, Singh et al. 2009, Bhushan, Walesky et al. 2014). Various signaling mediators have been identified that prevent progression of liver injury and promote tissue repair following APAP induced toxicity. For instance, IL6 KO mice exhibited prolonged liver injury accompanied by inhibited liver regeneration. Pretreatment with IL6 in these mice improved liver injury and restored liver regeneration following APAP toxicity(James, Lamps et al. 2003). Loss of TNFR1 and VEGFR1 in mice reduced hepatocyte proliferation and contributed to exaggerated liver injury following APAP toxicity (Chiu, Gardner et al. 2003, Donahower, McCullough et al. 2006, Kato, Ito et al. 2011). Whereas, treatment with human recombinant VEGF in WT mice prevented progression of liver injury and improved liver regeneration (Donahower, McCullough et al. 2010). Two independent studies have found importance of stem cell factor (SCF) and its receptor c-kit (progenitor cell marker) in liver regeneration after APAP overdose in mice (Simpson, Hogaboam et al. 2003, Hu

and Colletti 2008). Depletion of SCF in mice with antibody or genetic deletion increased liver injury and mortality whereas SCF treatment increased hepatocyte proliferation and improved survival after APAP toxicity (Simpson, Hogaboam et al. 2003, Hu and Colletti 2008). The role of liver regeneration in favorable outcome (survival without transplant) has also been demonstrated in patients. Two retrospective studies using APAP overdosed patient samples identified elevated α-fetoprotein (AFP) levels and hypophosphatemia as an indicator of patient survival and correlated results with improved liver regeneration in those patients (Schmidt and Dalhoff 2002, Schmidt and Dalhoff 2005).

Further, two reports correlated increased β-catenin activation with cell cycle progression and higher liver regeneration (Apte, Singh et al. 2009, Bhushan, Walesky et al. 2014). Bhushan et al. used incremental dose model in mice with 300 and 600 mg/kg APAP dose. In this model, mice treated with 300 mg/kg APAP showed liver injury accompanied by compensatory increase in regeneration, while mice treated with 600 mg/kg APAP showed injury progression and significantly inhibited liver regeneration. APAP600 animals showed reduced activation of Wnt/β-catenin pathway, a pro-regenerative mechanism, and sustained activation of cell cycle inhibitor p21, anti-proliferative mechanism. Next, β-catenin overexpression in mice improved liver regeneration following APAP600 dose. Another study using a GSK 3 inhibitor increased activation of β-catenin signaling and liver regeneration following APAP600 treatment in mice (Bhushan, Poudel et al. 2017). These studies highlighted importance of active β-catenin signaling following APAP toxicity in promoting liver regeneration and improving outcome.

These studies emphasize that stimulating liver regeneration in APAP-induced ALF patients is a plausible therapeutic option. However, mechanisms that regulate liver regeneration are not entirely known. Studying these mechanisms will provide better understanding of the dynamics of liver regeneration and may lead to improved therapeutic intervention for ALF patients.

1.4 DNA DAMAGE RESPONSE (DDR) AND ITS ROLE IN APAP TOXICITY

Cellular DNA is constantly subjected to assault from exposure to endogenous (e.g. metabolic ROS, replicative stress) and environmental (e.g. chemicals, radiation) stress (Kawanishi, Hiraku et al. 2006, Valko, Rhodes et al. 2006). Estimations suggest that a cell could experience up to 10^5 DNA lesions per day due to various insults (Lindahl and Barnes 2000). The maintenance of DNA integrity and stability is an essential task to both cellular function and survival, as uncorrected insult could cause mutation and possibly a disease. To efficiently manage DNA damage resulting from various insults, a broad cellular response is elicited and it is referred to as DDR. DDR is an integrated signaling network that senses DNA damage, replication stress and transduces this information to induce protective mechanisms in the cell and influence cellular responses to DNA damage (Fig. 2). In response to DNA damage, the pathways of DDR co-ordinate cell cycle progression with DNA repair, chromatin remodeling, transcriptional programs and other metabolic adjustments including cell death (Zhou and Elledge 2000, Ciccia and Elledge 2010). Proteins involved in DDR can be

categorized in four groups including damage sensors, signal transducers, mediators and outcome effectors. The sensor proteins such as PARP sense the DNA damage and generate signals in order to recruit and activate transducer proteins. These signals are amplified and propagated by transducers such as kinases (ATM, CHK1) and mediator proteins (53BP1, H2AX). This initiates a signaling cascade to co-ordinate effector proteins such as p53 (Sulli, Di Micco et al. 2012). Mediator proteins can also recruit DNA repair effector proteins to the damaged DNA site, which are responsible for repairing the damaged DNA (Javle and Curtin 2011, Sulli, Di Micco et al. 2012).

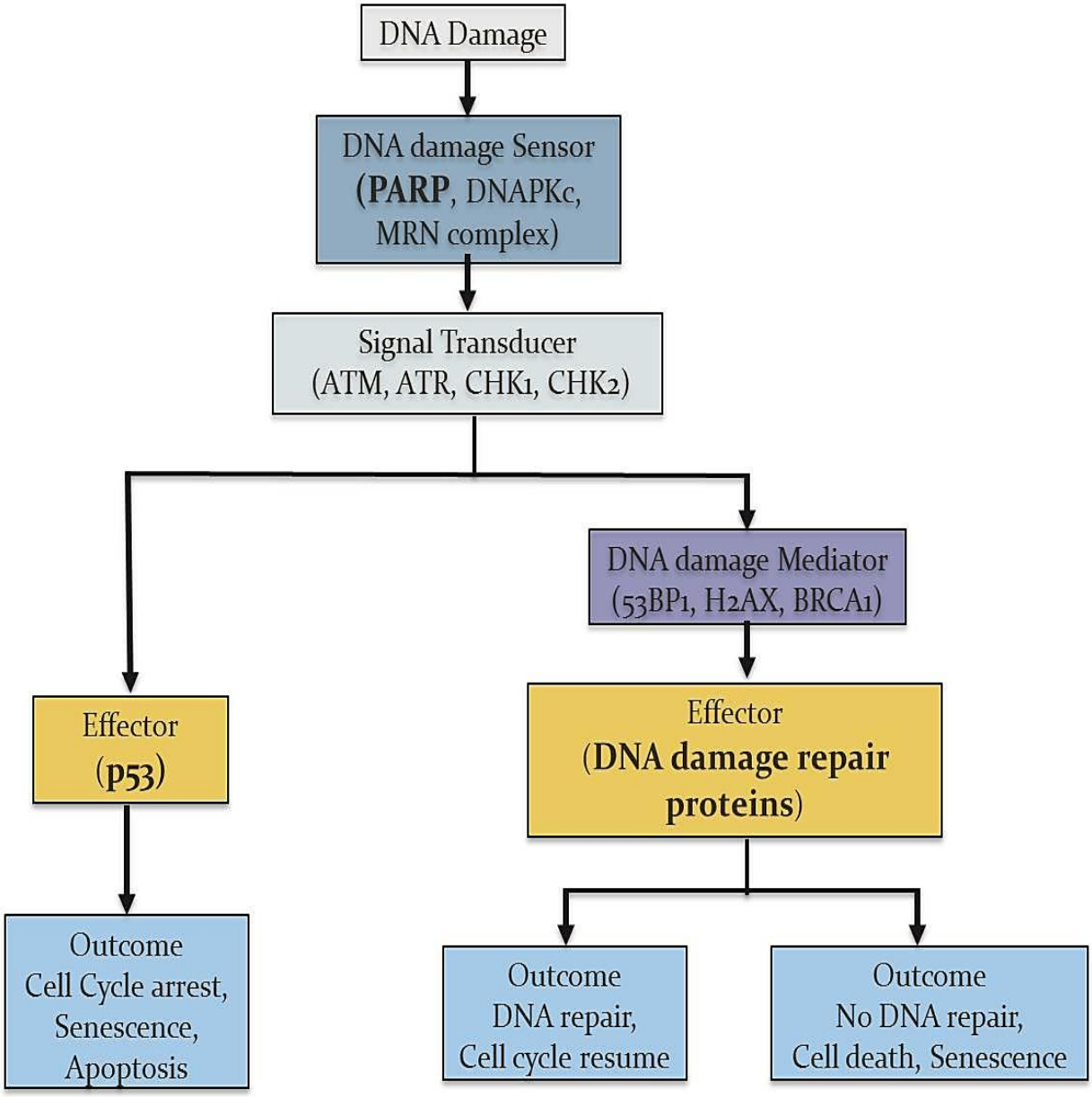


Fig.1.4.1. Schematic representation of double strand break DNA damage signaling and repair pathway. After detection of DNA damage by sensor proteins (PARP, MRN, DNAPKc), this signal is amplified and diversified by multiple transducer proteins (ATM, ATR, CHK1, CHK2) to mediator proteins (53BP1, H2AX, BRCA1) and effector proteins (DNA repair proteins and p53). These downstream effector proteins regulate various cellular functions (DNA repair, cell cycle arrest, senescence, cell death) PARP: PolyADPribose polymerase, MRN : Mre11 RAD500 NBS1 complex, DNAPKcs: DNA dependent protein kinase catalytic subunit, ATM: Ataxia telangiectasia mutated, ATR: ATM related, CHK1, CHK2: Checkpoint kinase 1 and 2, 53BP1: p53 binding protein 1, BRCA1: Breast cancer 1

APAP toxicity involves a sequential chain of events beginning with formation of a reactive metabolite (NAPQI), increased ROS, mitochondrial damage, nuclear DNA fragmentation and necrotic cell death. To compensate for necrotic cell death, hepatocytes undergo proliferation and the liver regenerates following APAP toxicity. However, if severe DNA fragmentation, ROS or replicative stress after APAP overdose can elicit DDR is not well studied. Previously, one study showed that APAP causes increased phosphorylation of histone H2AX at Ser 139 as a marker of DNA double strand break in inner medullary collecting duct (p1rIMCD) cells. Increased toxicity of APAP when combined with caffeine in p1rIMCD cells was speculated due to inhibition of DNA damage repair (Cai, Dmitrieva et al. 2003). Several studies have investigated role of DDR sensor protein PARP in pathophysiology of APAP overdose (Shen, Kamendulis et al. 1992, Kroger, Dietrich et al. 1997, Cover, Fickert et al. 2005). PARP1 is activated after APAP mediated DNA damage and its activation did not contribute to APAP toxicity in mice (Cover, Fickert et al. 2005). However, involvement of PARP in DNA repair and liver regeneration post APAP toxicity is not yet studied.

1.5 DOUBLE STRAND BREAK (DSB) REPAIR AND ITS ROLE IN APAP PATHOPHYSIOLOGY

DNA lesions that elicit the DDR can be classified based on structural manifestation including single strand break (SSB) and double strand break (DSB). These can be further classified based on chemical nature of insult as bulky adduct formation, base mismatch, base insertion or deletion, base alkylation, base oxidation. Repair mechanisms are specific to the type of lesion and the phase of the cell cycle. The most subtle changes, such as oxidative lesions, alkylation and SSBs are repaired through the base excision repair (BER) mechanism. Missmatch repair (MMR) replaces mispaired DNA bases with correct bases. Bulkier DNA single strand lesions are repaired through the nucleotide excision repair (NER) mechanism (Polo and Jackson 2011, Lord and Ashworth 2012). DSB is considered the most deleterious lesion and is repaired through non-homologous end joining (NHEJ) at all phases of the cell cycle or through homologous recombination (HR) during S and G2 phases (Chapman, Taylor et al. 2012). During HR, an undamaged copy of DNA that shares sequence homology with broken DNA is used to repair the break. In contrast, NHEJ ligates two broken DNA ends, without the requirement for sequence homology (Jackson 2002).

DDR following DNA DSB involves post-translational modifications of histones and histone-binding proteins near damaged site. The earliest modification is Poly(ADP-ribosyl)ation (also called PARylation) of lysine residues in histones, binding of chromatin modifiers NuRD (nucleosome remodeling and deacetylase) and polycomb complexes to PAR chains (Javle and Curtin 2011). These chromatin modifiers enable recruitment and

activity of the RING finger protein 8 (RNF8) and RING finger protein 168 (RNF168) ubiquitin ligases, which are also implicated in DNA repair (Ciccia and Elledge 2010, Bekker-Jensen and Mailand 2011, Polo and Jackson 2011). Another important histone modification and a hallmark of DSB is phosphorylation of histone H2AX on Ser139 (γ-H2AX) by apical kinases such as ATM, ATR, DNAPkcs. This event is followed by dephosphorylation of the Tyr142 residue by EYA phosphatse. The Tyr142 residue on H2AX is constitutively phosphorylated by WSTF kinase in the absence of damage. Dephosphorylation of Tyr142 is required for binding of MDC1- sensor protein, to γ-H2AX (Cook, Ju et al. 2009, Xiao, Li et al. 2009). Further MDC1 and NBS1 anchor activated ATM spreads H2AX phosphorylation to adjacent nucleosomes. Activated ATM also phosphorylates MDC1, which triggers recruitment of RNF8, to initiate an ubiquitylation cascade, which is amplified by RNF168. This facilitates the recruitment of genome caretakers such as BRCA1, RAP80, RAD18, PTIP, 53BP1 etc. (Lukas, Lukas et al. 2011). BRCA1 recruitment at DSB site favors the repair through HR while 53BP1 favors NHEJ. DNA end resection is another important deciding factor for DSB repair pathway choice as it is required for homology searching during HR. CtIP mediates end resection. Interaction of CtIP with BRCA1 and its end resection activity is dependent on CDK1 mediated phosphorylation, which is higher during S and G2 phase of cell cycle. In contrast, during G1 phase 53BP1 and RIF1 inhibit BRCA1 recruitment to DSB site, hence favoring the NHEJ pathway. 53BP1 also prevent the 5' end resection and hence promote the binding of Ku70/Ku80 flanking the DSB (Zhou and Elledge 2000, Rothkamm, Kruger et al. 2003, You and Bailis 2010).

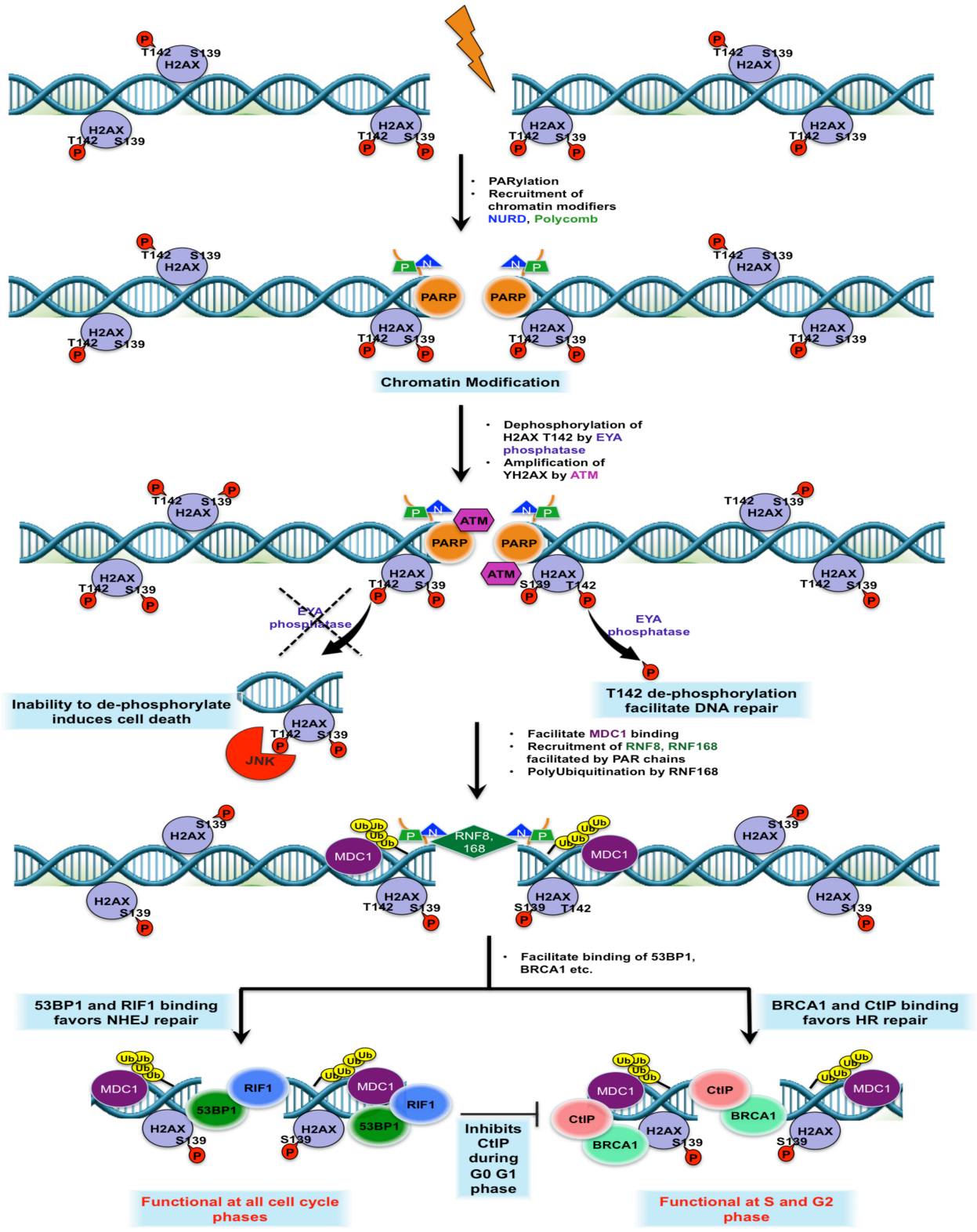

H2AX
T142
S139
P
PARP
ATM
PARylation
Recruitment of chromatin modifiers NURD, Polycomb
Chromatin Modification
Dephosphorylation of H2AX T142 by EYA phosphatase
Amplification of YH2AX by ATM
EYA phosphatase
Inability to de-phosphorylate induces cell death
JNK
T142 de-phosphorylation facilitate DNA repair
Facilitate MDC1 binding
Recruitment of RNF8, RNF168 facilitated by PAR chains
PolyUbiquitination by RNF168
MDC1
RNF8, 168
Ub
Facilitate binding of 53BP1, BRCA1 etc.
53BP1 and RIF1 binding favors NHEJ repair
BRCA1 and CtIP binding favors HR repair
RIF1
53BP1
CtIP
BRCA1
Inhibits CtIP during G0 G1 phase
Functional at all cell cycle phases
Functional at S and G2 phase

Fig. 1.5.1 Schematic showing initiation of DSB repair. Following DNA DSB histone H2AX get phosphorylated at Ser139 (γ-H2AX) and chromatin modulators (PARP, NuRD) modifies chromatin favorable to DNA repair. EYA phosphatase dephosphorylate H2AX-Tyr 142 residue and ATM amplifies γ-H2AX. Binding of MDC1 and RNF8/168 mediated ubiquitination facilitate recruitment of 53BP1 (during all cycle phases) or BRCA1 (during S/G2 phase). 53BP1 binding mediates NHEJ repair while BRCA1 mediates HR repair. PARP: PolyADPribose polymerase, PARylation: Poly ADP Ribosylation, NuRD: nucleosome remodeling and deacetylase complex, EYA: Eyes Absent phosphatase, MDC1: Mediator of DNA Damage Checkpoint 1, RNF8,168: Ring Finger Protein 8, 168, ATM: Ataxia Telangiectasia Mutated, 53BP1: p53 binding protein, BRCA1: Breast Cancer 1 protein, RIF1: Replication Timing Regulatory Factor 1, CtIP: the CtBP (carboxy-terminal binding protein) interacting protein.

1.5.1 NONHOMOLOGOUS END JOINING REPAIR PATHWAY

During NHEJ, Ku70/80 heterodimer detects and binds to the extreme termini of the DSB through its high affinity for double stranded DNA ends. It is required to propagate signal to the subsequent NHEJ factors (Fig. 3). Binding of the KU heterodimer facilitate recruitment of a serine threonine protein kinase - DNA-dependent protein kinase catalytic subunit (DNA-PKcs), of the phosphatidyl inositol 3-kinase-like protein kinase (PIKK) family. DNA-PKcs and Ku form a holoenzyme and hold juxtaposed DNA ends in a complex. Formation of the holoenzyme enhances DNA PKcs kinase activity, leading to phosphorylation of several NHEJ associated proteins including KU70, KU80, XRCC4, XLF, Artemis, and LIG4 (Hartlerode and Scully 2009). These factors then process the juxtaposed DNA ends while repairing the DSB. If the DSB results in non-ligatable ends containing 3’ phosphate, 3’ phosphoglycolate or 5’ hydroxyl etc., these must be converted to 3’ hydroxyls and 5’ phosphates or removed. Polynucleotide kinase/phosphatase (PNKP), a DNA 3’ phosphatase/5’ kinase processes these non-ligatable ends before ligation. Nucleases such as exonuclease 1(Exo1) and Artemis may trim the 3′ and 5′ single-stranded overhangs to reveal DNA sequence microhomology (Ma, Pannicke et al. 2002). After DNA end processing any short nucleotide gaps are filled by DNA polymerases μ and λ by adding nucleotides. X-ray cross-complementing gene 4 (XRCC4) and XRCC4-like factor (XLF or Cernunnos) don’t have catalytic activity but interact with PNKP and DNA ligase IV (LIG4). Once complementary ends have been generated, LIG4 joins the end in presence of a

complex containing XRCC4 and XLF (Zhou and Elledge 2000, Rothkamm, Kruger et al. 2003, Lieber 2010, You and Bailis 2010, Polo and Jackson 2011).

Finally, γ-H2AX must be dephosphorylated once the DNA is repaired to restore the genome. During DDR signaling cascade effector protein p53 gets activated which halts the cell cycle while damaged DNA is repaired. WIP1 phosphatase is a target of p53 and it dephosphorylates γ-H2AX (Moon, Nguyen et al. 2010). WIP1 can also dephosphorylate MDM2 and facilitate MDM2 mediated p53 ubiquitination and degradation. WIP1-p53-MDM2 form a feedback mechanism to terminate the DDR.

In response to partial hepatectomy (PHX) or liver injury, remaining hepatocytes undergo cell division rapidly. That increases DNA replication associated risk of DNA damage (Saintigny, Delacote et al. 2001). A recent study has shown that genetic deletion of nucleostemin in mice results in increased DNA double strand breaks in regenerating liver following PHX (Lin, Ibrahim et al. 2013). However there is no investigation done to study role of DNA damage in liver regeneration following APAP toxicity.

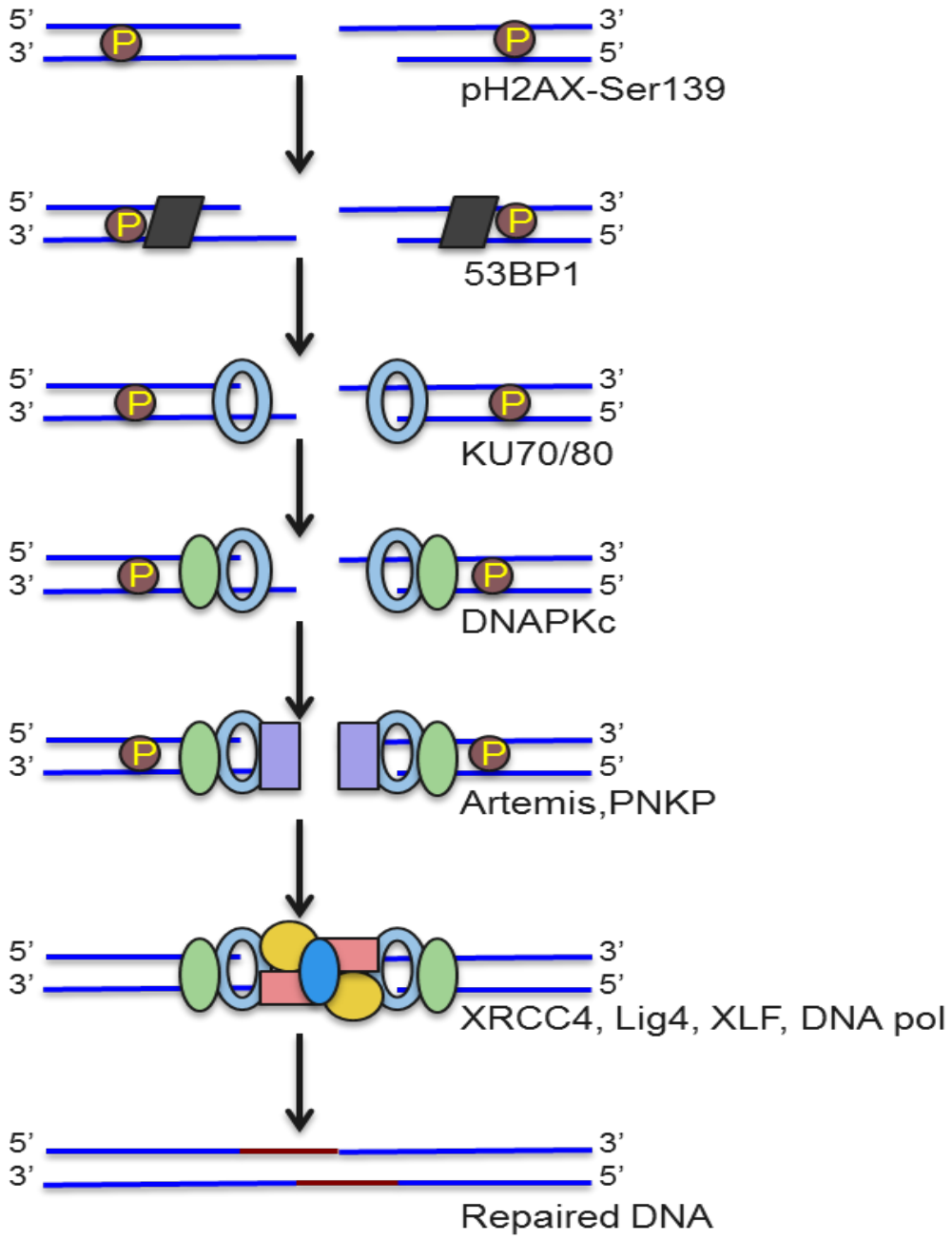


Fig.1.5.1.1. Schematic representation of Double strand break repair by NHEJ pathway. DNA repair process is initiated by binding of 53BP1 (p53 binding protein 1) at DNA ends then recruiting Ku70/80 and two DNA-PK_{CS} (DNA dependent protein kinase catalytic subunit) molecules. Activated DNA PK help recruit proteins involved in DNA end processing e.g. Artemis, PNKP (Polynucleotide kinase 3' phosphatase) to form ligatable termini. The break is then repaired by the Lig4(ligase 4), XRCC4(X-ray repair cross-complementing protein 4), XLF (XRCC4 like factor) and DNA pol (DNA polymerase) complex.

1.6 P53 AND ITS ROLE IN APAP PATHOPHYSIOLOGY

DDR pathway coordinates cell cycle checkpoint activation and DNA repair proteins by arresting the cell cycle, thus allowing time for repair processes. The key effector protein that co-ordinate these processes is p53. It plays a critical role in deciding cell fate following DNA damage. Widely known as the “cellular gatekeeper”, p53 is a tetrameric transcription factor which binds as dimers of dimers to sequence-specific p53 response elements and stimulates or represses transcription of many protein-coding and non-protein-coding genes including microRNAs and lincRNAs (McLure and Lee 1998, He, He et al. 2007). Cellular levels of p53 are primarily controlled by mouse double minute protein 2 (MDM2) through its ubiquitin-mediated proteasomal degradation (Haupt, Maya et al. 1997, Levine and Oren 2009). However, activity of MDM2 is inhibited by several mechanisms including ARF interference of Mdm2-p53 interaction, ATM, c-Abl mediated phosphorylation, and CBP/p300-mediated acetylation that results in increased p53 levels (Kruse and Gu 2009, Shi and Gu 2012). p53 is stabilized by several post-translational modifications. In response to stress, more than 36 amino acids in p53 are post-translationally modified by phosphorylation, ubiquitination, acetylation, methylation, sumoylation, glycosylation or ribosylation (DeHart, Chahal et al. 2014). All these post-translational modifications lead to stabilization, accumulation and activation of p53 transcriptional function. After DNA damage Ser-15 and Ser-20 residues of p53 are phosphorylated by a number of kinases including ATM, ATR, DNAPKc, Chk1, Chk2(Kastan and Lim 2000, Ou, Chung et al. 2005, Zannini, Delia et al. 2014). Phosphorylation of p53 at Ser-15 prevents its

interaction with MDM2 inhibiting its ubiquitination and degradation. Stabilized p53 binds to p53 response elements in the presence of co-activators or co-repressors and regulates target gene expression. Following DNA damage, the activated p53 triggers cell cycle arrest until damaged DNA is repaired. If the DNA damage is beyond repair, p53 can induce cell senescence or apoptosis (Haupt, Berger et al. 2003, Carvajal and Manfredi 2013, Qian and Chen 2013).

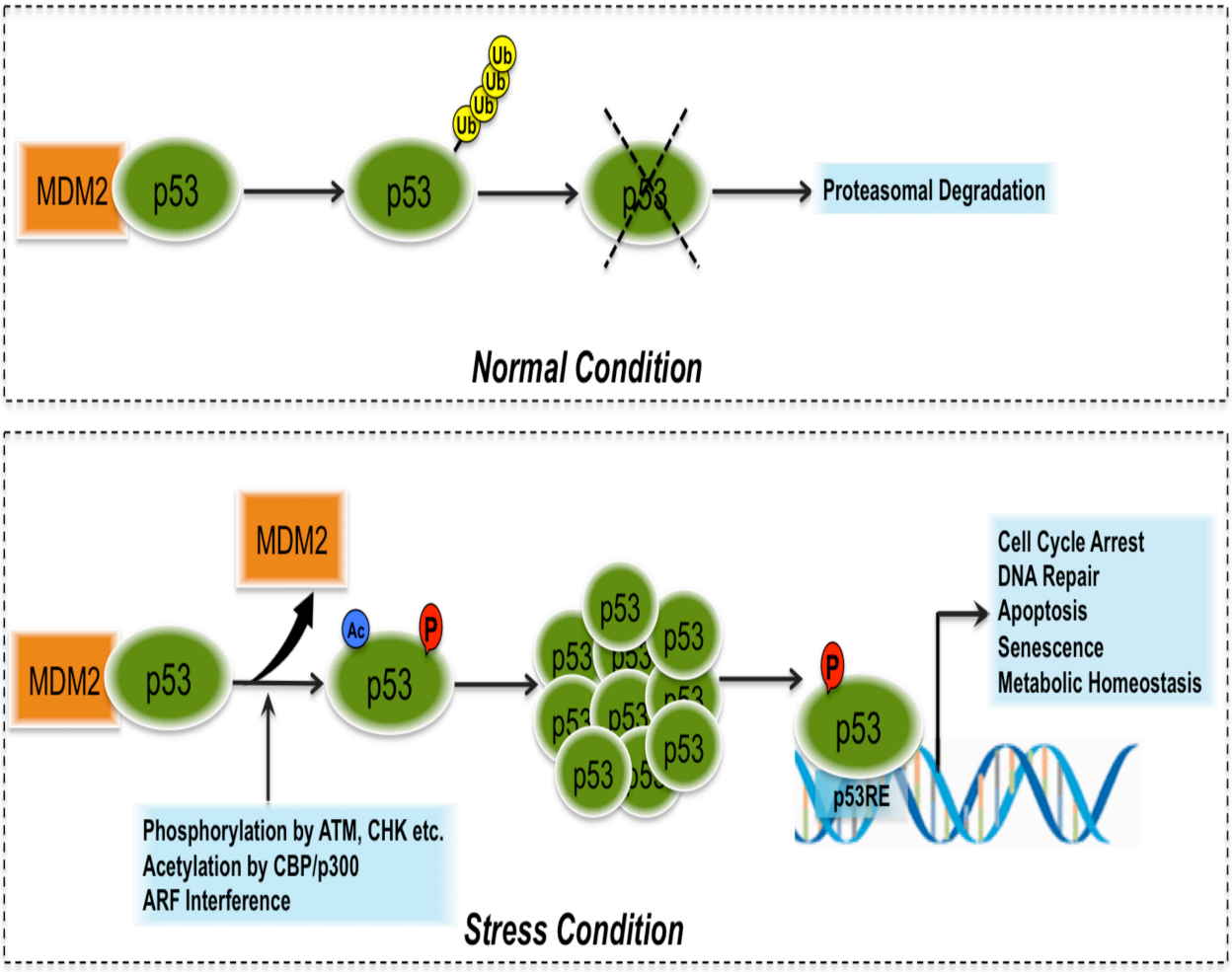


Figure 1.6.1 Schematic of p53 activation pathway. During normal conditions, p53 is degraded through MDM2 mediated ubiquitination. Stress induces stabilaization of p53 by ATM, CHK and other kinases mediated phosphorytion that results in activation or repression of p53 target genes. MDM2: Mouse double minute 2 E3 ubiquitin protein ligase.

In recent years a significant number of p53 responsive genes have been identified, which respond to various forms of cellular stresses including DNA damage, ROS, metabolic homeostasis (Kruiswijk, Labuschagne et al. 2015). p53 target genes involved in various cellular processes are listed in Table 1.6.1. The functions of p53 have been primarily studied in tumor models and cultured cancer cell lines. Although numerous studies have shown role of p53 in various pathophysiological conditions beyond cancer e.g. development, aging, obesity, diabetes, its role in compensatory cell proliferation after drug-induced liver injury is not known.

Previous studies in rodents using partial hepatectomy model have depicted a role of p53 in regulation of ploidy and cell proliferation during liver regeneration (Stepniak, Ricci et al. 2006, Kurinna, Stratton et al. 2010, Kurinna, Stratton et al. 2013, Jin, Hong et al. 2015, Zhang, Liu et al. 2015). p53 controls cell proliferation by binding a p53RE near FOXO3 transcription start site and maintains active expression of Foxo3 - a cell proliferation inhibitor - in quiescent mouse liver. However, during liver regeneration in mice following PHX, p53 binding and expression of Foxo3 is reduced (Kurinna, Stratton et al. 2010). Further, deletion of p53 rescues liver regeneration in mice lacking c-jun in liver, suggesting role of p53 in inhibiting liver regeneration (Stepniak, Ricci et al. 2006). In contrast, mice with C/EBPα-S193A mutation and low p53 expression failed to stop liver regeneration following PHX (Jin, Hong et al. 2015). As discussed in Chapter 1.5.1 WIP1 phosphatase is a target of p53. WIP1 can dephosphorylate MDM2 and facilitate MDM2 mediated p53 degradation results in feedback loop formation. Wip1 KO mice showed higher liver regeneration despite p53 expression and p53 deletion in Wip1 KO mice further increases regeneration indicating an anti-proliferative role of p53 in liver

regeneration (Zhang, Liu et al. 2015). Furthermore, p53KO mice fail to resolve polyploidy following PHX (Kurinna, Stratton et al. 2013).

A recent study showed that deletion or chemical inhibition of p53 increases APAP toxicity in mice indicating protective role of p53 in pathophysiology of APAP overdose (Huo, Yin et al. 2017). Previous studies using *in vitro* and *in vivo* models of APAP overdose have indicated a protective role of p53 in pathophysiology of APAP toxicity (Stamper, Garcia et al. 2015). Whereas these studies indicated that p53 may play a novel protective role in acute liver injury, they did not put forth any mechanistic details.

Table 1.6.1. Representative p53 target genes

Cellular Processes	**P53 Target Genes**
Cell Cycle Arrest	GADD45, p21, BTG2, mir34
Senescence	p21, PAI1, PML
Apoptosis	BAX, Survivin, PUMA
DNA repair	GADD45, DDB2, RRM2
Metabolic Homeostasis	SCO2, AMPK, PFK
ROS clearance	GPX, MnSOD, Sestrin, ALDH4, GAMT
Autophagy	TIGAR, AMPK,

1.7 PARP AND ITS ROLE IN APAP PATHOPHYSIOLOGY

Poly(ADP-ribose) (PAR) polymerase-1 (PARP-1) is a stress sensor and a stress response mediator protein (Luo and Kraus 2012, Filomeni, De Zio et al. 2015). PARP-1 uses NAD^+ to add ADP ribose units in PAR polymers on nuclear proteins such as histones, DNA repair proteins, transcription factors, and chromatin modulators (Kraus 2008, Luo and Kraus 2012). PAR glycohydrolase (PARG) hydrolyzes the covalently attached polymers to free PAR or mono(ADP-ribose). PARP-1 can bind to intact and damaged DNA. It is activated during genotoxic stress facilitating DNA repair, chromatin modification, and transcription. In the presence of low DNA damage, PARP1 functions as a survival factor, PARylates and remodels DNA damage. Hyperactivation of PARP depletes NAD^+ levels and subsequent cellular ATP resulting necrotic cell death (Swindall, Stanley et al. 2013, Weaver and Yang 2013).

The role of PARP has been studied in DNA repair after arsenic toxicity. Studies show that arsenite directly inhibits PARP enzyme activity and prevents DNA repair, leading to toxicity (Sun, Zhou et al. 2014, Luo, Li et al. 2015). Previous studies show that PARP is activated within 6-12 hours after APAP overdose in mice and PARP activation was believed to play role in promoting injury. However, PARP inhibition using PARP KO mice or specific PARP inhibitor do not prevent APAP mediated liver injury. In fact, PARP KO developed exaggerated injury when compared to WT mice, suggesting a protective role in injury development after APAP overdose (Cover, Fickert et al. 2005). However,PARPs role in DNA repair and liver regeneration after APAP overdose is not known.

1.8 STATEMENT OF PURPOSE

Overdose of APAP is the cause of almost 50% of all ALF cases in the US, 70% in the UK, 40% in Sweden and 15% in Germany (Ellis 1998, Ostapowicz, Fontana et al. 2002, Bernal 2003, Larson, Polson et al. 2005, Wei, Bergquist et al. 2007, Bernal and Wendon 2013). Despite being the major cause of ALF in Western world, therapeutic options for APAP induced ALF are extremely limited. There is a critical need to develop novel therapeutic strategies for APAP induced ALF cases. Recent studies using experimental models and ALF patient samples demonstrated that liver regeneration is a critical determinant of final outcome (Schmidt and Dalhoff 2002, Chiu, Gardner et al. 2003, James, Lamps et al. 2003, Simpson, Hogaboam et al. 2003, Schmidt and Dalhoff 2005, Donahower, McCullough et al. 2006, Hu and Colletti 2008, Apte, Singh et al. 2009, Donahower, McCullough et al. 2010, Kato, Ito et al. 2011, Bhushan, Walesky et al. 2014, Bhushan, Poudel et al. 2017). Thus, stimulating liver regeneration in patients of APAP-induced ALF holds great therapeutic potential. However, development of novel regenerative therapies for ALF is hampered because the mechanisms of liver regeneration after APAP-induced ALF are less understood. The long-term goal of Apte laboratory is to develop regenerative therapies based on improved liver regeneration for APAP-induced ALF. APAP mediated liver injury involves generation of ROS, release of endonucleases, severe DNA fragmentation leading to necrosis in centrilobular region (McGill, Sharpe et al. 2012). In response to injury, healthy hepatocytes surrounding the necrotic zone undergo rapid cell proliferation and liver regenerates the injured mass (Mehendale 2005, Jaeschke and Bajt 2006, Apte, Singh et al. 2009, Bhushan, Walesky

et al. 2014). Hence, rapidly dividing hepatocytes surrounding the necrotic zone may have high propensity for DNA insult (Saintigny, Delacote et al. 2001). This DNA insult may trigger the DDR to repair the damage and allow cell cycle progression making it a critical step in deciding whether regeneration will be 'timely' or 'delayed'. Hence, the objective of this dissertation work was to investigate role of DDR in liver regeneration and identify targets with therapeutic potential.

We used an incremental dose model consisting of a regenerative low dose and non-regenerative high dose of APAP to delineate the mechanisms that inhibit liver regeneration following APAP-induced ALF. Our hypothesis was that DDR should be impaired and checkpoint activation should be prolonged at high dose APAP relative to low dose APAP. Thus, differential regulation of DNA damage repair pathways and checkpoint activation mediated by p53 after APAP-induced toxicity could be analyzed and potential targets to stimulate regeneration after APAP overdose could be identified. Further, p53 regulates several cellular processes including DDR. Hence, we hypothesized that p53 plays critical role in pathophysiology of APAP toxicity such that it can connect liver injury with regeneration. This was investigated using p53KO mice.

CHAPTER II: INVESTIGATING ROLE OF DNA DAMAGE RESPONSE PATHWAY IN LIVER REGENERATION FOLLOWING ACETAMINOPHEN OVERDOSE

2.1 ABSTRACT

Acetaminophen (APAP) overdose is a leading cause of acute liver failure (ALF) with limited treatment options. Previous studies have shown that liver regeneration following APAP induced ALF is a deciding factor in patient outcome. A recent study from our laboratory using an incremental dose model involving a regenerating (300 mg/kg, APAP300) and a non-regenerating (600 mg/kg, APAP600) dose of APAP in mice have revealed several pro- regenerative pathways that can improved survival after APAP overdose. Here we report the role of DNA damage and repair mechanisms in inhibition of liver regeneration in the non-regenerating dose of APAP. Liver injury, regeneration and microarray-based global gene expression changes were studied in male C57BL6 mice over time course of 0 to 96 hours following treatment with either APAP300 or APAP600. The ingenuity pathway analysis of microarray data revealed significant differences in DNA damage, replication and checkpoint related pathways in regenerating and non-regenerating doses of APAP. Phosphorylation of histone H2AX (pH2AX), a marker of the DNA double strand break (DSB), was significantly prolonged in mice treated with APAP600 than APAP300. In APAP300 treated mice H2AX was rapidly dephosphorylated at Tyr 142, an indication of beginning of the DNA repair process. However, HA2X phosphorylation was sustained in APAP600 for the entire timecourse. Expression of several DNA repair proteins was substantially lower in APAP600. Poly (ADP) ribose polymerase (PARP) activation, involved in DNA repair, was significantly higher in APAP300 treated mice as compared to APAP600 treated mice. Activation of p53, the major cell cycle checkpoint protein, was significantly higher

in APAP600 as demonstrated by substantially higher expression of its target genes. Taken together, these data illustrate that DNA damage occurs in high dose APAP toxicity and lack of prompt DSB repair after APAP overdose leads to prolonged growth arrest and proliferative senescence resulting in inhibited liver regeneration.

2.2 INTRODUCTION

Acetaminophen (APAP) is widely used analgesic and antipyretic drug present in several over the counter and prescription medications. It is safe at therapeutic doses of ≤ 4 gm per day. However; overdose of APAP can cause acute liver injury (ALI) and even acute liver failure (ALF).(Shiffman, Rohay et al. 2015) Overdose of APAP is the cause of almost 50% of ALF cases in the US with close to 35% mortality (Ostapowicz, Fontana et al. 2002, Larson, Polson et al. 2005). Despite being the major cause of ALF in Western world, therapeutic options for APAP induced ALF are extremely limited. Several studies in patients and rodents have demonstrated that stimulation of liver regeneration improves survival and prognosis after APAP overdose(James, Lamps et al. 2003, Schmidt and Dalhoff 2005, Hu and Colletti 2008, Apte, Singh et al. 2009, Donahower, McCullough et al. 2010, Bhushan, Walesky et al. 2014). Although these studies implicate enhanced liver regeneration in the APAP-induced ALF patients as a plausible therapeutic option, the mechanisms are not entirely known. Especially, the role of DNA damage response (DDR) in regulation of liver regeneration after APAP induced ALI has not been investigated.

DDR involves proteins that sense DNA damage and trigger a repair response to protect the cell. Sensor proteins in DDR detect the damage and send the signal to mediator and effector proteins via activation of apical kinases. Mediator proteins recruit DNA repair effector proteins at the damaged DNA site, which then carry out the repair process(Zhou and Elledge 2000, Sulli, Di Micco et al. 2012). One of the major effector proteins in DDR is p53 that activates cell cycle checkpoints and induce cell cycle arrest

till damage is repaired. However, if damage is beyond repair it can activate the cell death pathway(Carvajal and Manfredi 2013). Previous studies have shown that APAP injury results in nuclear DNA fragmentation preventing the cell proliferation by inducing cell cycle arrest(Ray, Sorge et al. 1990, Shen, Kamendulis et al. 1992, Bhushan, Walesky et al. 2014).

We investigated the role of DDR in liver regeneration after APAP toxicity using an incremental dose model that includes a regenerating (300 mg/kg, APAP300) and a non-regenerating (600 mg/kg, APAP600) dose in mice(Bhushan, Walesky et al. 2014). Our studies indicated that APAP overdose results in dose dependent DNA damage but at higher doses the DNA repair mechanisms fail resulting in initiation of cellular senescence and inhibition of liver regeneration. These studies have revealed novel mechanisms connecting cellular injury to initiation of liver regeneration after APAP overdose.

2.3 MATERIALS AND METHODS

Animals, Treatment and Tissue Harvesting.

All animal experiments were performed in compliance with protocols approved by the Institutional Animal Care and Use Committee at University of Kansas Medical Center. The details of incremental dose model have been previously published(Bhushan, Walesky et al. 2014). Briefly, male C57BL/6 mice were fasted overnight and injected with either 300 or 600 mg/kg APAP intraperitoneally (i.p. dissolved in warm saline). Mice (n = 5 to 7) were sacrificed at 0, 3, 6, 12, 24, 48, 72 and 96 hours after APAP treatment and blood and livers were collected. Parts of liver tissue were processed separately to obtain paraffin sections, frozen sections, RNA samples, nuclear, cytoplasmic and RIPA total protein extracts as described previously(Wolfe, Thomas et al. 2011). Liver injury was assessed by serum alanine aminotransferase (ALT) activity. Liver regeneration was assessed using PCNA analysis as described before.

Antibodies.

The following antibodies were used for analyses: (#4588) KU70, (#9532) PARP, (#9718) p-H2AX Ser139, (#2524) p53, (#9284) p-p53 S15 from Cell Signaling Technologies (Danvers, MA), (SC9051) DNAPkc, (SC1485) KU80, (SC166488) XLF, (SC8285) XRCC4, (SC28232) DNA Lig4, (SC642) BRCA1 from Santacruz Biotech. (#07-1590) p-H2Ax Tyr 142 from EMD Millipore Corporation (Billerica, MA), (PP-H1415-

00) HNF4α from Perseus proteomics, (nb100-904) 53BP1 from Novus Biolabs and (#1020) PAR from Tulips Biolabs. All Alexa fluor secondary antibodies were purchased from Invitrogen, Thermo Fisher and HRP conjugated secondary antibodies were purchased from Cell Signaling Technologies (Danvers, MA).

Western Blotting.

Protein estimation of RIPA and nuclear extracts was done by BCA method and Western blot analysis was performed using pooled protein extracts as described before(Wolfe, Thomas et al. 2011).

Immunofluorescence staining.

Fresh-frozen liver sections (5 μm thick) were used to detect pH2AX Ser 139 immunofluorescence as described before(Walesky, Gunewardena et al. 2013).

Real Time PCR.

Total RNA was isolated from APAP300 and APAP600 livers using Trizol method according to the manufacturer's protocol (Sigma, St. Louis, MO) and converted to cDNA as previously described (Apte, Singh et al. 2009). Gene expression of various genes was determined by comparing mRNA levels from APAP treated groups at different time point with 0 hour control group using Real Time PCR analysis. SYBR Green technology

was used for Real time PCR analysis on the Applied Biosystems Prism 7300 Real-time PCR Instrument according to manufacturer's protocol. 18s gene expression in the same samples was used for data normalization. Primers used for real time PCR are listed in Table 2.3.1.

Table 2.3.1. Primers used in this study

Gene	Forward (5'-3')	Reverse (5'-3')
GADD45α	CCGAAAGGATGGACACGGTG	TTATCGGGGTCTACGTTGAGC
GADD45β	CAACGCGGTTCAGAAGATGC	GGTCCACATTCATCAGTTTGGC
GADD45γ	GGGAAAGCACTGCACGAACT	AGCACGCAAAAGGTCACATTG
BIRC5	AAGGAATTGGAAGGCTGGG	TTCTTGACAGTGAGGAAGGC
BTG2	ATGAGCCACGGGAAGAGAAC	GCCCTACTGAAAACCTTGAGTC
PAI-1	TTCAGCCCTTGCTTGCCTC	ACACTTTTACTCCGAAGTCGGT

Microarray Analysis.

Gene array experiments were conducted on pooled liver samples (n=3 per group) from four timepoints (0, 3, 12, 24, 48 hours after APAP administration). Ingenuity

Pathway Analysis (IPA) of gene array data was conducted as described before (Walesky, Gunewardena et al. 2013).

Statistical Analysis.

Data are shown as mean ± SEM. Student's T-test was used for statistical analysis. Difference between groups was considered statistically significant at $P<0.05$ and indicated by * in graphs.

2.4 RESULTS

Sustained liver injury and inhibited liver regeneration following higher dose of APAP

Liver injury after APAP300 and APAP600 treatment was assessed using serum ALT and histopathological analysis of liver tissue over 0 to 96 hours time course(Bhushan, Walesky et al. 2014). At both doses, serum ALT activity was increased and peaked at 12 hours after treatment. In APAP300 treated mice, serum ALT activity regressed after 24 hours and returned to normal by 72 and 96 hours. However, in APAP600 treated mice ALT activity remained high up to 24 hours and later decreased but was persistently higher until 96 hours after APAP treatment as compared to APAP300 (Fig. 2.4.1A). All mice receiving APAP300 dose recovered from injury, whereas mice with APAP600 treatment showed 25% lethality and remaining mice had sustained injury up to 96 hours(Bhushan, Walesky et al. 2014).

To determine the difference in liver regeneration after two doses of APAP, we determined expression of PCNA in mouse liver(Bhushan, Walesky et al. 2014). Western blot analysis of PCNA revealed significantly delayed and reduced cell proliferation after APAP600 dose as compared to APAP300 dose (Fig. 1B and 1C). In APAP300 group, significant increase in PCNA was observed from 24 hours upto 72 hours. However, in APAP600 group, PCNA expression was delayed untill 48 hours and it was significantly lower than APAP300 group.

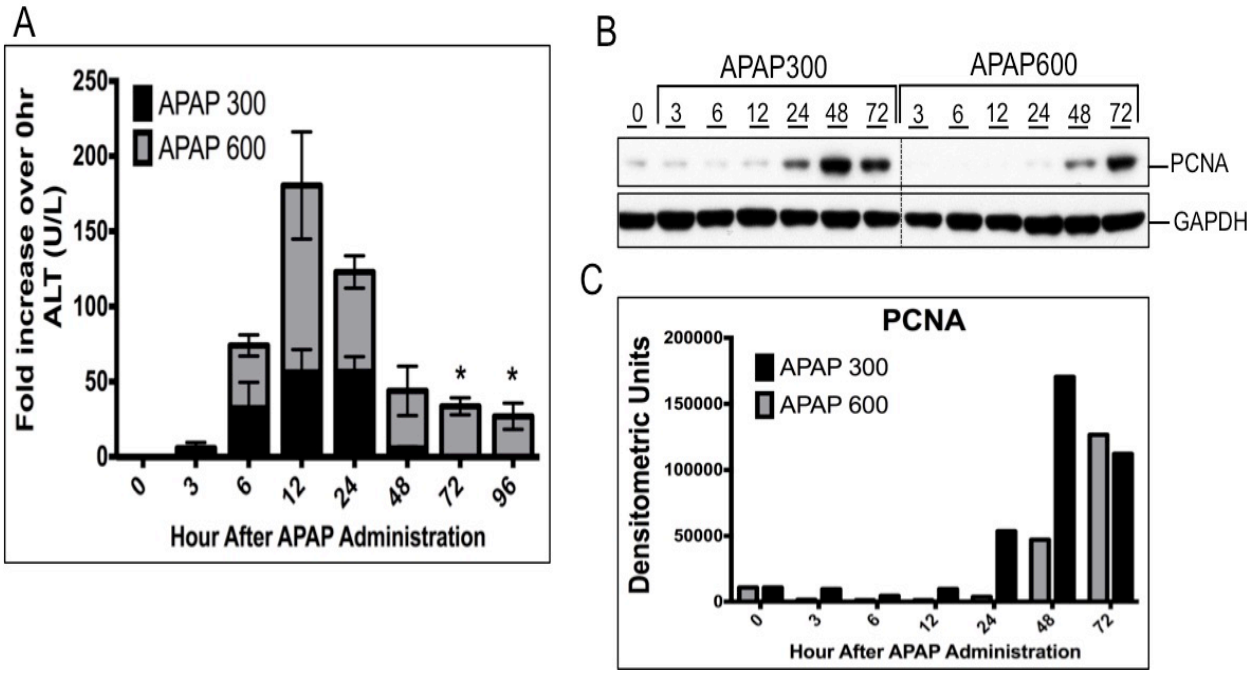
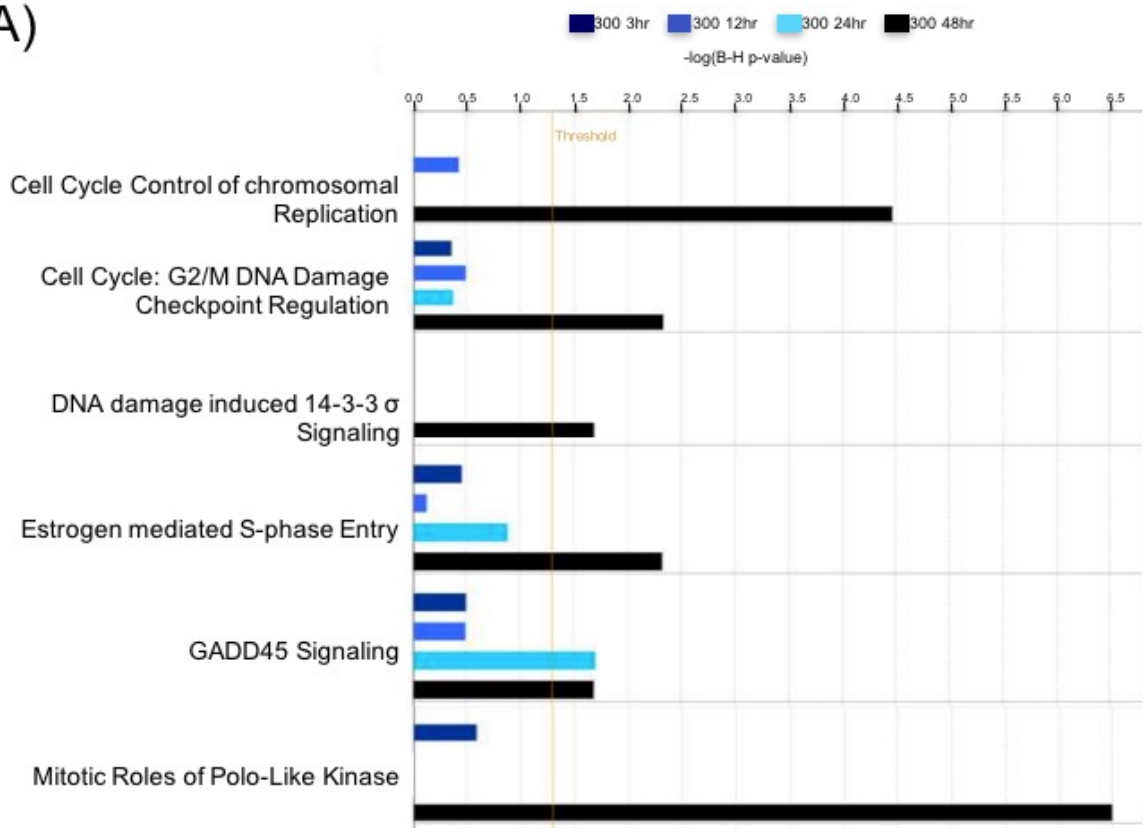


Figure 2.4.1. Sustained liver injury and inhibited liver regeneration following higher dose of APAP. (A) Liver injury analysis by serum ALT levels after APAP treatment. Shown as fold increase in ALT levels compare to 0 hour. (B) Western blot analysis of PCNA in whole liver extract. (C) Densitometric analysis of PCNA Western blot. * $p<0.05$ (APAP300 vs APAP600)

IPA Analysis Reveals Differences In DNA Damage And Repair Pathways Between APAP 300 And APAP 600

We performed global microarray analysis to delineate mechanisms that inhibit liver regeneration in APAP600 group vs APAP300. Microarray data obtained were analyzed using web-based application Ingenuity Pathway Analysis (IPA). Canonical pathway analysis revealed significant differences in several pathways related to cell cycle checkpoint and DNA damage signaling as shown in Fig 2.4.2 A-B. In the microarray data, many genes that were altered after APAP when compared to 0 hour control were common in APAP300 and APAP600 group. Hence, we compared genes that were uniquely altered (up or down regulated) in APAP300 or APAP600 relative to 0 hour. As shown in Fig. 2.4.2 C canonical pathway analysis using unique genes showed that DNA damage, repair and replication pathways were significantly altered in APAP300 relative to APAP600.

(A)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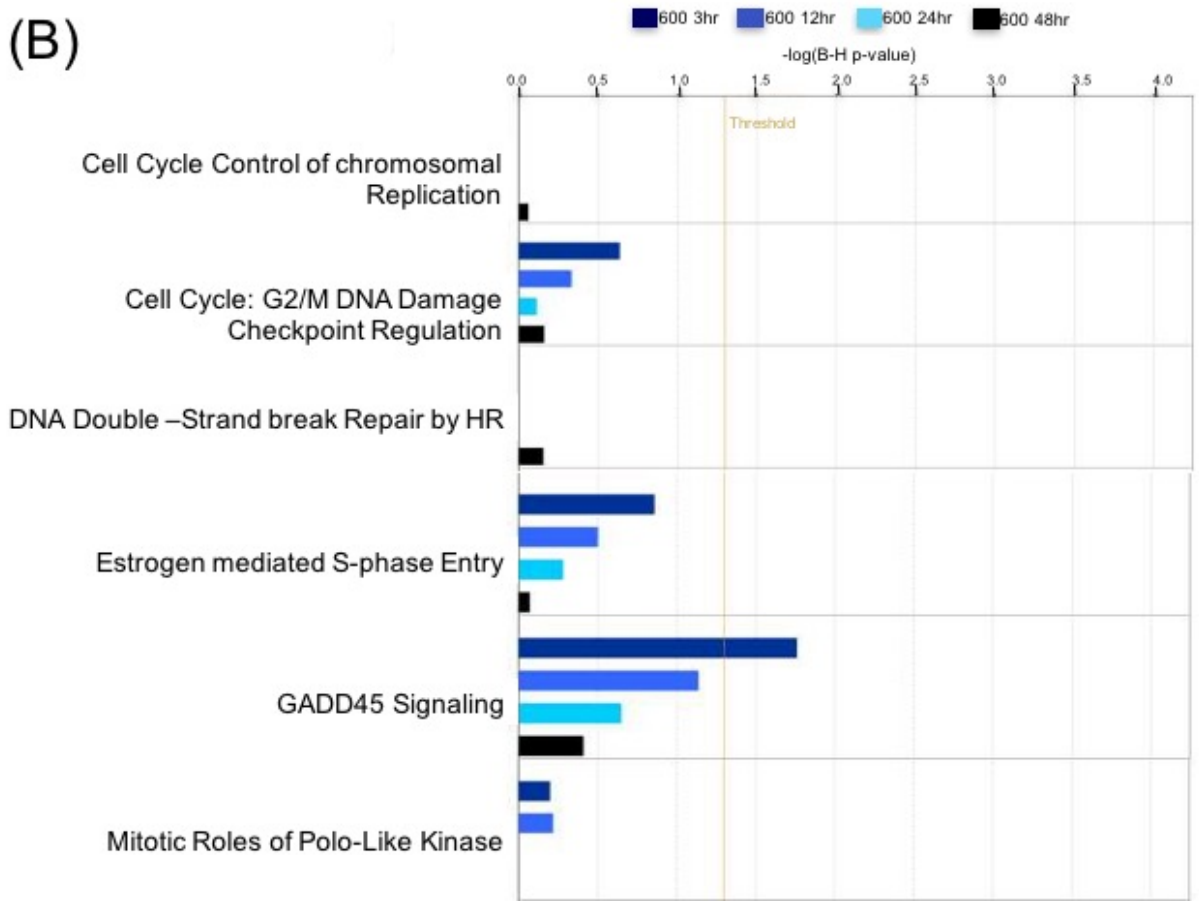
5.5
6.0
6.5
Threshold
Cell Cycle Control of chromosomal Replication
Cell Cycle: G2/M DNA Damage Checkpoint Regulation
DNA damage induced 14-3-3 σ Signaling
Estrogen mediated S-phase Entry
GADD45 Signaling
Mitotic Roles of Polo-Like Kinase

(B)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600 24hr
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-log(B-H p-value)
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2.5
3.0
3.5
4.0
Threshold
Cell Cycle Control of chromosomal Replication
Cell Cycle: G2/M DNA Damage Checkpoint Regulation
DNA Double –Strand break Repair by HR
Estrogen mediated S-phase Entry
GADD45 Signaling
Mitotic Roles of Polo-Like Kinase

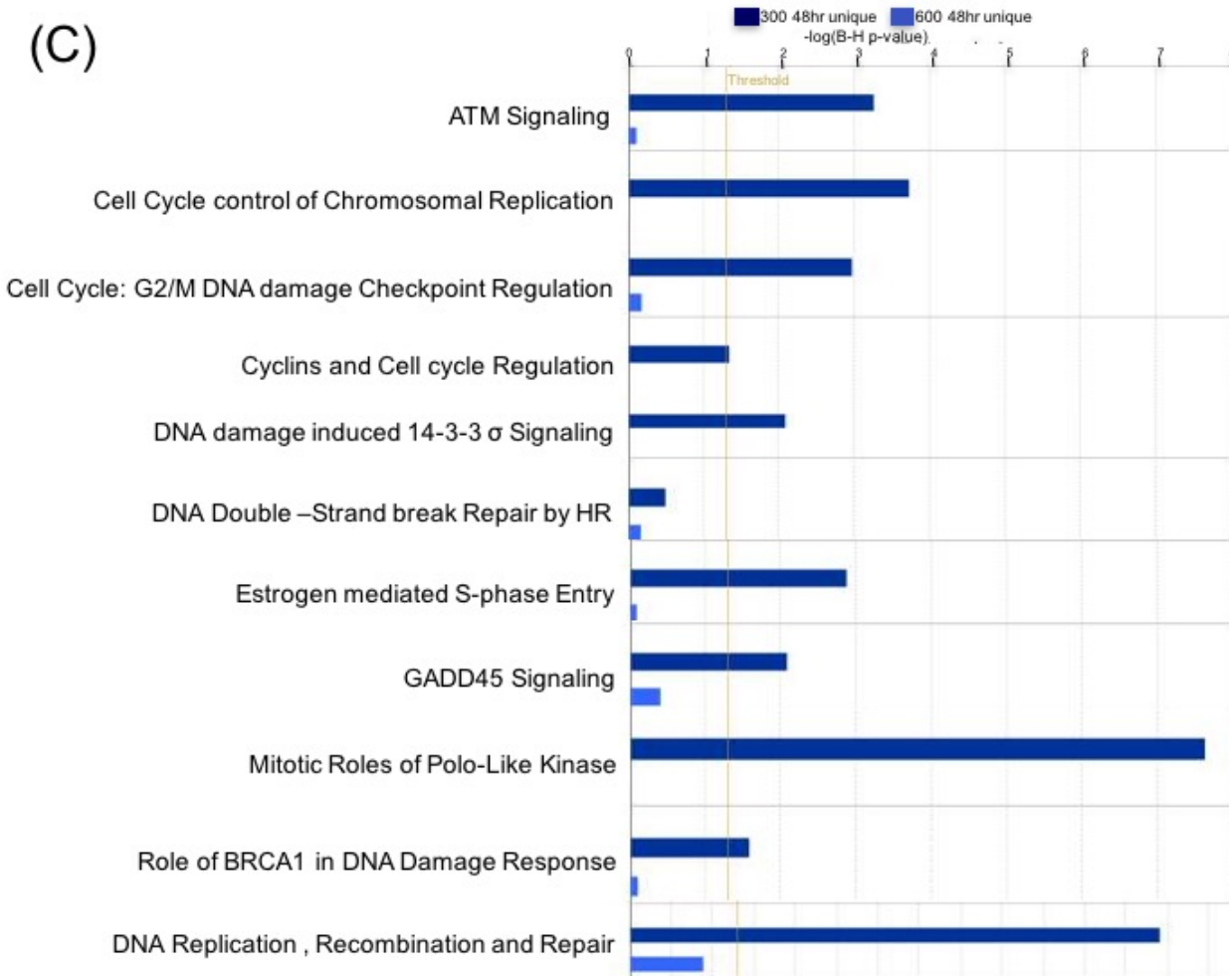


Figure 2.4.2. IPA analysis of microaaray data obtained from APAP 300 and APAP 600 pooled liver samples obtained at 0, 3, 24, 48 hours after APAP treatment. Gene expression changes 3 fold and higher were selected for IPA analysis. (A) Canonical Pathway analysis of APAP 300, (B) Canonical Pathway analysis of APAP 600, (C) Canonical Pathway analysis of APAP 300 and APAP 600 at 48 hours based on unique gene changes.

Prolonged DNA DSB and reduced repair protein expression after higher dose of APAP

The microarray data indicated that DNA damage, repair and replication pathways were significantly different between dosage groups. Moreover, DNA damage could result from replication stress. Therefore, to examine the mechanism underlying delayed and attenuated cell proliferation in mice exposed to a higher APAP dose regarding DNA replication, we considered the most lethal form of DNA damage i.e. DNA double strand break (DSB). DSB was determined using Western blot analysis and immunofluorescence detection of Ser 139 phosphorylation of histone 2A.X (pH2AX), a hallmark of DSB. At both APAP300 and APAP600 doses, Ser 139 pH2AX expression was observed from 6 hours (Fig. 2.4.3 A). In APAP300 group pH2AX-Ser139 expression peaked at 12 hours, remained high at 24 hours and returned to control level at 48 and 72 hours. In APAP600 group pH2AX Ser 139 induction peaked at 12 hours and remained high up to 72 hours. To determine which cells exhibit pH2AX after APAP overdose, we performed double immunofluorescence staining with pH2AX and hepatocyte marker HNF4α. Immunofluorescence staining revealed that hepatocytes immediately surrounding APAP induced necrotic zone were positive for DSB (Fig. 2.4.3 B).

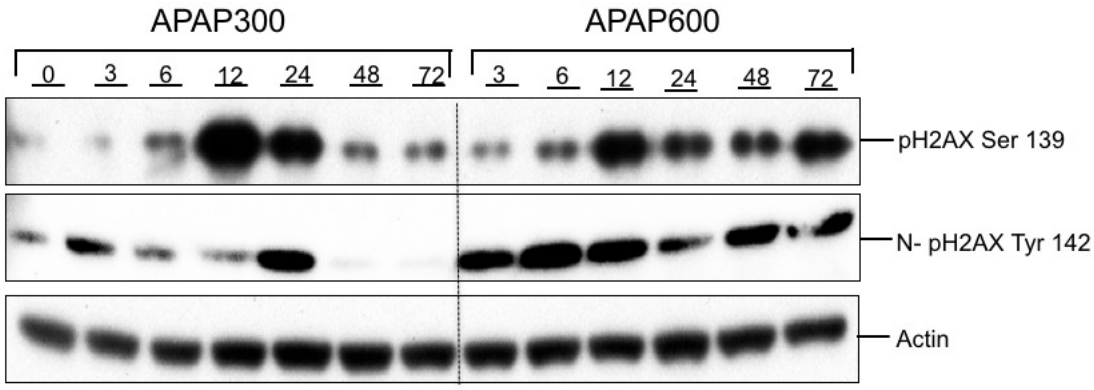
Next we looked at DNA DSB repair proteins including another modification of H2AX i.e. de-phosphorylation at Tyr 142 that facilitates recruitment of repair proteins(Cook, Ju et al. 2009, Xiao, Li et al. 2009). Western blot analysis of nuclear extracts showed significantly decreased levels of pH2AX Tyr142 in APAP300 group

indicating initiation of DNA repair. In contrast, APAP600 group mice had higher levels of pH2AX Tyr142 throughout the time course (Fig. 2.4.3 A). BRCA1 and 53BP1 are critical mediator proteins involved in DDR, which can interact with broken DNA ends and help binding of DNA repair effector proteins at the damaged DNA site(Jackson 2002, Panier and Boulton 2014). A marked increase in 53BP1 and BRCA1 protein levels was seen from 12 hours up to 72 hours after APAP300 treatment as compared to 0 hour control. In contrast, 53BP1 and BRCA1 protein expression was down regulated after APAP600 treatment (Fig. 2.4.3 C).

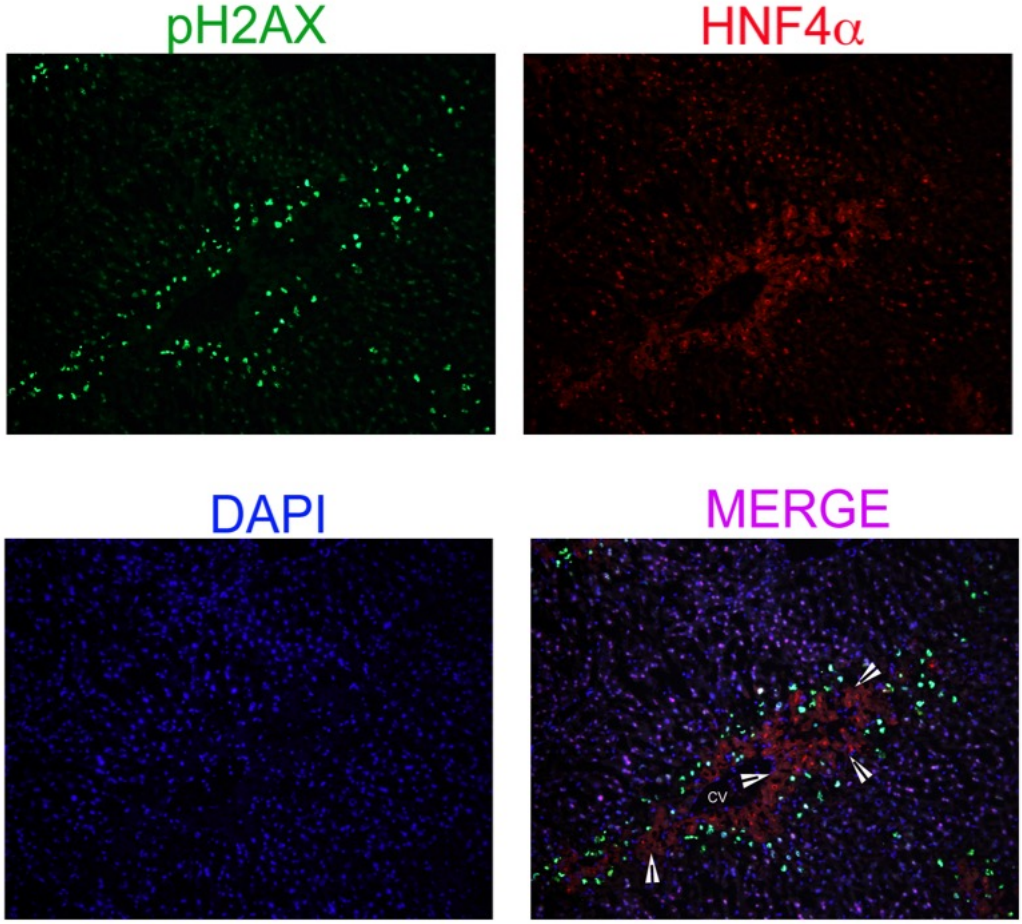
We have previously demonstrated that in APAP600 group hepatocytes were arrested at G0/G1 phase(Bhushan, Walesky et al. 2014). In G0/G1 phase of the cell cycle, DSB is repaired mainly by non-homologous end joining (NHEJ)(Chapman, Taylor et al. 2012). Therefore, we studied NHEJ repair pathway proteins to further examine the difference in DNA repair between APAP300 and APAP600 groups. We determined expression of proteins involved in NHEJ repair including KU70, KU80, DNA Pkc, XRCC4, XLF, and DNA Lig4 using Western blot analysis (Fig. 2.4.3 D). The data indicated significant upregulation of XRCC4, XLF, DNA Pkc and Lig4 in APAP300, all of which were downregulated in APAP600 group as compared to the 0 hour control (Fig. 2.4.3 D). We did not observe differences in KU70 and KU80 protein levels between APAP300 and APAP600 groups.

These data suggest that after higher dose of APAP there is reduced DNA repair protein expression and inadequate chromatin modification resulting in impaired DSB repair.

A


APAP300
APAP600
0
3
6
12
24
48
72
3
6
12
24
48
72
pH2AX Ser 139
N- pH2AX Tyr 142
Actin

B


pH2AX
HNF4α
DAPI
MERGE
CV

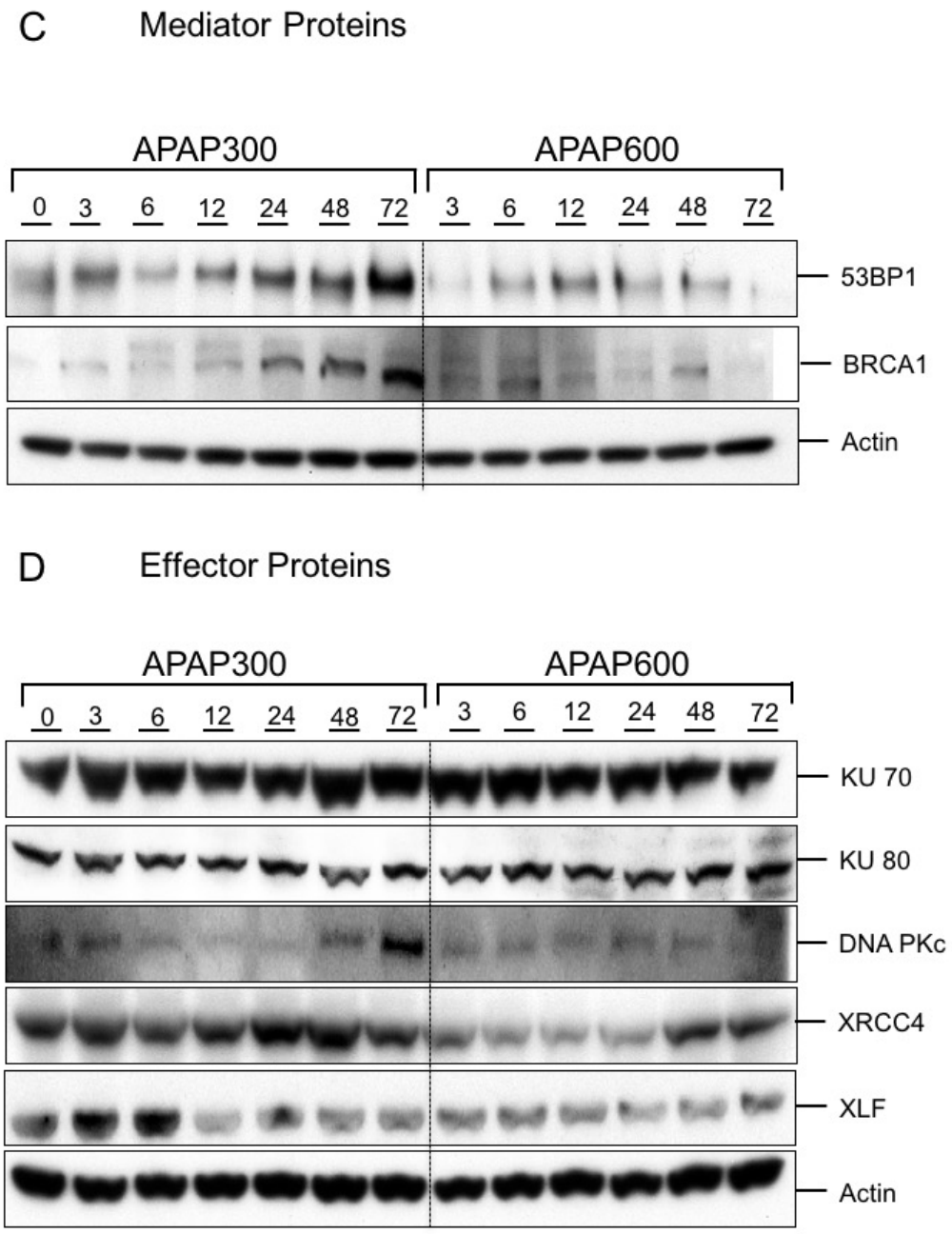


Figure 2.4.3. Prolonged DNA DSB and reduced repair protein expression after higher dose of APAP. A) Immunoblot analysis of phos-H2AX Ser 139 using total liver extract and phos-H2AX Tyr 142 using nuclear extract. (B) Representative immunofluorescence staining for pH2AX Ser139 (green), HNF4α (red) and DAPI (blue) for cell nuclei. Arrowheads are pointing to necrotic cells. (C) Immunoblot analysis of DNA repair mediator proteins 53BP1, BRCA1in total liver extract (D) Immunoblot analysis of DNA repair effector proteins KU70, KU80, DNAPkc, XRCC4, XLF, Lig4 using total liver extract.

Reduced PARP activation following APAP600 dose of APAP

Another critical protein in DDR is poly (ADP) ribose polymerase (PARP-1) that senses DNA damage and mediates the stress response by poly-ADPribosylation of nuclear proteins. It results in chromatin remodeling, which favors DNA repair(Krishnakumar and Kraus 2010). We did not observe differences in total PARP1 protein expression between APAP300 and APAP600 mice. However, nuclear PARP1 (N-PARP1) was significantly downregulated in APAP600 treated mice as compare to APAP300 treatment (Fig. 2.4.4 A). Next, we determined PARP activation by staining for PARylated proteins using immunohistochemistry (Fig. 2.4.4 B). Following APAP300 dose, PARP activation was observed in a time-dependent manner. No PAR staining was evident untill 12 hours (data not shown), but significant nuclear PAR staining was evident from 12 to 72 hours after APAP300 treatment. PAR staining intensity significantly increased at 24 hours, sustained till 72 hours and disappeared by 96 hours after APAP300 treatment. On the contrary, in APAP600 treated mice, at 12 and 24 hours very few cells stained positive for PAR with low intensity. At 48 hours many PAR positive cells were observed however staining intensity was weak as compare to APAP300. PAR staining disappeared by 72 hours following APAP600 treatment. These data indicate that PARP activation is significantly higher and sustained following APAP300 treatment however it is delayed and weak following APAP600 treatment.

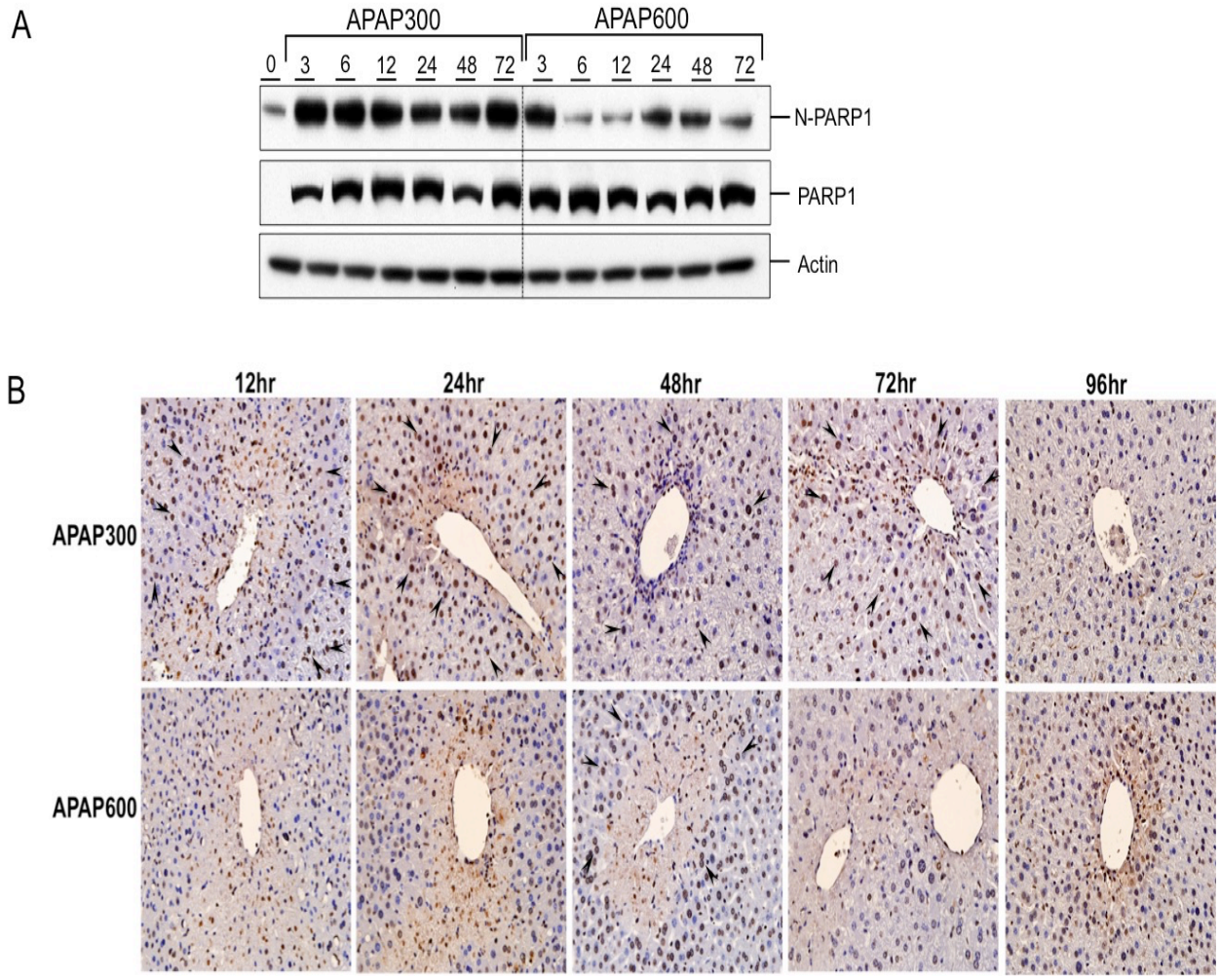


Figure 2.4.4. Delayed activation of PARP following APAP600 treatment. (A) Immunoblot analysis of PARP1 in total liver extract and nuclear extract. (B) Immunohistochemical analysis of PARylated proteins.

Increased transcriptional activation of p53 at APAP600 exposure

p53 is the major effector protein of DDR pathway, which can activate cell cycle checkpoint and arrest the cell cycle till damage is repaired. Stabilization and activation of p53 protein has been shown to play an important role in many cellular processes such as cell cycle arrest, cell senescence, cell death, cell metabolism etc(Riley, Sontag et al. 2008, Vousden and Prives 2009). We determined p53 activation using Western blotting for p53 and mRNA for several p53 target genes after APAP overdose. Western blot analysis of APAP300 and APAP600 samples indicated marked increase in p53 stabilization after APAP treatment (Fig. 2.4.5 A). Interestingly, p53 protein levels were significantly higher in APAP600 group as compare to APAP300 from 6 hours up to 72 hours. Similarly, Ser 15 phosphorylation of p53, which indicates activation of p53, was significantly higher in APAP600 at all time points (Fig. 2.4.5 A). Real time PCR analysis showed that expression of several p53 responsive genes involved in cell cycle inhibition (GADD45α, GADD45β, GADD45γ, BTG2) (Fig. 2.4.5 B), cell senescence (PAI1) (Fig. 2.4.5 C) and cell death (repression target - BIRC5) (Fig. 2.4.5 D) corroborated with increased p53 activity. A marked increase in all cell cycle inhibitor and cell senescence gene expression was seen in both groups after APAP treatment. In APAP300 group, mRNA levels of all these genes were significantly reduced from 24 hours to 96 hours. However, these cell cycle inhibitor and senescence gene expressions was sustained and significantly higher in APAP600 group. BIRC5, gene associated with inhibition of cell death and negatively regulated by p53, was significantly up regulated in APAP300 group at later time points, in contrast it remained repressed throughout the time course after APAP600 treatment. These data indicate that sustained activation of p53 after

APAP600 treatment results in cell cycle arrest, replicative senescence and may be cell death.

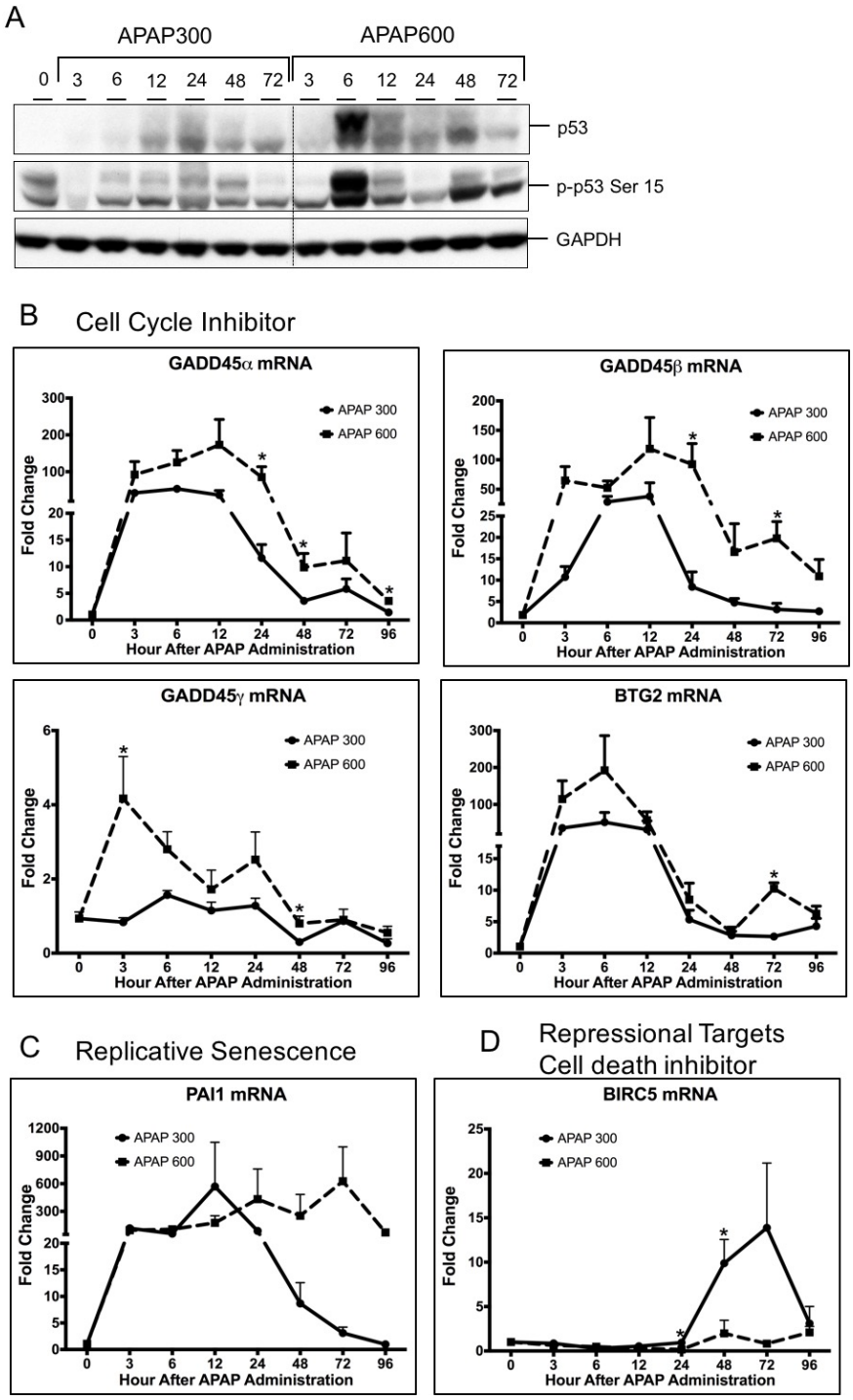


Figure 2.4.5. Activation of p53 is higher following APAP600 treatment. (A) Western blot analysis of p53 and phospho-p53 Ser15 using total liver extract. Real time PCR analysis of p53 target genes regulating (B) cell cycle inhibition Gadd45α, Gadd45β, Gadd45γ, BTG2 (C) replicative senescence PAI1 and (D) cell death inhibition BIRC5 * $p<0.05$ (APAP300 vs APAP600)

2.5 DISCUSSION

APAP is a safe analgesic and antipyretic drug when taken at recommended daily dose. It is safely metabolized in liver and excreted in urine. However, overdose of APAP causes ALI and even ALF, which is a number one cause of ALF in USA and UK.(Larson, Polson et al. 2005, Bernal and Wendon 2013) The mechanism of APAP toxicity involves generation of ROS, release of endonucleases, extensive DNA fragmentation and subsequent cell necrosis(Jaeschke and Bajt 2006). In response to injury, healthy hepatocytes surrounding the necrotic zone divide rapidly and help repair the injured liver(Bajt, Knight et al. 2003, Apte, Singh et al. 2009, Bhushan, Walesky et al. 2014). In previous study we have demonstrated that liver regeneration is stimulated rapidly following treatment with 300 mg/kg of APAP (regenerating dose) but it is significantly delayed and blunted after a 600 mg/kg of APAP (non-regenerating dose)(Bhushan, Walesky et al. 2014). The main reason behind this is the cells that surround the necrotic zone, which normally undergo proliferation to fuel liver regeneration are arrested in mice treated with the higher non-regenerating dose of APAP. In this study, we determined if this cell cycle arrest at non-regenerating doses is due to enhanced DNA damage and blunted DNA repair processes. Our data indicate that DNA damage occurs following both the regenerating (APAP300) and non-regenerating (APAP600) doses of APAP but DNA repair process is significantly inhibited following treatment with non-regenerating dose of APAP. Furthermore, our immunofluorescence data demonstrated that the extensive DNA damage observed at later time points is in the hepatocytes immediately next to the necrotic zone. These are

the hepatocytes that are required to proliferate in order to ensue liver regeneration. Whereas previous studies have shown that DNA damage is part of necrotic cell death after APAP, our data are the first to demonstrate that DNA damage and subsequent lower DNA repair inhibit liver regeneration, repair and recovery after APAP overdose.

Because we observed sustained DSB in non-regenerating animals, we further studied whether DSB repair is inactive and cells are arrested due to failure to replicate damaged DNA in APAP600. De-phosphorylation of p-H2AX at Tyr 142 is one of the chromatin modifications that facilitate DSB repair(Cook, Ju et al. 2009, Xiao, Li et al. 2009) was significantly deregulated following treatment with the non-regenerating APAP600 dose. Additionally, higher dose of APAP suppressed expression of mediator proteins 53BP1 and BRCA1 (Fig. 2C). Expression of several DSB repair effector proteins was suppressed at non-regenerating doses. Because DSB repair is the collective effort of various proteins, lack of several critical proteins will result in delayed or completely suppressed DAB repair in APAP600 treated mice. Furthermore, dephosphorylation of H2AX at Tyr 142, which is required for easy access of repair proteins to DSB sites was significantly lower in APAP600 treated mice. This may have made damage site inaccessible for the repair protein in non-regenerating dose treated mice. These results collectively show that DSB repair is deregulated in non-regenerating animals leading to sustain DSB.

Further studies showed that APAP600 treated mice exhibited reduced nuclear PARP1 levels in spite of similar amounts of total PARP1 levels as compared to APAP300. It is known that normally PARP1 is present in the nucleus where it is involved

in protein PARylation(Schreiber, Dantzer et al. 2006, Gibson and Kraus 2012). Our data indicate that 6 hours following APAP600, PARP1 is rapidly removed from the nucleus. This was consistent with significantly decreased PARylation of nuclear proteins in APAP600. In agreement with previous study(Cover, Fickert et al. 2005), these data show that PARP activation is not associated with increased liver injury following APAP toxicity. However, these data suggest that PARP activation is a critical step in DSB repair following APAP overdose. Further studies are required to delineate the mechanism of nuclear export of PARP1 following higher dose of APAP.

p53 is a primary effector protein that plays a critical role in cell cycle regulation during DDR. Under stress conditions p53 is stabilized and activated through various posttranslational modifications. One such modification is phosphorylation at Ser 15 that leads to transcriptional activation of p53. Activated p53 regulates plethora of downstream gene expression involved in cell cycle inhibition and senescence(Riley, Sontag et al. 2008). Our data indicate significantly higher and sustained activation of p53 following APAP600 dose (Fig. 4A). The expression of p53 target genes (Cell cycle inhibitor- GADD45α, GADD45β, GADD45γ, cell senescence PAI1, p21) increased after both APAP300 and APAP600 but it was significantly higher in APAP600 dose at all time points. Previous studies indicate that moderate activation of p53 results in cell cycle arrest that permit cell to repair the DNA damage, however excessive and sustained activation of p53 results in replicative senescence and cell death(Vousden and Prives 2009). Further, BIRC5, a negative target of p53 was repressed at all time points in non-regenerative dose consistent with higher p53 activation. These data suggest that moderate activation of p53 at regenerative dose results in transient cell cycle arrest

whereas, sustained excessive activation of p53 at non-regenerative dose may cause prolonged growth arrest, replicative senescence or cell death. Further studies are required to demonstrate the exact role of p53 and some of these target genes in liver regeneration after APAP overdose.

In conclusion, our study indicates that DNA damage and repair response plays a critical role in deciding whether liver regeneration will be ‘timely’ or ‘delayed’ following APAP overdose. At high doses of APAP, DSB repair is impaired resulting in inhibited liver regeneration. This study is the first to highlights the complex signaling pathway involved in DNA DSB repair in regulation of liver regeneration following APAP induced ALI. These data also indicate that improving DNA repair may have therapeutic benefit after APAP overdose.

CHAPTER III: INVESTIGATING PLEIOTROPIC ROLE OF P53 IN LIVER INJURY AND REGENERATION FOLLOWING APAP OVERDOSE

3.1 ABSTRACT

p53 is the major cellular gatekeeper, which regulates diverse functions including proliferation, cell death, cell migration, metabolic homeostasis. The role of p53 in pathogenesis of drug-induced liver injury is poorly defined. We investigated the role of p53 in liver injury and regeneration after APAP overdose, the most common cause of acute liver failure in the Western world. Eight-week-old male WT and p53KO mice (C57BL/6J background) were treated with 300mg/kg APAP (APAP300) and the dynamics of liver injury and regeneration were studied over time course of 0 to 96 hour. Deletion of p53 resulted in a 3-fold higher liver injury than WT mice implying protective role for p53 in injury progression. Interestingly, despite higher liver injury p53KO mice recovered similarly as the WT mice due to faster liver regeneration. Deletion of p53 did not affect APAP bioactivation however it delayed clearance of APAP protein adducts from liver. Global Transcriptomic analysis revealed that p53KO mice had disrupted metabolic homeostasis, induced inflammatory and proliferative signaling. p53KO mice showed prolonged steatosis, hypoglycemia, impaired GSH transulfuration and reduced expression of mitochondrial complex proteins correlating with prolonged liver injury. Deletion of p53 also resulted in increased expression of cytokines such as IL1β and IL10 in liver. Further, p53KO mice displayed delayed but rapid liver regeneration than WT mice due to sustained AKT, ERK and mTOR signaling. These studies show that p53 plays a pleotropic role after APAP overdose where it prevents progression of liver injury by maintaining mitochondrial and metabolic homeostasis and also regulates initiation of liver regeneration through inflammatory and proliferative signaling.

3.2 INTRODUCTION

p53 functions as a cellular gatekeeper involved in regulation of various stress responses including cell proliferation, migration, metabolism, autophagy, DNA repair, senescence, immune response and stem cell reprogramming. Because of these multifaceted functions, deregulation of p53 is implicated in various diseases such as cancer, obesity, diabetes, ischemia, and aging (Tyner, Venkatachalam et al. 2002, Muller, Vousden et al. 2011, Kenzelmann Broz and Attardi 2013, Menendez, Shatz et al. 2013, Aloni-Grinstein, Shetzer et al. 2014, Kruiswijk, Labuschagne et al. 2015). Recent studies in rodents using partial hepatectomy model have depicted role of p53 in regulation of ploidy and cell proliferation during liver regeneration (Stepniak, Ricci et al. 2006, Kurinna, Stratton et al. 2010, Kurinna, Stratton et al. 2013, Jin, Hong et al. 2015, Zhang, Liu et al. 2015). Liver regeneration is essential for patient survival following surgical resection or acute and chronic liver injury secondary to drugs, toxins and viruses (Mehendale 2005). However, role of p53 in liver homeostasis and regeneration following drug induced liver injury is not known.

Overdose of acetaminophen (APAP), the commonly used antipyretic and analgesic agent, is the leading cause of Acute Liver Failure (ALF) in the Western world (Larson, Polson et al. 2005, Bernal and Wendon 2013, Shehab, Lovegrove et al. 2016). The mechanism of liver injury after APAP overdose involves formation of a reactive metabolite N-acetyl-p-quinoneimine (NAPQI), which depletes cellular glutathione and covalently binds to protein. These initial events lead to oxidative stress, activation of c-Jun N-terminal kinase (JNK), mitochondrial damage and release of cytochrome C,

Apoptosis inducing factor (AIF) and endonuclease that cause nuclear DNA fragmentation and eventually hepatocyte necrosis (Jaeschke and Bajt 2006). The replacement of necrotic cells and restoration of liver function occurs through liver regeneration. Several studies have shown that enhanced liver regeneration following APAP induced liver injury improves final outcome (Schmidt and Dalhoff 2005, Bhushan, Walesky et al. 2014).

Whereas recent studies have implicated p53 in pathogenesis of APAP induced liver injury, the exact role of p53 in initiation and progression of injury and the subsequent liver regeneration after APAP overdose is not clear (Stamper, Garcia et al. 2015, Huo, Yin et al. 2017). In this study, we investigated role of p53 in liver injury and regeneration using APAP overdose in WT and p53 KO mice. Our findings reveal that p53 regulates metabolic homeostasis, inflammatory response and initiation of cell proliferation and disruption of p53 signaling culminates in progression of liver injury yet faster recovery.

3.3 MATERIALS AND METHODS

Animals, Treatments and Tissue Harvesting.

Two-month-old male WT ($p53^{+/+}$) and p53KO ($p53^{-/-}$) mice on C57BL/6J background purchased from Jackson Laboratories were used in these studies. All mice were housed in Association for Assessment and Accreditation of Laboratory Animal Care-accredited facilities at the University of Kansas Medical Center under a standard 12hour light/dark cycle with access to chow and water *ad libitum*. All animal experiments were performed with approved Institutional Animal Care and Use Committee protocols at University of Kansas Medical Center. Acute liver injury was induced by injecting 300 mg/kg APAP intraperitoneally (i.p. dissolved in saline) following 12hour fasting as described previously (Bhushan, Walesky et al. 2014). Following APAP treatment mice (n = 3 to 5) were sacrificed at 0, 1, 12, 24, 48 and 96 hours and blood and livers were collected. Serum samples obtained from blood were used for ALT and glucose level measurement using the Infinity ALT (GPT) and the Infinity Glucose kit (Thermo Scientific; Middletown, VA) as per the manufacturer's protocol. Parts of liver tissue were processed separately to obtain paraffin sections, frozen sections, RNA samples, nuclear, cytoplasmic and RIPA total protein extracts as described previously (Borude, Edwards et al. 2012).

Immunohistochemistry and staining procedures.

Hepatocyte proliferation was determined using PCNA immunohistochemical staining and liver necrosis was determined by H&E staining as previously described (Borude, Edwards et al. 2012). Fresh-frozen liver sections (5 μm thick) were used to stain lipid droplets using Oil Red O as described before (Borude, Edwards et al. 2012).

Western Blotting and Real time PCR.

Protein estimation and Western blot analysis was performed using pooled protein extracts as described before (Borude, Edwards et al. 2012). The antibodies used in this study are listed in Supplemental Table 3.3.1. Total RNA isolation from WT and p53KO liver, reverse transcription to cDNA and Real time PCR analysis by SYBR Green technology was performed as previously described (Bhushan, Walesky et al. 2014). 18s gene expression in the same samples was used for data normalization and Messenger RNA (mRNA) levels were determined by comparing post APAP treatment time points to 0hour control of respective group. Primers used for real time PCR are listed in Table 3.3.2.

Table 3.3.1. Antibodies used in this study

Name	Catalog Number
PCNA	Cell Sign-2586
p-JNK	Cell Sign -4668
JNK	Cell Sign -3708
Cyclin D1	Cell Sign -2978
p-pRb	Cell Sign -9308
p21	Santa cruz-6246
GAPDH	Cell Sign -2118
CYP2E1	Abcam- 19140
SREBP2	Thermo- PA1338
TFAM	Cell Sign -8076
NRF1	Cell Sign -12381
Mitochondrial Complex	Mitosci- MS604
p-AKT	Cell Sign -9271
AKT	Cell Sign -4691
p-ERK	Cell Sign -4376
p-EGFR	Cell Sign -1068
EGFR	Cell Sign -4267
p-mTOR Ser 2448	Cell Sign -2971
p-mTOR Ser 2481	Cell Sign -2974
mTOR	Cell Sign -2983

p-P70S6K	Cell Sign -9234
P70S6K	Cell Sign -9202

Table 3.3.2. Primers used in this study

Gene	Forward (5'-3')	Reverse (5'-3')
Acot1	ATACCCCCTGTGACTATCCTGA	CAAACACTCACTACCCAACTGT
Cd14	CTCTGTCCTTAAAGCGGCTTAC	GTTGCGGAGGTTCAAGATGTT
Cyp7a1	GGGATTGCTGTGGTAGTGAGC	GGTATGGAATCAACCCGTTGTC
Fasn	GGAGGTGGTGATAGCCGGTAT	TGGGTAATCCATAGAGCCCAG
Fdps	GGAGGTCCTAGAGTACAATGCC	AAGCCTGGAGCAGTTCTACAC
Fos	CGGGTTTCAACGCCGACTA	TTGGCACTAGAGACGGACAGA
Hmox1	AAGCCGAGAATGCTGAGTTCA	GCCGTGTAGATATGGTACAAGGA
Elovl3	TTCTCACGCGGGTTAAAAATGG	GAGCAACAGATAGACGACCAC
Ly6d	GCCTGGGCACTTCGATGTC	TGAGTTTGCACACTCTTTCC
Igfbp3	CCAGGAAACATCAGTGAGTCC	GGATGGAACTTGGAATCGGTCA
Acot2	GTTGTGCCAACAGGATTGGAA	GCTCAGCGTCGCATTTGTC
Afp	CTTCCCTCATCCTCCTGCTAC	ACAAACTGGGTAAAGGTGATGG
Cd36	GCGCATGATTAATGGCACAG	GATCCGAACACAGCGTAGATAG
Scd1	CTACAAGCCTGGCCTCCTGC	GGACCCCAGGGAAACCAGGA
Pdk4	AGGGAGGTCGAGCTGTTCTC	GGAGTGTTCACTAAGCGGTCA
Stat1	TCACAGTGGTTCGAGCTTCAG	GCAAACGAGACATCATAGGCA
Insig2	GGAGTCACCTCGGCCTAAAAA	CAAGTTCAACACTAATGCCAGGA

G0s2	TAGTGAAGCTATACGTTCTGGGC	GTCTCAACTAGGCCGAGCA
Fgf21	CTGCTGGGGGTCTACCAAG	CTGCGCCTACCACTGTTCC
Lpin1	CATGCTTCGGAAAGTCCTTCA	GGTTATTCTTTGGCGTCAACCT
Gadd45α	CCGAAAGGATGGACACGGTG	TTATCGGGGTCTACGTTGAGC
Gadd45β	CAACGCGGTTCAGAAGATGC	GGTCCACATTCATCAGTTTGGC
Gadd45γ	GGGAAAGCACTGCACGAACT	AGCACGCAAAAGGTCACATTG
Bax	TGAAGACAGGGGCCTTTTTG	AATTCGCCGGAGACACTCG
Birc5	AAGGAATTGGAAGGCTGGG	TTCTTGACAGTGAGGAAGGC
Btg2	ATGAGCCACGGGAAGAGAAC	GCCCTACTGAAAACCTTGAGTC
Cpt1a	CTCCGCCTGAGCCATGAAG	CACCAGTGATGATGCCATTCT
Gpx1	AGTCCACCGTGTATGCCTTCT	GAGACGCGACATTCTCAATGA
Gpx3	CCTTTTAAGCAGTATGCAGGCA	CAAGCCAAATGGCCCAAGTT
Nanog	TCTTCCTGGTCCCCACAGTTT	GCAAGAATAGTTCTCGGGATGAA
Sco2	AGCTCTCTCAGTTCAAACCCC	GCAGTCTAGTTCTTAGCCCAGG
Sod2	TGCTCTAATCAGGACCCATTG	CATTCTCCCAGTTGATTACATTCC
Tgfβ	AGCTGGTGAAACGGAAGCG	GCGAGCCTTAGTTTGGACAGG
Il1β	GAAATGCCACCTTTTGACAGTG	CTGGATGCTCTCATCAGGACA
Il10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG

Hepatic GSH and APAP protein adducts measurement.

Total hepatic GSH levels were measured in liver homogenates using the commercially available colorimetric assay kit (Sigma) as described before (Bhushan, Borude et al. 2013). APAP protein adducts were measured in liver and serum samples using HPLC method as previously described (McGill, Lebofsky et al. 2013).

Transcriptome and Pathway analysis.

Total RNA isolated from WT and p53KO livers at 12, 24 and 48hour post APAP treatment were used for global transcriptomic analysis. Equal amount of RNA was pooled from 3 representative samples at each time point from both groups. RNA Integrity was determined on the Agilent Bioanalyzer 2100 using the RNA6000 Nano assay kit VII and global transcriptomic analysis was performed using Affymetrix Clariom D array at the Genomics Core Facility of the University of Kansas Medical Center (Kansas City, KS). Hierarchical clustering and heatmap analyses of differential expression data of transcripts were done as described previously (Walesky, Edwards et al. 2013). Ingenuity Pathways Analysis (IPA, Ingenuity Systems) was used for functional pathway analysis of differentially expressed transcripts.

Mitochondrial DNA measurement.

Genomic and mitochondrial DNA was isolated using commercially available kit (Qiagen). Real time PCR analysis was used to quantify relative copy numbers of genomic DNA and mitochondrial DNA (mito DNA) in WT and p53KO mice livers from 0-96 hours time course. LPL as nuclear DNA target and ND1 as mito DNA target were used for mito DNA content measurement. Exponential amplification efficiency of each primer pair was confirmed using series of dilution and 5ng of DNA was used in final PCR reaction. Mito DNA content in each sample was calculated as described previously (Rooney, Ryde et al. 2015). Relative mito DNA fold change was calculated by dividing mito DNA value of a sample by average mito DNA value in WT at respective time point.

Statistical Analysis.

All data are shown as mean ± SEM. Student's t-test was used for statistical analysis. Difference between groups was considered statistically significant at $P<0.05$ and indicated by * in graphs.

3.4 RESULTS

p53 deletion results in progression of APAP induced injury and faster recovery.

APAP treatment resulted in significant liver injury in both WT and p53KO as demonstrated by histopathological (Fig. 3.4.1A) and serum ALT (Fig. 3.4.1B) analysis. Liver sections stained with H&E showed necrotic cell injury at 12 and 24 hours in WT animals, which reduced by 48 hours. Serum ALT levels corroborated the histopathological findings. Liver injury was similar in WT and p53KO mice till 12 hours. However, at 24 hours p53KO mice exhibited significant higher liver injury as demonstrated by increase in necrotic area and 3-fold higher serum ALT. Interestingly, despite very high liver injury, p53KO mice recovered similarly to WT at 48 hours after APAP treatment. JNK activation (phosphorylation) is a critical step in APAP pathophysiology. Western blot analysis of p-JNK revealed prolonged JNK activation after APAP treatment in p53KO than WT group (Fig. 3.4.1C).

p53 deletion results in delayed but faster liver regeneration.

WT mice showed moderate increase in cell proliferation by 24 hours accompanied by increased expression of cell cycle proteins Cyclin D1, p-pRb, PCNA (Fig. 3.4.1C-D). PCNA staining of liver sections also revealed increased cell proliferation surrounding necrotic zone by 24 hours in WT group (Fig. 3.4.1D). On the contrary, p53KO animals showed delayed onset of cell proliferation as demonstrated by delay in

appearance of PCNA positive cells and delayed increase in Cyclin D1 expression till 48 hours after APAP treatment (Fig. 3.4.1D). At 48 hours post APAP, Cyclin D1, p-pRb and PCNA protein levels were comparable to WT (Fig. 3.4.1C). Interestingly, expression of p21, a cell cycle inhibitor, was higher in p53KO group at 24 hours than WT, which rapidly declined at 48hour after APAP treatment (Fig. 3.4.1C).

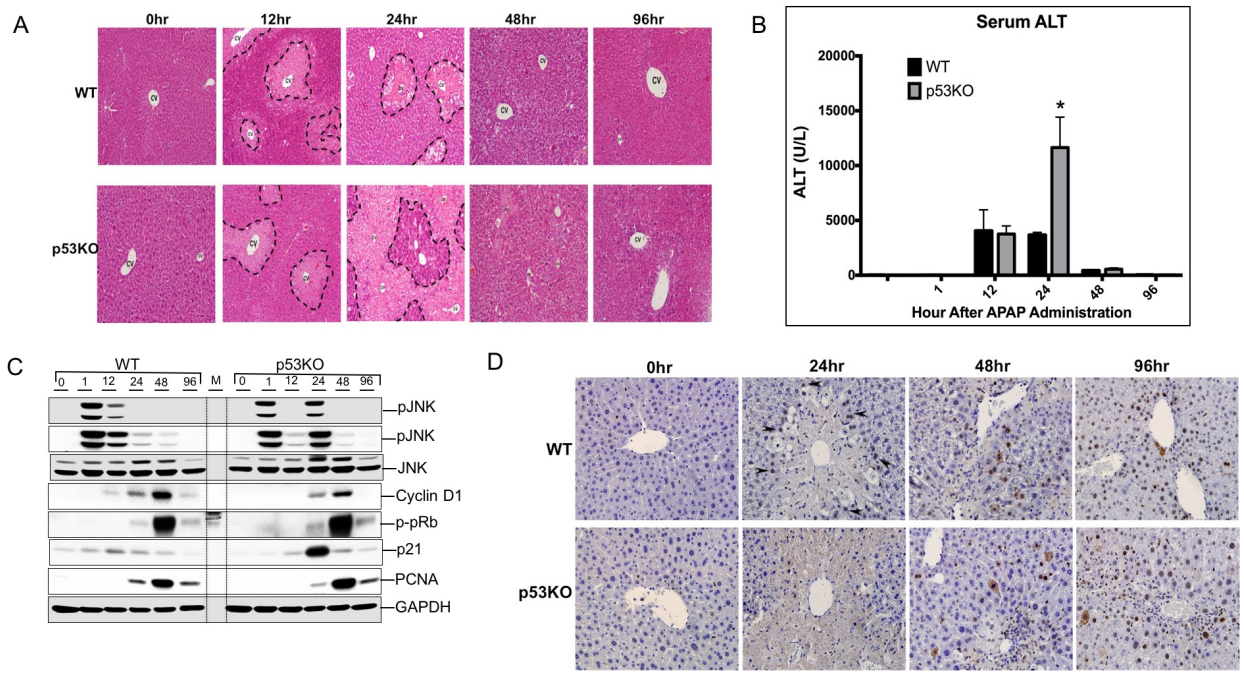


Figure 3.4.1. Deletion of p53 aggravates APAP induced acute liver injury yet promotes faster recovery. WT and p53KO mice were treated with APAP300 dose, serum and livers were harvested and processed post treatment for histological and biochemical analysis. (A) Representative photomicrographs of H&E stained livers. Dotted line shows necrotic area. (B) Serum ALT levels. (C) Western blot analysis of p-JNK, JNK, Cyclin D1, p-pRb, p21, PCNA using total liver extracts from the respective groups. (D) Representative photomicrographs of PCNA stained livers. Arrowheads point to PCNA positive cells. All images are 400X * indicates $p<0.05$ (WT vs p53KO)

p53 deletion does not change APAP bio activation but delays APAP-adduct clearance from liver.

Because we observed higher injury in p53KO mice, we measured total hepatic GSH, CYP2E1 and APAP adducts (McGill, Lebofsky et al. 2013, Ni, McGill et al. 2016), which are involved in initiation of APAP toxicity, to determine whether changes in bioactivation of APAP can explain the higher injury. CYP2E1 protein expression was not different between two groups (Fig. 3.4.2A). Total GSH levels before APAP treatment were similar and depleted GSH to same extent 1 hour after APAP administration in both groups. Interestingly, GSH replenishment, known to be involved in injury progression was not altered by p53 deletion as well (Fig. 3.4.2B). Also, we did not observe any significant difference in APAP adduct formation in liver at 1 hour post APAP300 treatment in p53KO mice compared to WT mice (Fig. 3.4.2C inset). All these data indicate that deletion of p53 in mice does not affect APAP bio activation. However, we observed a decrease in adducts clearance in p53KO mice than WT mice (Fig. 3.4.2C). WT mice were able to remove 30% of total adducts (formed at 1 hour) from liver by 12 hours. In contrast, p53KO mice cleared only 17% of adducts at 12 hours post-APAP treatment. By 24 hours p53KO were able to remove liver adducts to same extent as WT mice. This delay in liver adduct clearance was accompanied by delayed serum adduct secretion in p53KO mice (Fig. 3.4.2D). In WT mice maximum (62% of total serum adducts secreted from 1 through 24 hours) adduct secretion in serum was observed at 12 hours, however, in p53KO mice maximum adduct secretion (69%) was delayed up till 24 hours after APAP treatment (Fig. 3.4.2D).

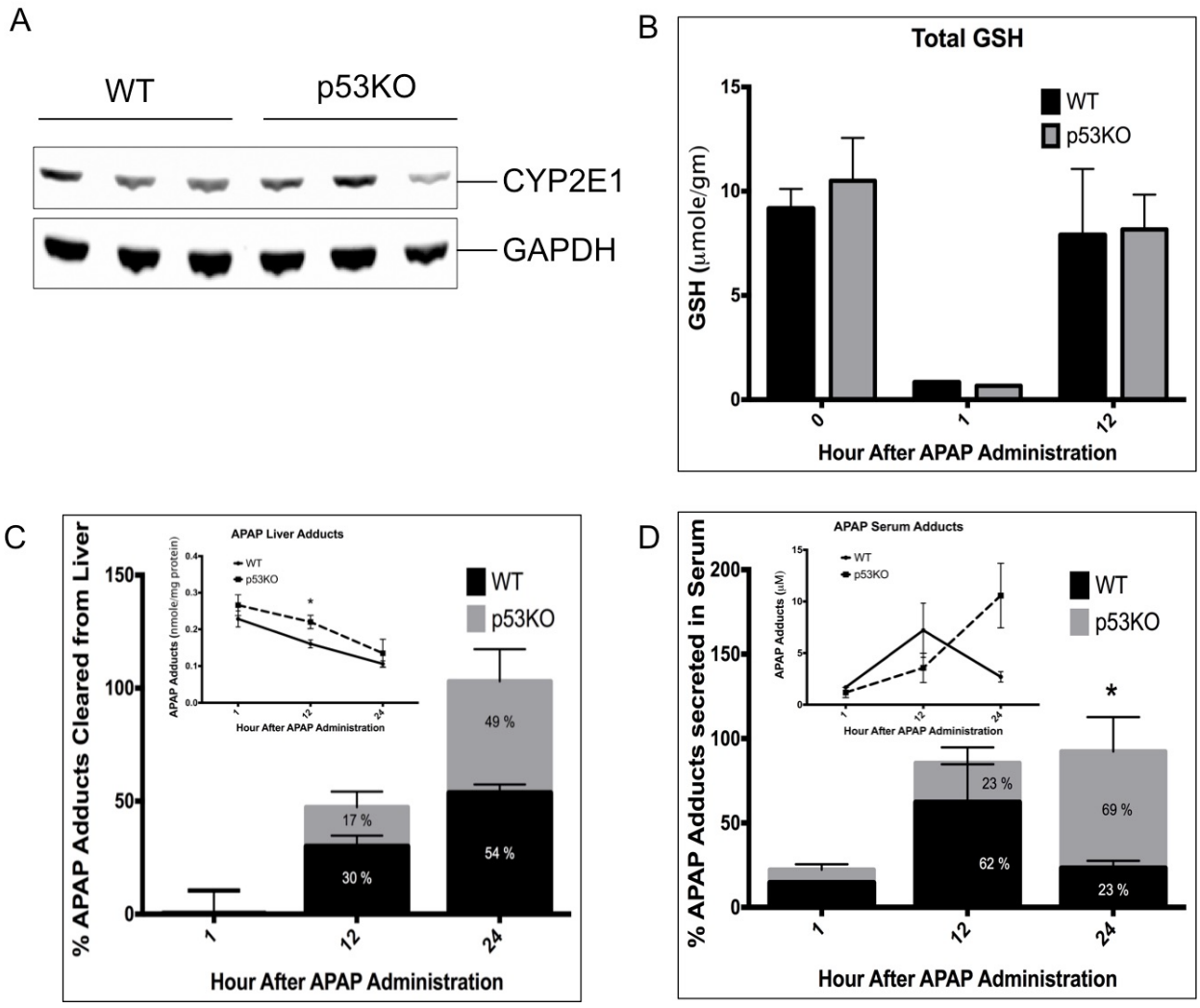


Figure 3.4.2. p53 deletion does not change APAP bioactivation but delays APAP-adduct clearance from liver. (A) Immunoblot of CYP2E1 using total liver extract from untreated WT and p53KO mice (B) Hepatic total GSH levels. (C) Bar graphs showing clearance of APAP-Cys adducts in liver, inset shows actual liver adduct levels, (D) bar graph showing clearance of APAP-Cys adducts from serum, inset shows actual adduct levels. * indicates $p<0.05$ (WT vs p53KO)

Transcriptomics analysis revealed expression patterns within each group.

We performed global transcriptomic analysis to delineate mechanisms through which p53 regulates progression of liver injury and initiation of regeneration. We did not observe any difference in liver injury between WT and p53KO mice till12 hours. However, significant difference was observed at 24 hours in injury progression and initiation of liver regeneration after APAP treatment. Therefore, we chose three time points including 12, 24 and 48 hours for transcriptome analysis. p53KO transcript expression was normalized to WT at respective time points. We selected all (3495) transcripts with ≥2 fold up or down regulated expression at least at one time point. Next, we sorted coding (676) and noncoding (1439) transcripts individually among 3495 transcripts and performed hierarchical clustering analysis on their differential expression data as shown in (Fig. 3.4.3A-C). Dendrograms generated by cluster analysis of all selected transcripts (Fig. 3.4.3A), coding transcripts (Fig. 3.4.3B) and noncoding transcripts (Fig. 3.4.3C) revealed two clusters of expression pattern. Cluster 1 consists of WT 48, p53KO 48 and WT 24, while Cluster 2 consists of WT 12, p53KO 12 and p53KO 24. Within Cluster 1, transcript expression pattern of WT 48 and p53KO 48 was closely related indicating similarity of both groups at pathophysiological level, which is consistent with injury and recovery data. Interestingly, WT 24 transcript expression pattern varied slightly but was very similar to WT 48 and p53KO 48. This shows that WT group begin to express Cluster 1 pattern at 24 hours and continues till 48 hours after APAP treatment. However, deletion of p53 delayed transcript expression pattern from 24 hours to 48 hours, which is also consistent with observations of liver regeneration. Within Cluster 2, expression pattern of WT 12 and p53KO 12 was closely related

indicating similar pathophysiological condition in both groups. p53KO 24 expression pattern is related to WT 12 and p53KO 12 but varied highly in magnitude which is also consistent with observations of liver injury. Finally, transcript expression pattern of WT 24 and p53KO 24 was significantly contrasting in all transcripts (Fig. 3.4.3A), coding (Fig. 3.4.3B) and noncoding transcript (Fig.3.4.3C). This is consistent with the three-fold difference in liver injury and delayed initiation of liver regeneration observed in p53KO mice as compared to WT mice at 24 hours time point. Further, selection of up and down regulated transcripts at individual time point with expression ≥2 fold in p53KO than WT revealed that majority of transcripts were significantly affected at 24 hours (Fig.3.4.3D). These data show that p53KO mice respond similarly to WT mice till 12 and 48 hours time points after APAP treatment but differ significantly at 24 hours after APAP administration.

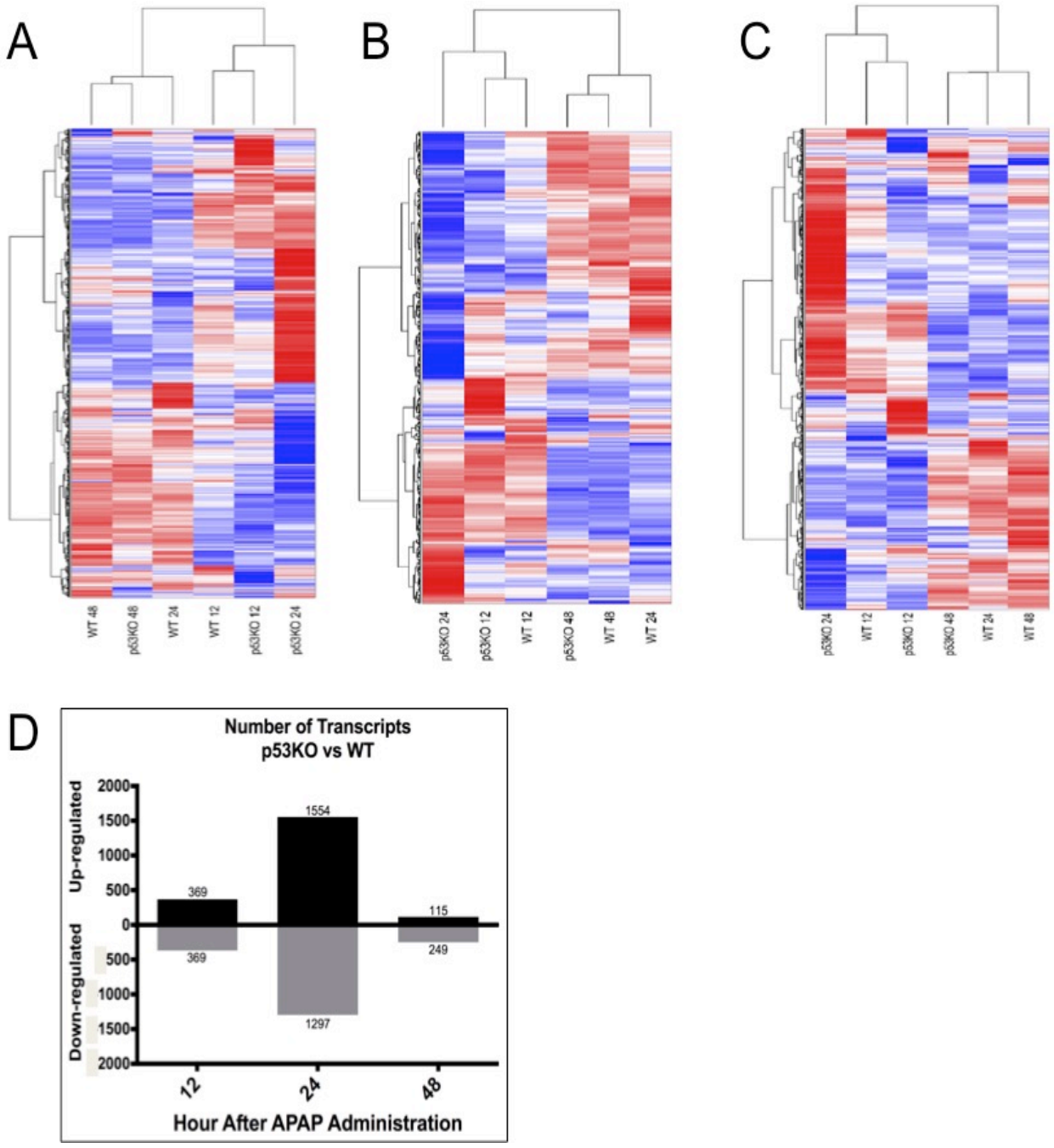
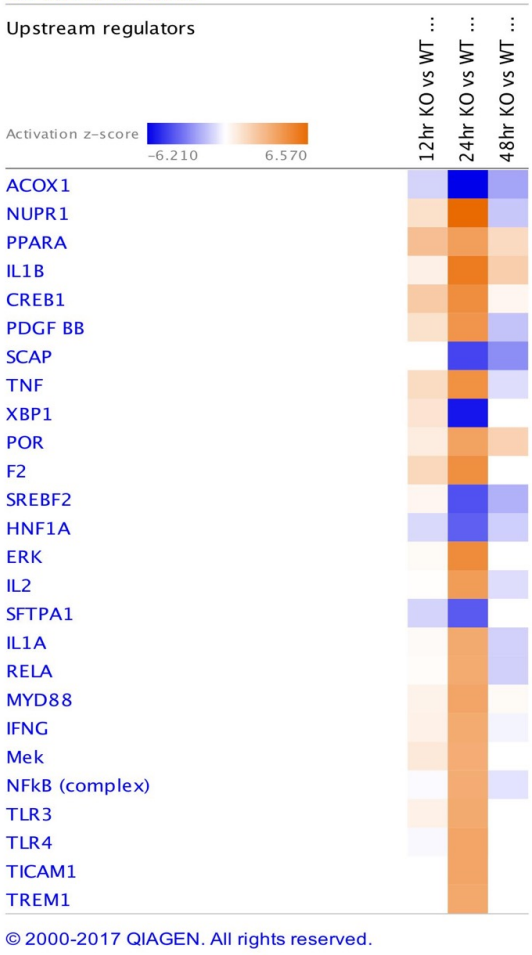


Figure 3.4.3 Transcriptomic expression patterns in WT and p53KO mice liver post APAP300 treatment. Global transcriptome analysis was performed using Affymetrix Clariom D array. Bi-weight (log2) values were used for cluster analysis and presented as heatmaps of (A) All selected transcripts (B) only coding transcripts (C) only non coding transcripts. Every group title represents the genotype and the time point post APAP300 treatment. (D) Number of transcripts in p53KO at respective time point with ≥2 fold change difference.

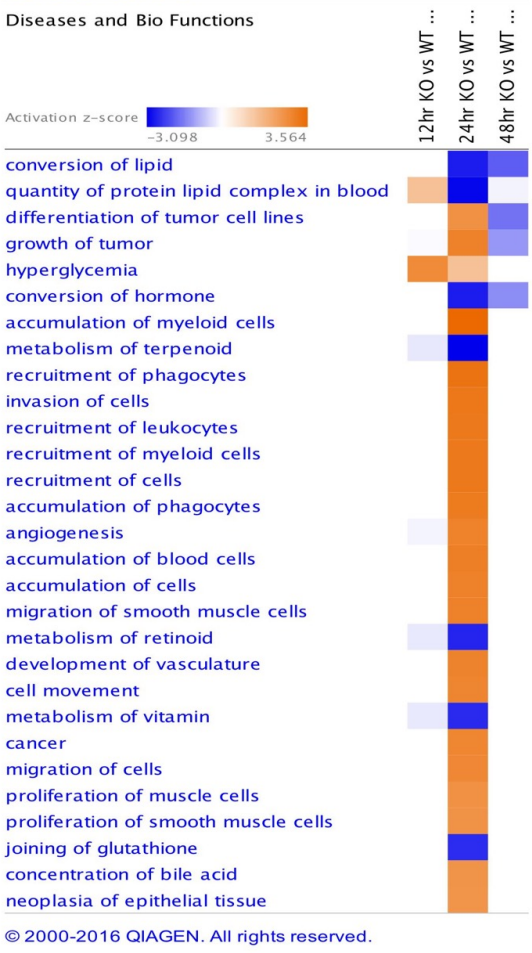
Functional analysis of transcriptomic data revealed changes in metabolic homeostasis, inflammation and proliferation.

The transcript expression data were further analyzed using Core Analysis in IPA (Ingenuity Pathway Analysis) followed by comparison analysis between three time points. This analysis revealed differentially regulated upstream regulators, biofunctions and canonical pathways (Fig. 3.4.4A-C, Table 3.4.1). This analysis revealed that transcript expression changes can be categorized into three groups: disrupted metabolic homeostasis (upstream regulators- ACOX1, PPARα, SREBP2), increased inflammatory signaling (upstream regulators- IL1β, TNFα, IL1A, NF-κB complex and TLRs) and proliferative signaling (upstream regulators- CREB1, ERK, TNFα, NUPR1). Next, we validated transcriptomic gene expression data of selected genes chosen from these three categories using real time PCR analysis (Fig. 3.4.4D and E).

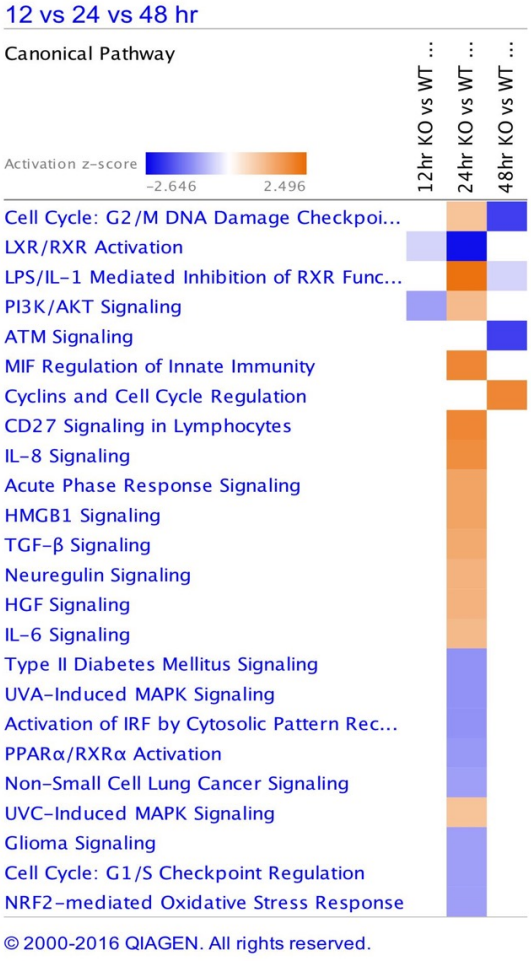
A



B



C


12 vs 24 vs 48 hr
Canonical Pathway
12hr KO vs WT ...
24hr KO vs WT ...
48hr KO vs WT ...
Activation z-score
-2.646
2.496
Cell Cycle: G2/M DNA Damage Checkpoi...
LXR/RXR Activation
LPS/IL-1 Mediated Inhibition of RXR Func...
PI3K/AKT Signaling
ATM Signaling
MIF Regulation of Innate Immunity
Cyclins and Cell Cycle Regulation
CD27 Signaling in Lymphocytes
IL-8 Signaling
Acute Phase Response Signaling
HMGB1 Signaling
TGF-β Signaling
Neuregulin Signaling
HGF Signaling
IL-6 Signaling
Type II Diabetes Mellitus Signaling
UVA-Induced MAPK Signaling
Activation of IRF by Cytosolic Pattern Rec...
PPARα/RXRα Activation
Non-Small Cell Lung Cancer Signaling
UVC-Induced MAPK Signaling
Glioma Signaling
Cell Cycle: G1/S Checkpoint Regulation
NRF2-mediated Oxidative Stress Response
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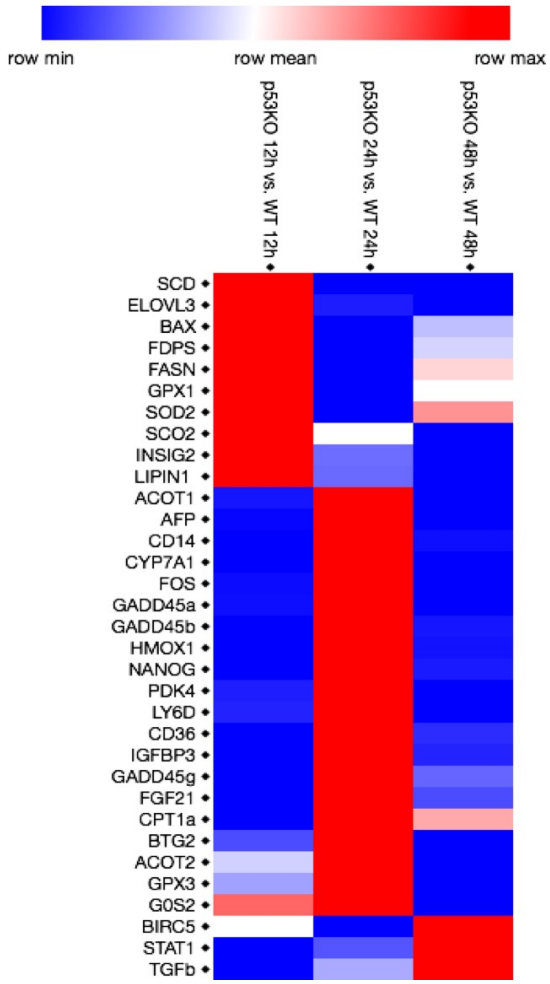
D


row min
row mean
row max
p53KO 12h vs. WT 12h
p53KO 24h vs. WT 24h
p53KO 48h vs. WT 48h
SCD
ELOVL3
BAX
FDPS
FASN
GPX1
SOD2
SCO2
INSIG2
LIPIN1
ACOT1
AFP
CD14
CYP7A1
FOS
GADD45a
GADD45b
HMOX1
NANOG
PDK4
LY6D
CD36
IGFBP3
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CPT1a
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ACOT2
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G0S2
BIRC5
STAT1
TGFb

E

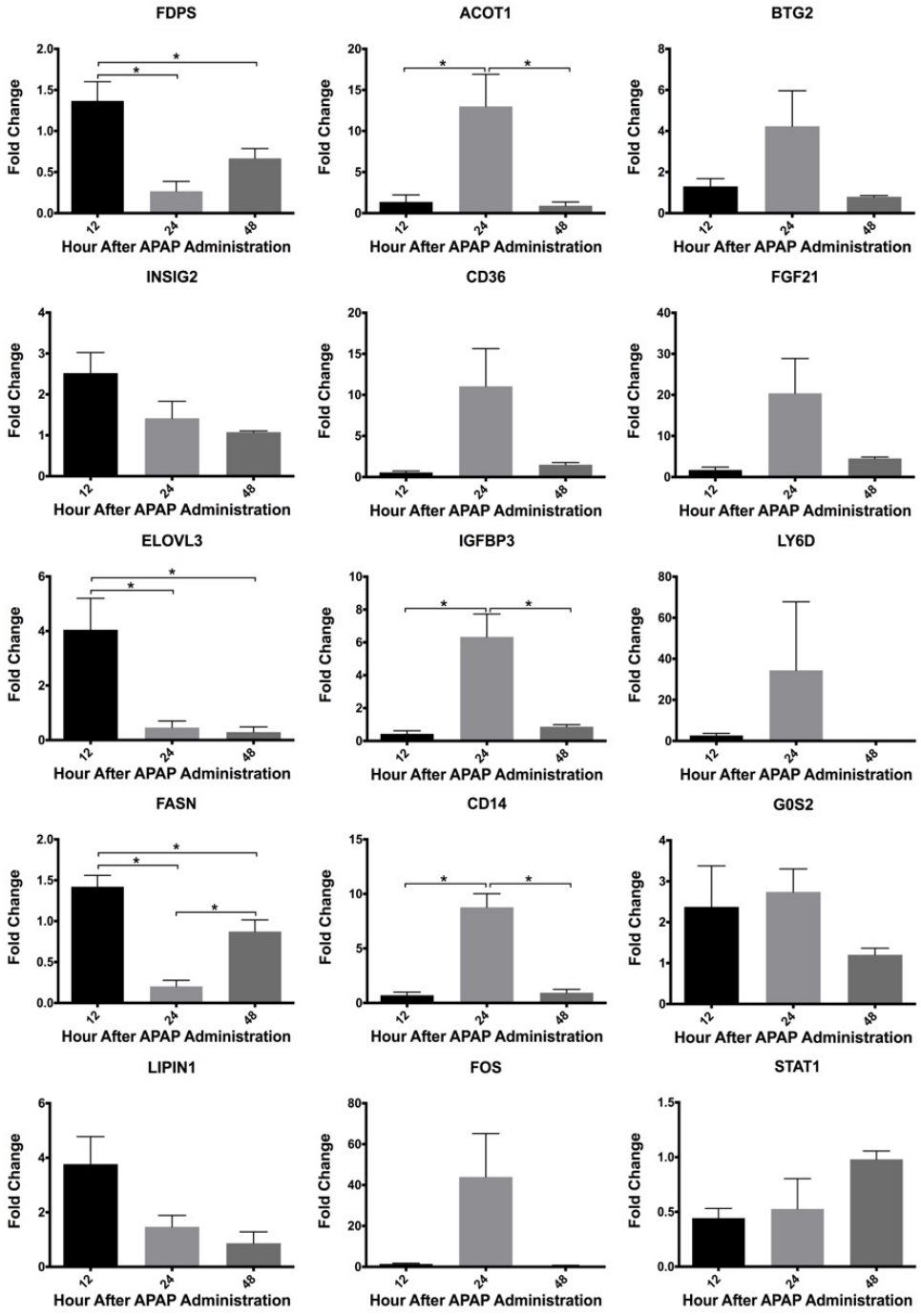

Figure 3.4.4. Functional analysis on transcriptomic data using IPA. Comparison analysis in the form of heatmap of top (A) Upstream regulators (B) Diseases and bio functions (C) Canonical Pathways, post APAP300 treatment in p53KO mice vs WT mice. (D) Validation of transcriptomic data by Real Time PCR analysis of representative genes. (E) Individual graphs of gene expression fold change values determined by Real Time PCR. Gene expression in p53KO mice relative to WT mice at respective timepoints. ANOVA was used to calculate statistical significance * $p<0.05$

Table 3.4.1 Top Upstream regulators in p53KO mice as revealed by IPA

Upstream Regulator	Z Score 12hour	24hour	48hour
ACOX1	-1.05	-6.2	-2.2
NUPR1	1.4	6.5	-1.3
PPARα	2.8	4.2	1.6
IL1b	0.7	5.8	2.1
CREB1	2.3	5	0.4
PDGF BB	1.3	4.6	-1.5
SCAP	0	-4.5	-2.8
TNF	1.5	4.8	-0.8
XBP1	1.2	-5.7	0
POR	0.8	4.1	2
F2	1.7	5	0
SREBF2	0.4	-4.2	-2
HNF1A	-0.9	-4	-1.2
ERK	0.3	5	0
IL2	0.1	4.3	-0.8
SFTPA1	-1.1	-4	0
IL1A	0.2	3.7	-1.1
RELA	0.2	3.7	-1.2

MYD88	0.6	3.9	0.2
IFNG	0.6	3.7	-0.3
MEK	1	3.6	0
NFκB (Complex)	-0.1	3.6	-0.7
TLR3	0.6	3.8	0
TLR4	-0.2	4	0
TICAM1	0	4	0
TREM1	0	4	0

Deletion of p53 induced prolonged steatosis and hypoglycemia following APAP300 treatment in mice.

The transcriptomic data indicated that p53KO mice had changes in metabolic homeostasis after APAP administration. Therefore, we analyzed hepatic lipid content and serum glucose levels. Oil Red O staining of liver sections revealed that APAP treatment induced steatosis at 1 hour and 12 hours and it was resolved by 24 hours in WT mice (Fig. 3.4.5A). p53KO mice liver showed slightly higher steatosis at 0 hour than WT, which further increased after APAP treatment, remained significantly higher than WT mice till 24 hours and disappeared at 48 hours. Steatosis resolution was delayed by 24 hours in p53KO mice as compared to WT (Fig. 3.4.5A). SREBP2 is the major lipid sensor in the hepatocytes. The prolonged steatosis in p53KO mice was accompanied by prolonged SREBP2 expression and cleavage (Fig. 3.4.5B). Further, p53KO mice exhibited more decrease in serum glucose levels post APAP300 treatment as compared to WT mice (Fig. 3.4.5C).

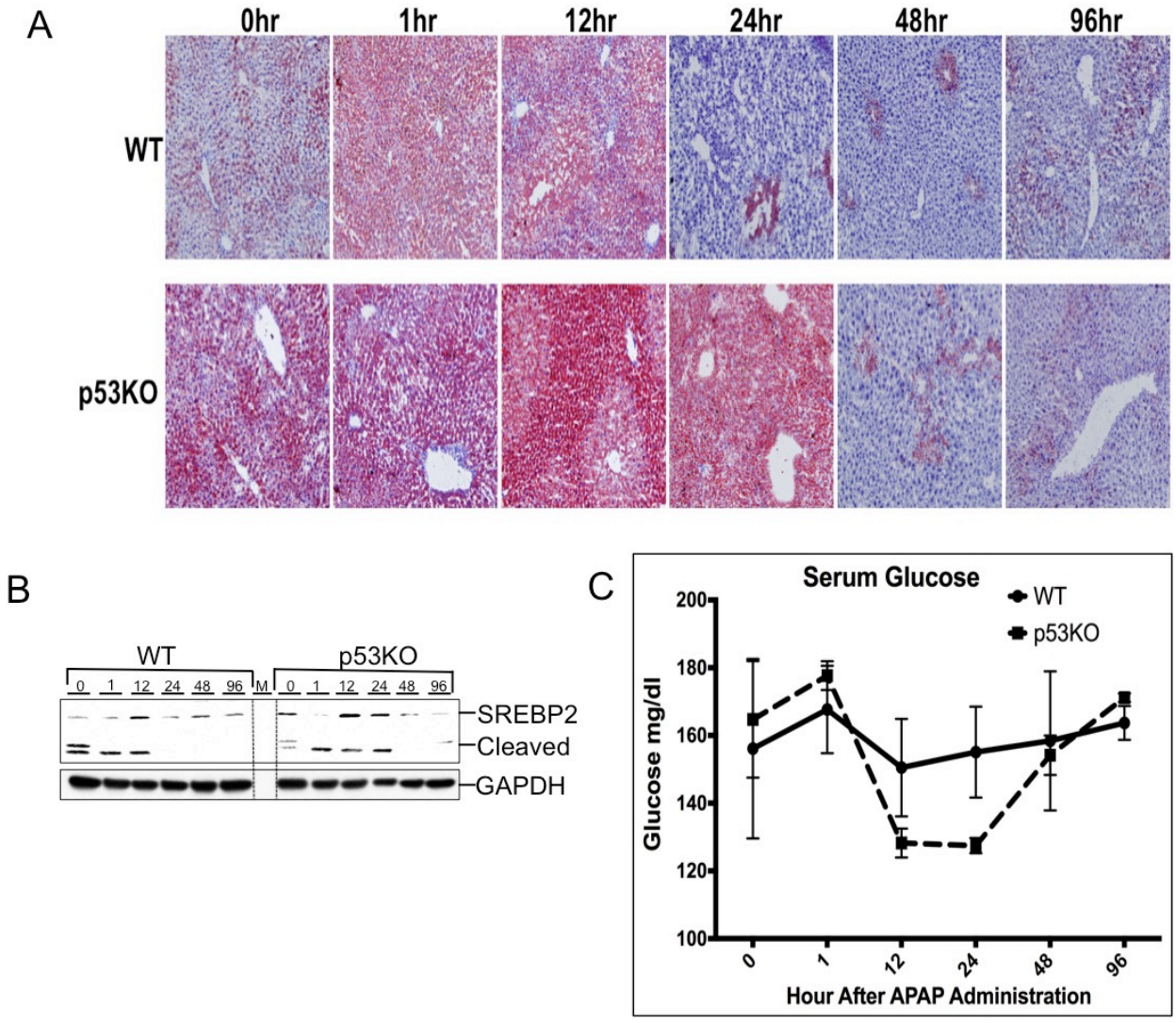

Figure 3.4.5. Prolonged steatosis and hypoglycemia in p53KO mice liver post APAP300 treatment. (A) Representative photomicrographs of Oil Red O-stained frozen liver sections from WT and p53KO mice (B) Western blot analysis of SREBP2 and cleaved SREBP2 using total liver extracts from the respective groups (C) Serum glucose levels.

Deletion of p53 reduced expression of mitochondrial complexes and induces ROS.

Mitochondria are central to APAP-induced injury (McGill, Sharpe et al. 2012). Studies have shown role of p53 in mitochondrial biogenesis and function (Park, Wang et al. 2009). To investigate effect of p53 deletion post APAP treatment on number of mitochondria we measured mitochondrial DNA content in mice liver. p53KO mice tend to have reduced mitochondrial copy number than WT till 24 hours, but at 24 hours a marked increase in mitochondrial copy number was observed (Fig. 3.4.6A). Further, Western blot analysis of mitochondrial complexes revealed that p53KO mice exhibited significantly low expression of complex I, II, and V up to 24 hours than WT mice (Fig. 3.4.6B and C). TFAM and NRF1 are the two transcription factors that regulate expression of mitochondrial complexes (Kelly and Scarpulla 2004). Protein expression of NRF1 was not significantly different between two groups however; TFAM levels were low initially during the first 12 hours after APAP treatment but were induced at 24 hours after APAP treatment in p53KO than WT correlating with induction of complex I, II, and V at 24 hours (Fig. 3.4.6B and C).

We further studied if p53KO mice have increased oxidative stress at 24 hours by measuring hepatic total GSH level. Indeed, hepatic GSH level was decreased in p53KO mice at 24 hours than WT mice (Fig. 3.4.6D upper panel). Low GSH levels can result from increased ROS and reduced GSH synthesis. One of the target genes of p53 is an enzyme called guanidinoacetate methyltransferase (GAMT), which is involved in GSH synthesis by maintaining cysteine pool (Lu 2000). We measured mRNA levels of GAMT

expression, which were highly up regulated at 24 hours after APAP treatment in the WT mice. Interestingly, p53KO mice had reduced expression of GAMT throughout the time course than WT and at 24hour GAMT expression was significantly down regulated than WT mice correlating with decreased GSH levels at 24hour than WT mice (Fig. 3.4.6D lower panel).

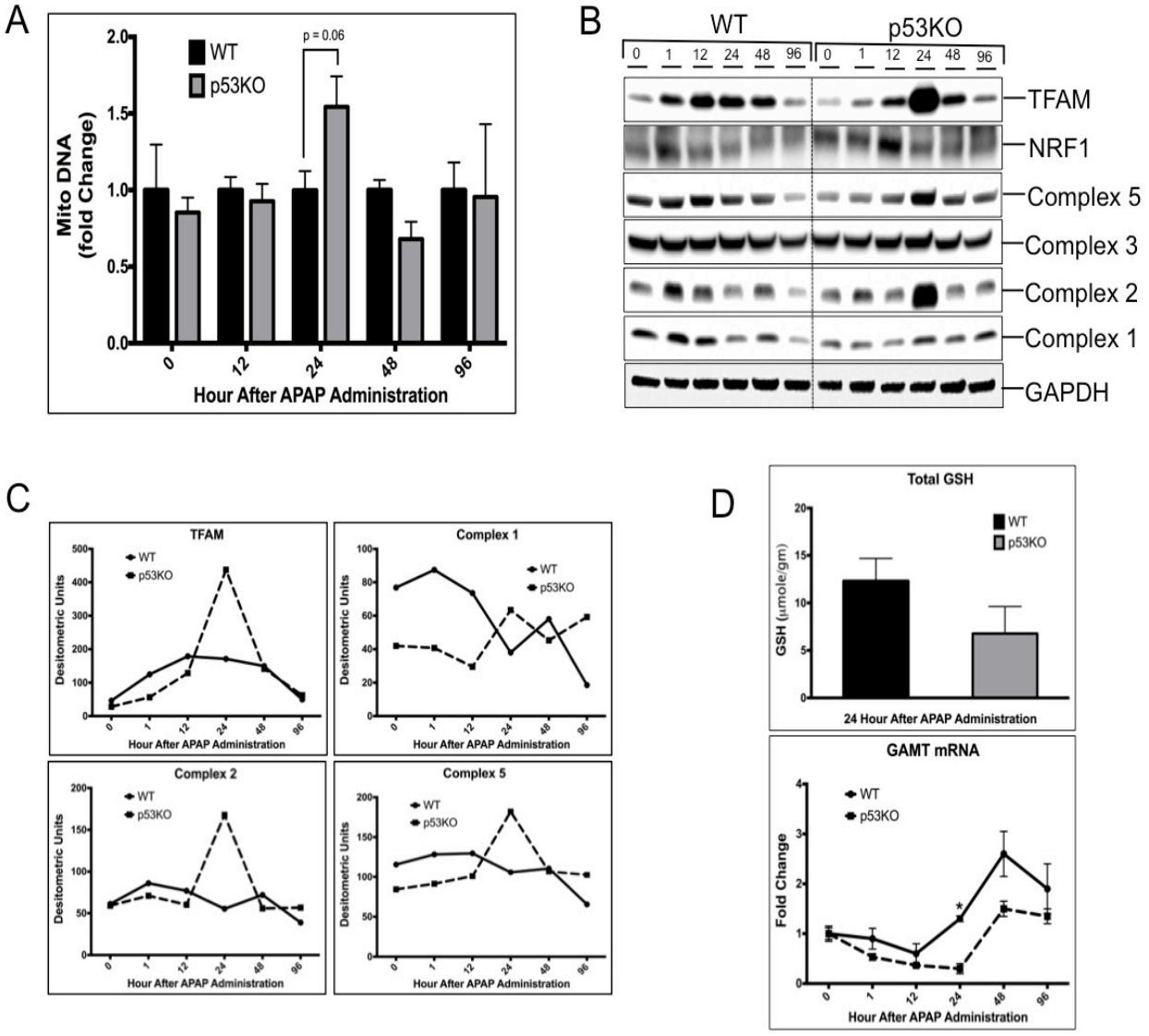

Figure 3.4.6. Reduced mitochondrial complexes and increased ROS during injury phase following APAP300 treatment in p53KO mice. (A) Determination of mitochondrial DNA content. (B) Immunoblot of mitochondrial complexes (I), (II), (III), (V) and their regulatory transcription factors TFAM and NRF1 using total liver extracts from the respective groups. (C) Quantitative densitometry analysis of immunoblot of TFAM, Complex (I), (II) and (V). (D) Hepatic total glutathione levels at 24hour (upper panel) and Real Time PCR analysis of GAMT mRNA (lower panel).

p53KO mice exhibit increased inflammation after APAP treatment

Our data on liver injury (serum ALT and H&E) and repair (PCNA staining and cell cycle markers) revealed that despite 3-fold higher liver injury, p53KO mice still recover at the same time as WT mice. We hypothesized that faster recovery despite very high injury in p53KO mice is due to higher inflammatory and proliferative signaling. Real time PCR analysis revealed that IL1β mRNA levels were induced at 1 and 12hour and declined by 24hour post APAP300 in WT animals (Fig. 3.4.7A). Deletion of p53 resulted in further induction of IL1β mRNA levels at 1hour, 12hour and at 24hour it was significantly higher than WT mice (Fig. 3.4.7A). Likewise, IL10 mRNA levels were induced at 1 and 12hour and reduced by 24hour post APAP300 treatment in WT animals. Deletion of p53 resulted in significant induction in IL10 mRNA at 1hour and 24hour after APAP300 treatment (Fig. 3.4.7B). Previous studies have shown importance of both cytokines IL1β and IL10 in liver injury repair (Jaeschke, Williams et al. 2012). These data indicate that p53 deletion augments cytokine signaling which may result in faster recovery.

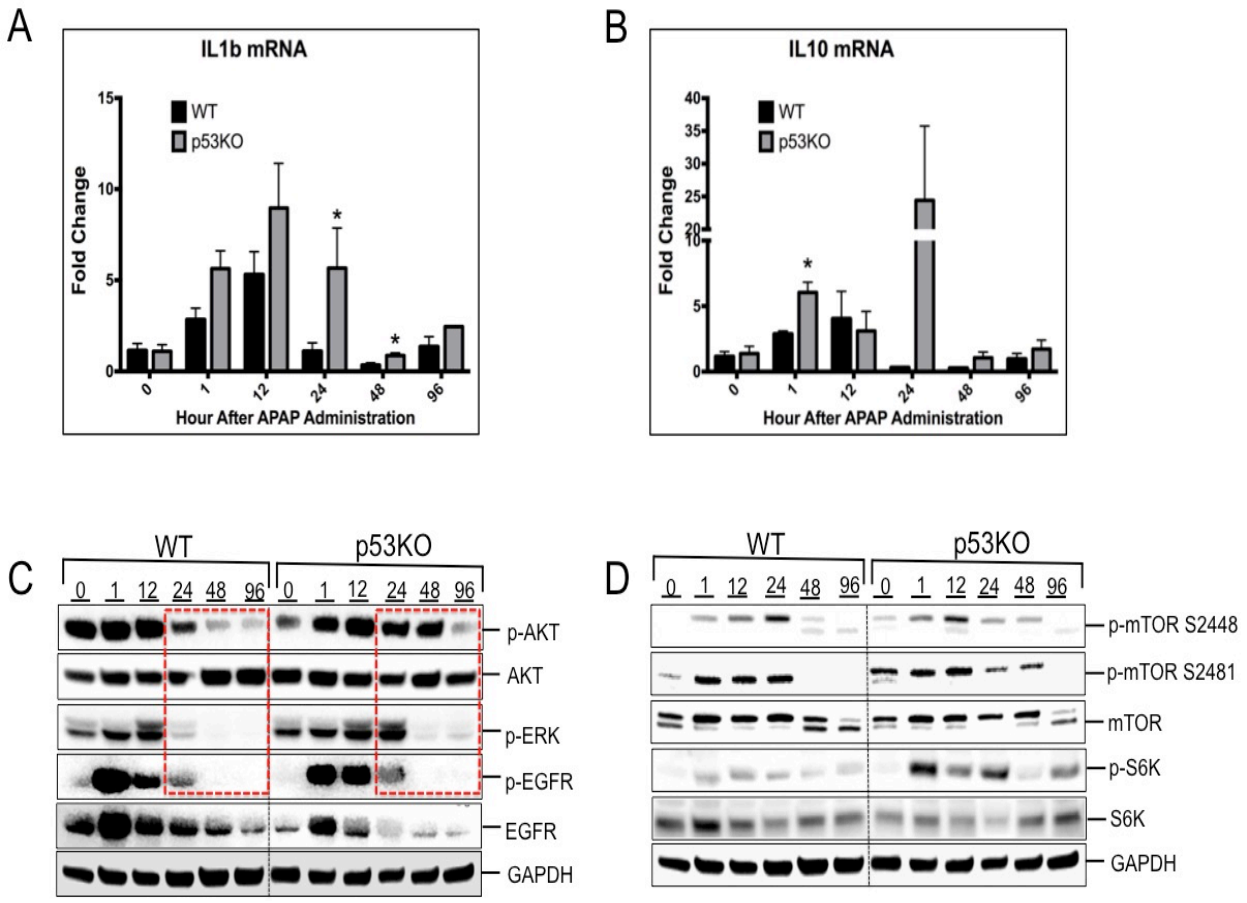

Figure 3.4.7. Deletion of p53 augments inflammatory and proliferative signaling during recovery phase following APAP300 treatment in mice. Real time PCR analysis of inflammatory genes (A) IL1β and (B) IL10. Western blot analysis of proliferative signaling consisting (C) p-AKT, AKT, p-ERK, p-EGFR, EGFR and (D) mTOR signaling, p-mTOR Ser2448, p-mTOR Ser2481, mTOR, p-p70S6K and p70S6K using total liver extracts from the respective groups. * $p<0.05$ (WT vs p53KO)

Enhanced liver regeneration in p53KO mice after APAP overdose

Analysis of cell proliferation (PCNA) and cell cycle (Cyclin D1) showed that p53KO mice exhibit an initial delay followed by a robust liver regeneration leading to timely recovery despite three-fold higher liver injury. We further probed the mechanisms of this enhanced regenerative response in p53KO mice by measuring activation of key pathways involved in liver regeneration. Western blot analysis of AKT showed significantly higher and prolonged activation in p53KO after APAP treatment (Fig. 3.4.7C). Likewise, deletion of p53 resulted in prolonged phosphorylation of ERK till 24hour after APAP300 treatment. p53 is known to regulate EGFR at transcriptional level (Ludes-Meyers, Subler et al. 1996). Consistent with this we observed reduced expression of EGFR total protein in p53KO mice at all time points than WT mice. However, p53KO mice showed sustained EGFR phosphorylation following APAP300 treatment (Fig. 3.4.7C). These data indicate that p53 deletion resulted in sustained proliferative signaling via AKT and ERK, which may enhance faster regeneration.

Finally, we studied mTOR pathway, which plays a critical role in lipid homeostasis, cell proliferation, mitochondrial biogenesis, all major processes identified by our global transcriptomic analysis. p53 is known to inhibit mTOR through AMPK, PTEN, TSC2 expression (Feng, Hu et al. 2007), hence we hypothesized that deletion of p53 will increase activation of mTOR pathway. Western blot analysis of total mTOR protein showed no significant difference at all time points in both WT and p53KO groups. APAP300 treatment induced phosphorylation of mTOR (activation) in both

groups however phosphorylation was prolonged in p53KO group (Fig. 3.4.7D). Phosphorylation of S6K - downstream target of mTOR was significantly higher and sustained in p53KO mice throughout time course than WT mice indicating higher and prolonged activation of mTOR pathway.

3.5 DISCUSSION

p53 is primarily known as a tumor suppressor because of it's ability to inhibit cell proliferation. However, there is increasing evidence that p53 can regulate various cell responses such as migration, metabolism, redox balance, senescence, and stem cell reprogramming (Tyner, Venkatachalam et al. 2002, Muller, Vousden et al. 2011, Kenzelmann Broz and Attardi 2013, Menendez, Shatz et al. 2013, Aloni-Grinstein, Shetzer et al. 2014, Kruiswijk, Labuschagne et al. 2015). Recently Huo et. al. have shown protective role of p53 in APAP induced liver injury (Huo, Yin et al. 2017) but the exact mechanisms of this protection are not known. Our study strengthened this finding and further revealed various mechanisms by which p53 regulate injury progression as well as initiation of liver regeneration.

The pathogenesis of APAP induced ALF can be divided into three phases including initiation of injury (formation of a reactive metabolite NAPQI and protein adducts) progression of injury (cellular signaling, oxidative stress, mitochondrial damage leading to cell death) and regeneration/recovery phase (cell proliferation, angiogenesis and wound healing) (Mehendale 2005). Our data show that liver injury in WT and

p53KO mice is similar till first 12 hours. However, liver injury progresses rapidly in p53KO mice resulting in 3-fold higher liver injury at 24 hours post APAP. These data indicate that p53 protects from progression of APAP-induced liver injury. Whereas the mechanisms of initiation of injury are well studied, the mechanisms of progression of APAP-initiated liver injury are not known. Our study indicates that p53 activation is a crucial component of protection against these mechanisms.

We did not observe any difference in mechanisms of initiation of injury (CYP2E1, APAP adduct formation, GSH depletion or recovery etc.) (Reid, Kurten et al. 2005, Jaeschke and Bajt 2006), which is consistent with no difference in initial injury. Interestingly, p53KO mice exhibited reduced clearance of adducts from liver. APAP adduct formation has been shown to damage mitochondria and autophagy helps in removal of those adducts (Ni, McGill et al. 2016). Accumulating evidence supports p53's role in facilitating autophagy under stress condition (Crighton, Wilkinson et al. 2006, Kenzelmann Broz and Attardi 2013, Kruiswijk, Labuschagne et al. 2015). It is plausible that reduced autophagy may have resulted in delayed adduct removal in p53KO mice.

To determine the underlying mechanisms of increased injury and rapid repair in p53KO mice, we performed global transcriptomic analysis on three crucial time points namely 12, 24 and 48 hours after APAP treatment. Our transcriptomic analysis revealed disruption in metabolic homeostasis, increased inflammatory and proliferative signaling in p53KO mice. Consistently, p53KO mice exhibited higher and sustained steatosis, SREBP2 cleavage and prolonged hypoglycemia after APAP300 treatment correlating with progression of injury till 24hour. Malnutrition and lipid imbalance are known

predisposing factors for increased risk and severity of APAP induced injury (Fromenty 2013, Michaut, Moreau et al. 2014). Several studies in cancer cells have shown that p53 can limit glycolysis flux and promote fatty oxidation (Bensaad, Tsuruta et al. 2006, Kruiswijk, Labuschagne et al. 2015, Parrales and Iwakuma 2016). These data further indicate that p53 mediated metabolic homeostasis is disrupted in p53KO mice making them more vulnerable to APAP induced liver injury.

Another aspect that can contribute to progression of APAP induced injury and lipid homeostasis is number of functional mitochondria (McGill, Sharpe et al. 2012). Our data show that p53KO mice tend to have lower number of functional mitochondria as shown by lower mtDNA copy number and significantly reduced mitochondrial complexes till 24 hours. APAP overdose causes mitochondrial damage reducing capacity of liver to maintain homeostasis, and reduced mitochondrial complexes could further affect the homeostasis. Sustained steatosis along with less functional mitochondria in p53KO mice may have caused higher oxidative stress leading to depletion of hepatic GSH levels at 24 hours. Interestingly, we also observed deficiency in hepatic trans sulfuration pathway as demonstrated by reduced GAMT levels, a key enzyme in replenishing cellular cysteine pool required for GSH synthesis (Lu 2000, Schnackenberg, Chen et al. 2009). The decrease in GAMT in p53KO mice is consistent with the observation that it is a p53 target gene. Taken together, increased steatosis, higher oxidative stress and reduced GSH synthesis during progression phase results in higher progression of injury in p53KO mice.

Previous studies have shown that DAMPs released from necrotic cells after APAP overdose activate Kupffer cells (KCs) through TLR receptors leading to increased cytokine and chemokines production (Jaeschke, Williams et al. 2012). Pro-inflammatory cytokines and chemokines (IL1β, TNFα, MCP1 etc.) (Williams, Farhood et al. 2010) activate and recruit neutrophils and monocytes. Monocyte derived M2 macrophages get recruited in liver and secrete anti- inflammatory cytokines such as IL10. These activated phagocytes can remove dead cells from liver and initiate wound healing process (Bourdi, Masubuchi et al. 2002, Woolbright and Jaeschke 2017). Interestingly, IPA analysis revealed significant activation of inflammatory pathways in p53KO mice. Indeed IL1β and IL10 cytokine expression was significantly higher in p53KO group than WT. It is plausible that higher liver injury results in higher IL1β secretion, which further stimulates increased inflammatory cell infiltration in the liver. Increased expression of pro- resolving cytokine IL10 may induce higher M2 macrophage activation, which will further aid in rapid recovery.

Interestingly, despite very high injury, recovery in p53KO animals was similar to WT animals. PCNA and cell cycle protein data demonstrated delayed but rapid liver regeneration in p53KO mice indicating p53's role in initiation of liver regeneration. This is a significant observation because several acute toxicity studies with APAP and other toxic chemicals including thioacetamide and CCl_4 have shown that high injury results in extensive cellular stress and delay in liver regeneration (Mehendale 2005, Schmidt and Dalhoff 2005, Bhushan, Borude et al. 2013, Bhushan, Walesky et al. 2014). For example, previous study from our laboratory (Bhushan, Walesky et al. 2014) using incremental dose model (300 mg/kg or the regenerating dose and 600 mg/kg or the

non-regenerating dose of APAP) showed that resolution of injury after 600 mg/kg dose in WT mice was significantly impaired. In fact, ALT levels in the mice treated with 600 mg/kg APAP, the non-regenerating dose in that study were almost equal to levels observed in p53KO mice treated with 300 mg/kg dose in this study. However, the p53KO mice still recovered by 48 hours in contrast to the WT mice with similar injury. These data suggest that deletion of p53 improves recovery by boosting the liver regeneration.

Liver regeneration in response to injury has been shown to improve survival in rodent and human studies (Schmidt and Dalhoff 2005, Bhushan, Walesky et al. 2014). Several growth factor signaling pathways are known to stimulate liver regeneration. To study mechanism of rapid liver regeneration despite higher injury in p53KO animals, we looked at differential activation of several pro-mitogenic pathways involved in liver regeneration. These studies revealed significantly higher AKT, ERK and EGFR signaling in p53KO mice, all of which have been shown to stimulate hepatocyte proliferation and liver regeneration. Additionally, we also observed that mTOR pathway was highly activated in p53KO mice than WT. mTOR is known to inhibit autophagy process (Martina, Chen et al. 2012), increase lipid synthesis through S6K mediated SREBP cleavage, and increase cell proliferation (Laplante and Sabatini 2012). Previous studies have shown that p53 inhibits mTOR activity through direct transcriptional regulation of AMPK, TSC2, PTEN and IGFBP3 (Feng, Hu et al. 2007). It is plausible that during the injury progression phase following APAP overdose in WT mice; p53 activation leads to up regulation of these negative regulators of mTOR. Deletion of p53 led to higher and sustained activation of mTOR activity, which could result in down

regulation of autophagy mediated APAP adducts removal and disruption of lipid homeostasis leading to higher progression of APAP induced injury. It is also plausible that, during recovery phase, sustained mTOR and AKT activation may help faster liver regeneration in p53KO mice. Further studies are needed to delineate the exact role of mTOR signaling in p53KO mice after APAP.

Taken together these data indicate that transient activation of p53 following APAP induced liver injury is beneficial to prevent progression of injury, however sustained activation of p53 may affect liver regeneration and recovery. Future studies using stage specific activation (during injury phase) or inhibition of p53 (during regeneration phase) are required to further establish function of p53 in regulating injury progression and regeneration. Our studies have revealed novel pleiotropic role of p53 in linking the injury progression response to liver regeneration and intervention of these mechanisms may have a therapeutic benefit.

CHAPTER IV: CONCLUSIONS AND FUTURE DIRECTIONS

Drug-induced liver injury (DILI) is a significant clinical problem that also affects drug development process (Holt and Ju 2010). APAP mediated liver injury is the most prevalent cause of ALF in United States and many other countries (Ostapowicz, Fontana et al. 2002, Larson, Polson et al. 2005). Unlike several other drugs APAP induced toxic liver injury is not idiosyncratic reaction but occurs in dose-dependent manner that begins with mitochondrial dysfunction, ROS and extensive DNA fragmentation (Jaeschke 2015). Currently there are two main therapies for APAP-induced ALF. The primary treatment for APAP overdose patients is intravenous administration of N-acetylcysteine (NAC), a precursor of GSH and works mainly via enhancing GSH replenishment. However, it is most effective when delivered within 8 hours post APAP overdose. The majority of APAP-induced ALF patients are late presented reducing the effectiveness of NAC (Larson 2007, Fontana 2008, Lee, Larson et al. 2009). The other treatment option is liver transplantation for patients who progress to ALF. Unfortunately, transplantation has its own limitations including a lack of donor organs, high cost and life-long immunosuppression (Lee, Larson et al. 2009). Considering these extremely limited therapies at present, additional therapeutic options for APAP-induced ALF are urgently needed. Compensatory liver regeneration following DILI plays a critical role in survival (Mehendale 2005) (Jaeschke and Bajt 2006). Previous studies demonstrated that adequate stimulation of liver regeneration improves survival following APAP overdose (Mehendale 2005, Jaeschke and Bajt 2006, Apte, Singh et al. 2009, Bhushan, Walesky et al. 2014). Studies in ALF patients showed that increased liver regeneration indicated by elevated plasma α-fetoprotein (AFP) levels and hypophosphatemia, improves patient survival (Schmidt and Dalhoff 2002, Schmidt

and Dalhoff 2005). Further, in theory, it may be possible to modulate liver regeneration even at later stages of APAP-induced liver injury. Thus, it is imperative to develop novel pharmacological agents that improve liver regeneration in patients of APAP-induced ALF. In response to APAP-induced liver injury, intricate signaling stimulates proliferation of healthy hepatocytes to replace the dead cells and results in recovery (Bajt, Knight et al. 2003, Apte, Singh et al. 2009, Bhushan, Walesky et al. 2014). However, such compensatory liver regeneration response is only activated up to a threshold dose. Following exposure to doses higher than the regenerative threshold dose, liver regeneration is inhibited, recovery is delayed and can lead to death (Mehendale 2005, Bhushan, Walesky et al. 2014). The most important aspect of liver regeneration is cell proliferation where quiescent healthy hepatocytes enter cell cycle, progress through complete cell cycle and produce two daughter cells. This cell division/ cell cycle is governed by various factors including presence of adequate growth factors (HGF, EGF), cytokines (IL6, STAT3), metabolic homeostasis, intact DNA and feedback from growth stimulating as well as growth inhibiting signaling (Michalopoulos 2007, Albrecht 2009). The mechanisms that drive cell proliferation after APAP-induced liver injury are not completely known. Especially, the mechanisms that inhibit cell proliferation at very high dose exposures are not known. It has been postulated that several factors including lack of proper signal transduction either due to decreased pro-mitogenic ligands or disruption of intracellular signaling, depleted energy due to mitochondrial damage and significant DNA damage resulting in activation of cell cycle checkpoints may be involved.

Cellular DNA is vulnerable to damage from various factors including endogenous (ROS, replicative stress) and environmental (chemicals, radiation) stresses. Further, the risk of DNA damage increases during DNA replication phase of cell cycle (Saintigny, Delacote et al. 2001). Following surgical resection or chemical injury, hepatocytes undergo rapid proliferation to restore liver mass. That increases DNA replication associated risk of DNA damage (Saintigny, Delacote et al. 2001). Recent study has shown that genetic deletion of nucleostemin, a cell cycle modulator protein, in mice resulted in increased DNA double strand breaks in regenerating liver following PHX (Lin, Ibrahim et al. 2013). However, if DNA damage plays any role in liver regeneration following APAP induced liver injury remains unknown.

Our laboratory has used an incremental dose model comprising a lower regenerative dose (300 mg/kg) and a higher non-regenerative dose (600 mg/kg) to determine the mechanisms behind inhibited regeneration at high APAP over dose (Bhushan, Walesky et al. 2014). Both doses cause severe liver injury and dose-dependent activation of several proliferative pathways including EGFR, HGF and IL6 and anti-proliferative p21 pathway. However, compensatory liver regeneration was prompted only at lower dose but was inhibited at higher dose despite more than 50% viable hepatocytes (Bhushan, Walesky et al. 2014). These observations would suggest activation of anti-proliferative signaling at higher dose that counteracted the highly activated proliferative pathway leading to inhibition of liver regeneration and mortality. This study also revealed that decreased liver regeneration after non-regenerative dose of APAP is not due to lack of viable cell mass as there are over 50% surviving hepatocytes. It is most likely due to an intracellular stress in these surviving hepatocytes

that actively inhibits entry of these cells in cell cycle. The focus of the studies outlined in this dissertation was to study anti-proliferative mechanisms that can inhibit liver regeneration following APAP overdose. Understanding these mechanisms would provide insight into why some APAP induced ALF patients fail to spontaneously recover and need liver transplantation.

We performed microarray analysis to delineate differentially regulated anti-proliferative mechanisms using liver samples from both low and high dose of APAP treated mice. IPA analysis of microarray data revealed that DNA damage, DNA repair, replication, and cell cycle checkpoint pathways were significantly altered in lower dose animals as compared to higher dose. Consistently, APAP overdose caused DNA DSB, the most deleterious form of DNA damage, at both doses however it was sustained and prolonged only at the higher dose. More importantly, immunofluorescence detection revealed that hepatocytes surrounding necrotic zone exhibited DSB. Previous studies show that the hepatocytes that surround necrotic area undergo proliferation to replace dead cells that results in recovery from injury. Very interestingly, despite active growth factor (EGF, HGF) and cytokine (IL6, STAT3) mediated proliferative signaling at higher dose, hepatocytes appeared arrested either in G0 or in G1 phase of cell cycle. These data indicated to us that sustained DNA DSB could be hindering DNA replication and in turn liver regeneration at the higher, non-regenerative APAP dose. In response to DNA damage, the DDR pathway activates DNA repair process and p53, the DDR effector protein, arrests the cell cycle to provide time for DNA repair outcome (Sulli, Di Micco et al. 2012). DDR begins with detecting the DNA damage by sensor proteins such as PARP. PARP is activated in presence of DNA damage and, by PARylating nuclear

proteins, it facilitates recruitment of DNA repair mediator and effector proteins. Our data shows rapid and sustained activation of PARP when DNA DSB was detected at lower regenerative dose. However, PARP activation was significantly reduced and delayed at higher APAP dose. This observation is consistent with a previous study demonstrating that higher PARP activation is not associated with increased liver injury following APAP toxicity (Cover, Fickert et al. 2005). In fact, PARP activation appears to prevent sustained liver injury possibly via facilitating DNA DSB repair. Cell cycle analysis in liver from mice given the non-regenerative APAP dose demonstrated that hepatocytes were arrested in G0 and/or G1 phase. DSB repair non-homologous end joining (NHEJ) remains active through all phases of the cell cycle while the homologous recombination (HR) pathway, operates during S and G2 phases. Therefore, we investigated differential regulation of NHEJ pathway in both doses. Moreover, de-phosphorylation of Tyr142 residue of H2AX facilitates binding of repair proteins at DNA damage site (Cook, Ju et al. 2009, Xiao, Li et al. 2009). De-phosphorylation of H2AX Tyr142 and NHEJ repair protein expression was significantly deregulated at higher dose. These data collectively demonstrate that DNA DSB repair is impaired at higher dose due to reduced PARP activity (sensor) and repair protein expression (effector) leading to sustained DSB. Further investigation of DDR effector protein p53 revealed that p53 was activated at both low and higher dose of APAP, as DSB occurs at both doses. However, p53 activation was significantly prolonged at higher dose correlating with sustained DNA DSB. It is plausible that sustained DNA DSB resulted in prolonged p53 mediated growth arrest and replicative senescence at higher dose.

Overall, this study has revealed that timely repair of damaged DNA regulates liver regeneration following APAP overdose. This is a first study to demonstrate the dose-dependent increase in DNA damage, dose-dependent inhibition of DNA repair and their connection with inhibition of liver regeneration and recovery after APAP overdose. It also indicates that DDR is a vital link connecting liver injury and initiation of liver regeneration. Our work identified potential regulators in the DDR pathway that, if induced, could improve DNA repair and in turn, liver regeneration after APAP-overdose. These targets may be beneficial for potential development of regenerative therapies. In the future, induction of PARP activity and other DNA repair proteins in this model can be investigated in order to improve DNA repair following APAP toxicity.

Our work using incremental APAP dose model demonstrated that lack of prompt DNA DSB repair resulted in p53 mediated cell cycle arrest and replicative senescence following higher dose of APAP when compared to a lower APAP dose. p53 was activated at both lower and higher doses but its activation at the lower dose was transient when compared to its activation at the higher dose. This suggested that p53 activation regulates initiation of liver regeneration following APAP overdose. In order to explore that, we investigated the role of p53 in APAP toxicity using WT and p53KO mice treated with the lower, APAP300 dose. Surprisingly, deletion of p53 resulted in 3-fold higher injury than WT animals. This observation was consistent with another study (Huo, Yin et al. 2017) indicating a previously unknown protective role of p53 in injury progression following APAP overdose. Deletion of p53 did not affect bioactivation of APAP and APAP adduct formation, which was reflected in similar initial injury in WT and p53KO animals. Interestingly, the peak injury in p53KO animals (Avg. peak ALT: 11600)

following APAP300 dose was much higher than higher non-regenerative dose (Avg. peak ALT: 9500) give to WT animals in previous study. However, despite the very high injury, p53 KO mice showed full recovery from APAP exposure, presumably due to enhanced liver regeneration. In fact, deletion of p53 resulted in an initial delay in liver regeneration but once initiated p53 KO mice had significantly faster liver regeneration and complete recovery. These data collectively demonstrated that activation of p53 protects injury progression and orchestrate initiation of liver regeneration after APAP toxicity.

The role of p53 was further investigated using transcriptomic and molecular analysis. These studies revealed that deletion of p53 resulted in sustained activation of AKT and mTOR signaling, increased steatosis, decreased functional mitochondria, impaired adduct removal possibly through autophagy (Ni, McGill et al. 2016) and higher inflammation and cell proliferation. mTOR regulates inflammation, inhibit autophagy, increase lipid synthesis and cell proliferation (Laplante and Sabatini 2012, Martina, Chen et al. 2012). This observation is consistent with previous findings where p53 inhibited mTOR by direct transcriptional regulation of mTOR inhibitors - AMPK, TSC2, PTEN and IGFBP3 (Feng, Hu et al. 2007). Our work demonstrated that deletion of p53 led to higher and sustained activation of mTOR activity, which could result in down regulation of autophagy mediated APAP adducts removal and disruption of lipid homeostasis. GAMT is a p53 target gene and a key enzyme in replenishing cellular cysteine pool required for GSH synthesis (Lu 2000, Schnackenberg, Chen et al. 2009). Consistently, p53KO mice showed reduced GAMT levels indicating deficient GSH trans-sulfuration pathway. Moreover, p53KO mice showed less functional mitochondria and

hepatic GSH levels but increased GSSG levels when compared to WT mice. These observations collectively demonstrated that p53 activation during injury progression phase could promote autophagy mediated adduct removal as well as lipid homeostasis by inhibiting mTOR activation, and GSH replenishment in WT mice. However, in the absence of p53, impaired adduct removal, increased steatosis, reduced GSH synthesis, and higher oxidative stress culminate in higher progression of injury.

Interestingly despite very high injury deletion of p53 resulted in faster liver regeneration and complete recovery. Based on IPA and cytokine (IL1β and IL10) expression data we demonstrated that p53KO mice have significant activation of inflammatory pathways. Consistent with previous studies, increased IL1β and IL10 levels indicate activation and recruitment of neutrophils and monocytes. (Bourdi, Masubuchi et al. 2002, Woolbright and Jaeschke 2017). These activated phagocytes can remove dead cells from liver and initiate wound healing process by clearing path for regeneration. Moreover, deletion of p53 resulted in sustained activation of proliferative signaling such as mTOR, AKT, and ERK that correlated with delayed but rapid induction of PCNA positive cells. During recovery phase, higher monocyte recruitment as well as sustained mTOR and AKT activation may help faster liver regeneration in p53KO mice. Further studies are needed to delineate the exact role of mTOR signaling in liver regeneration after APAP.

Collectively, our data indicate that in absence of p53-mediated mTOR inhibition, APAP-induced liver injury progresses during injury development phase and induces liver regeneration during recovery phase. Thus, our study highlights p53 and its

downstream targets as potential therapeutic targets to improve liver regeneration following APAP-induced ALF. In the future, studies using stage-specific activation (during injury phase) or inhibition of p53 (during regeneration phase) will be performed to further establish the function of p53 in regulating injury progression and regeneration. Studies with an mTOR activator or p53 inhibitor in non-regenerative dose will be done to elucidate effect on liver regeneration. Further, more extensive p53 binding repertoire will be studied using ChIP sequencing to obtain more complete picture of its function in APAP pathophysiology.

CONCLUDING REMARKS

The studies outlined in this dissertation provide insight into novel anti-proliferative mechanisms that can inhibit liver regeneration following APAP induced ALF. The treatment options for ALF are extremely limited and recently several studies demonstrated the power of improved liver regeneration as a plausible therapeutic option. However, the mechanisms that inhibit liver regeneration after APAP-induced ALF have not extensively studied. Our previous study using incremental dose model with regenerative and non-regenerative dose of APAP identified several pro-regenerative pathways that improve recovery. Current work revealed the role of DNA damage as one of the anti-proliferative mechanisms that inhibit liver regeneration and recovery in non-regenerative dose. We demonstrated that APAP overdose caused DNA DSB leading to p53 mediated cell cycle arrest. However, timely repair of DSB releases cell cycle arrest and liver regenerates. On the contrary, failure to repair DNA damage leads to sustained activation of p53, replicative senescence and inhibited liver

regeneration at non-regenerative dose. Our work indicated that stimulation of DNA repair could be a viable approach to improve liver regeneration when spontaneous recovery is impaired following APAP toxicity. Another approach could be to inhibit p53 mediated replicative senescence. Indeed, our work demonstrated that deletion of p53 in mice enhances liver regeneration despite very high liver injury. Our work revealed a novel protective role of p53 in acute hepatotoxicity induced by APAP overdose. This suggests that transient activation of p53 following regenerative dose of APAP protects from injury progression. However, sustained p53 activation as seen in non-regenerative dose inhibits liver regeneration and recovery.

Overall, our studies have identified mechanisms that inhibit liver regeneration affecting recovery, which provide insight into why some patients following APAP toxicity fail to recover spontaneously. The mechanisms of liver injury are well studied here we uncovered another player in injury progression following APAP overdose. Furthermore, our work demonstrated a very novel and critical mechanism that orchestrate liver injury response to initiation of liver regeneration.

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