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NEW TARGET GENES FOR TUMOR SUPPRESSORS p53 AND p73 IN REGENERATING LIVER

Svitlana M. Kurinna

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by

Svitlana Mikhailivna Kurinna, M.S.

APPROVED:

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Michelle Barton, Ph.D., Supervisory Professor

_____

Sharon Dent, Ph.D.

_____

Guillermina Lozano, Ph.D.

_____

Elsa Flores, Ph.D.

_____

Jeffrey Frost, Ph.D.

APPROVED:

_____

Dean, The University of Texas

Graduate School of Biomedical Sciences at Houston

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IN REGENERATING LIVER

A

DISSERTATION

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
M. D. Anderson Cancer Center
Graduate School of Biomedical Sciences
in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

Svitlana Mikhailivna Kurinna, M.S.

Houston, Texas

May 2010

DEDICATION

I dedicate this work to my family whose love extended overseas to support me in every way.

Acknowledgements

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Publication No._____*

Svitlana Mikhailivna Kurinna

Supervisory Professor: Michelle Barton, Ph.D.

The p53-family of proteins regulates expression of target genes during tissue development and differentiation. Within the p53-family, p53 and p73 have hepatic-specific functions in development and tumor suppression. Despite a growing list of p53/p73 target genes, very few of these have been studied *in vivo,* and the knowledge regarding functions of p53 and p73 in normal tissues remains limited. $p53^{+/-}p73^{+/-}$ mice develop hepatocellular carcinoma (HCC), whereas overexpression of p53 in human HCC leads to tumor regression. However, the mechanism of p53/p73 function in liver remains poorly characterized. Here, the model of mouse liver regeneration is used to identify new target genes for p53/p73 in normal quiescent vs. proliferating cells. In response to surgical removal of ~2/3 of liver mass (partial hepatectomy, PH), the remaining hepatocytes exit G_0 of cell cycle and undergo proliferation to reestablish liver mass. The hypothesis tested in this work is that p53/p73 functions in cell cycle arrest, apoptosis and senescence are repressed during liver regeneration, and reactivated at the end of the regenerative response. Chromatin immunoprecipitation (ChIP), with a p73-antibody, was used to probe arrayed genomic sequences (ChIP-chip) and uncover 158 potential targets of p73-regulation in normal liver. Global microarray analysis of mRNA levels, at T=0-48h following PH, revealed sets of genes that change expression during regeneration. Eighteen p73-bound genes changed expression after PH. Four of these genes, *Foxo3, Jak1, Pea15,* and *Tuba1* have p53 response elements (p53REs), identified *in silico* within the upstream regulatory region. Forkhead transcription factor *Foxo3* is the most responsive gene among transcription factors with altered expression during regenerative, cellular proliferation. p53 and p73 bind a *Foxo3* p53RE and maintain active expression in quiescent liver. During liver regeneration, binding of p53 and p73, recruitment of acetyltransferase p300, and an active chromatin structure of *Foxo3* are disrupted, alongside loss of *Foxo3* expression. These parameters of *Foxo3* regulation are reestablished at completion of liver growth and regeneration, supporting a temporary suspension of p53 and p73 regulatory functions in normal cells during tissue regeneration.

TABLE OF CONTENTS

CHAPTER I: INTRODUCTION AND RATIONALE FOR THE STUDY

CHAPTER II: MATERIALS AND METHODS

List of Illustrations

List of Tables

CHAPTER I

INTRODUCTION

BACKGROUND AND RATIONALE FOR THE STUDY

1.   Transcription factors regulate regeneration and tumor development in liver.

1.1.   Physiology and biochemistry of liver: "Poor seed, good soil".

The liver is the largest solid organ in the body that serves essential functions, including carbohydrate and protein metabolism, lipogenesis, production of coagulation factors and bile acids, as well as the detoxification of compounds, absorbed by the intestine. The metabolic activity of liver is therefore essential for homeostasis; it requires a complex functional anatomy and tightly regulated biochemical processes. Two important characteristics of this complexity include highly developed cardiovasculature, represented in liver by a network of sinusoids, and a strong regenerative response to damaging agents. The major cell of liver tissue is the hepatocyte, one of the body's most versatile cells, which accomplishes multiple metabolic functions and retains the ability to proliferate in response to liver damage. A large blood supply and the ability of liver cells to proliferate predispose liver to a higher risk of cancer development. The fourth leading cause of cancer-related deaths, and the fifth most common solid tumor worldwide, is hepatocellular carcinoma (HCC). It is triggered in liver tissue by chronic inflammation, toxins intake, and hepatitis, resulting in aberrant proliferation of hepatocytes and activation of liver cancer stem cells (1, 2). Published studies of systemic chemotherapy report response rates of 0% to 25%, with no prolonged survival in patients with HCC (1). In some cases, partial hepatectomy (PH) is used to remove parts of liver lobes affected by HCC, but the overall response to liver resection in patients remains complicated (3, 4).

Back in 1889, Stephen Paget made an important observation that patients with breast cancer were predisposed for metastasis to the liver. He hypothesized that tumor cells (seeds) colonize distant organs with favorable environment (soil) (Paget, S. 1889, ref. in (5)). Since then, multiple studies confirmed that the liver is one of the most affected organs in patients with metastatic tumors. Often, liver is a terminal point of metastasized cancer, leading to a high mortality rate due to various conditions collectively known as liver failure. Extending Paget's "seed and soil" hypothesis, liver thus is a poor seed, but a good soil for cancer development. Infiltration of liver by metastatic colorectal cancer (CRC) causes up to 90% of all deaths from CRC (6). Importantly, hepatic metastases from CRC derive almost all of their blood flow from the arterial vasculature of affected liver lobes (7). If complete surgical resection, or if hepatospecific radiotherapy of liver-only CRC can be achieved, the survival probability increases significantly (6, 8). Whereas several types of cancer, like CRC, lymphomas, and pancreatic cancer, readily metastasize to liver, tumor growth in the latter does not infiltrate other organs (5, 6). Confinement of tumor growth to liver makes it an attractive therapeutic target, and many successful advances have already been made in this direction (6). Better understanding of mechanisms that distinguish normal hepatic growth and the development of liver cancer therefore remains a highly desirable goal of molecular hepatology.

1.2.   Common molecular mechanisms may regulate liver carcinogenesis and regeneration.

At present, liver resection and liver transplantation are considered the only curative measures for liver cancer. Both procedures are associated with high risk due to post-

hepatectomy complications (4, 9). A large number of liver transplantation candidates remain on the waiting list until they die from tumor progression or cirrhosis.

A significant research effort in creating bioartificial substitutes for liver using *ex vivo* cultured, immortalized human hepatocytes, in a filter unit connected to a patient had very limited success in clinical trials (10). Important issues such as finding a source of viable hepatocytes and maintaining viable and fully functional cells *ex vivo* throughout treatment directed research toward embryonic, induced pluripotent, and adult liver stem cells. Further understanding of molecular mechanisms that regulate liver development and regeneration is required for the new strategies in liver bioengineering and improvement of the patient's recovery after hepatectomy or liver transplantation.

Several intracellular signaling pathways have been studied in association with liver regeneration and liver cancer: PI3K/Akt-mediated growth factor signaling; transforming growth factor-beta (TGFβ)–mediated inhibition of cellular growth; Wnt/β-catenin pathway of cellular adhesion; Hippo/Yorki (Mst1/2/YAP) pathway of organ size control; regulation of the cell cycle by tumor suppressor proteins p53 and retinoblastoma (Rb) (11-14). Conceptually, liver regeneration and hepatocarcinogenesis are based on two major molecular events: (1) the activation of cytokine and growth factor-mediated signaling (*e.g.*, IL-6, IGF, VEGF), accompanied by the activation of oncogenes, such as c-Myc and β-catenin; and (2) the inactivation of tumor suppressors, such as p53 and Rb (12, 15). The immortalization of neoplastic cells, in the case of liver cancer, can be achieved by the activation of hTERT, a component of telomerase (16, 17). Loss of TGFβ signaling and activation of signal transducer and activator of transcription 3 (STAT3) contribute to the malignant transformation in hepatic tumors (18); similarly, cytokine-induced activation of

15

STAT3 and a decrease in TGFβ are observed during the initiation of hepatocyte proliferation during liver regeneration (13).

Growing evidence in the literature suggests that loss of p53 function contributes to the molecular pathogenesis of liver cancer, whereas restoration of p53 expression clears liver tumors in mice and humans *in vivo* (19-21). A significant increase in p53 protein levels was observed in Ataxia telangiectasia mutated (ATM) knock-out (KO) mice, rendering them resistant to the development of HCC (22). Importantly, the induction of p53 in liver cancer cells induces senescence and a decrease in tumor size without induction of cell death that might cause the inflammatory response and/or liver failure. Thus, activation of p53 might be the best approach for HCC treatment. However, very little is known about the mechanism of p53-mediated anti-proliferative functions in liver.

1.3.   p53 family of tumor suppressors have hepatoprotective functions *in vivo*.

The p53-family of proteins regulates expression of target genes that promote cell cycle arrest and apoptosis, which may be linked to growth control during liver regeneration, as well as to tumor suppression. These functions are executed through the extra-nuclear (mitochondrial) and the intra-nuclear (transcriptional) activity of p53 and family members p63 and p73 (represented by p53 on Diagram 1). p53 target genes, inhibitors of cell cycle *CDKN1A* (*p21*) and *CDKN1B* (*p27*) and the *TP53* gene itself, are mutated in HCC, or inactivated at the level of protein function (12). *TP53* alterations are generally associated with larger, less differentiated tumors and poor survival (23), whereas restoration of *wt Trp53* function clears liver carcinomas in mice (20). The reactivation of p53 protein via

inactivation of p53 ubiquitin ligase MDM2, as well as the overexpression of the positive regulator of p53 ARF might be a treatment strategy for HCC (24). Despite the clinically established correlation between the status of p53 and hepatocarcinogenesis, little is known about mechanisms of p53-mediated tumor suppression in liver.

p53 is member of a larger family of tumor suppressors, which includes p53, p63, and p73 and their multiple isoforms, with primary functions as transcription factors in mammalian cells (25). Comparison of $p53^{-/-}$, $p63^{-/-}$, and $p73^{-/-}$ mice, as well as studies in tissue culture, demonstrate that while trans-activating (TA) p63 and TA-p73 regulate many of the same functions controlled by p53, these proteins clearly play unique roles *in vivo* during development and disease (26). Tumor predisposition is the most obvious phenotype of $p53^{-/-}$ mice, which predominantly develop lymphomas and sarcomas within 3-5 months of age (27). However, knockout of all p63 isoforms ($p63^{-/-}$) results in embryonic lethality due to epithelial malformations (28). $p73^{-/-}$ mice have neurological, pheromonal and inflammatory abnormalities and die in early adulthood (29). Interestingly, when p73 is depleted together with p53 ($p53^{+/-}$; $p73^{+/-}$), there is an obvious phenotype in liver. The $p53^{+/-}$; $p73^{+/-}$ mice develop hepatocellular carcinoma (HCC) at 5-7 months of age, suggesting a pivotal and cooperative role for p53 and p73 in regulation of hepatic gene expression. In human HCC samples, alternatively spliced p73 transcripts, which may be associated with inactive or dominant negative isoforms of p73 (30), are increasingly expressed compared to healthy liver tissue (31). Approximately 90% of $p53^{+/-}$;$p73^{+/-}$ mice with HCC have loss of heterozygocity in *Trp73*, further emphasizing the importance of tissue-specific functions of p73 in liver (32). Very few studies elucidate roles for p53 and TA-p73 as transcriptional regulators in quiescent vs. proliferating hepatocytes during liver regeneration or in liver

cancer cells. Most of p53 and TA-p73 target genes are studied in tissue culture, and very little is known about p53 and TA-p73-mediated transcription in normal, quiescent liver or in proliferating liver cells *in vivo*.

Studies of p53 function in different cell types strongly suggest that p53 is activated in response to genotoxic stress in a tissue- and stress-dependent manner (33). MacCallum *et al*. compared the cellular responses of *wt* and p53 KO mice to γ–irradiation and observed a difference in accumulation of p53 among examined tissues, with liver showing no increase of p53 protein levels (34). More recently, another study of irradiated *wt* and p53 KO mice confirmed the lack of p53-mediated response to ionizing radiation in hepatocytes, and showed apoptosis in all irradiated *wt* tissues except liver (35). Recently, an induction of phosphatase in regenerating liver, PRL-1, observed in liver cancer, was shown to downregulate p53 via proteosomal degradation (36). A marker of metastatic liver tumors, *PRL-3*, is a p53 target involved in cell cycle regulation (37). Despite the relative resistance of the liver to irradiation and p53-mediated apoptosis, a high rate of *TP53* mutations in HCC, along with described here observations, clearly indicates a hepatoprotective role of p53. These results suggest a specific function of p53 in liver that is different from all other tissues and cell types. However, very limited research has been done to elucidate functions of p53 in normal liver *in vivo*. Based on the existing evidence for transcriptional activity of p53 family members p53 and TA-p73 in normal liver, as well as for their role in growth suppression of cultured cancer cells, a hypothesis was formulated to suggest that p53 and TA-p73 activate expression of tumor suppressor genes in normal quiescent adult liver. We hypothesized that p53 and p73 function in normal quiescent liver cells by targeting genes responsible for activation of cell cycle arrest, apoptosis and senescence. These functions of

p53/p73 are repressed during liver regeneration, and reactivated at the end of the regenerative response. A model of liver regeneration in mice, complemented by studies of cultured cells, was used to test p53 and TA-p73 transcriptional activity in quiescent vs. proliferating liver cells.
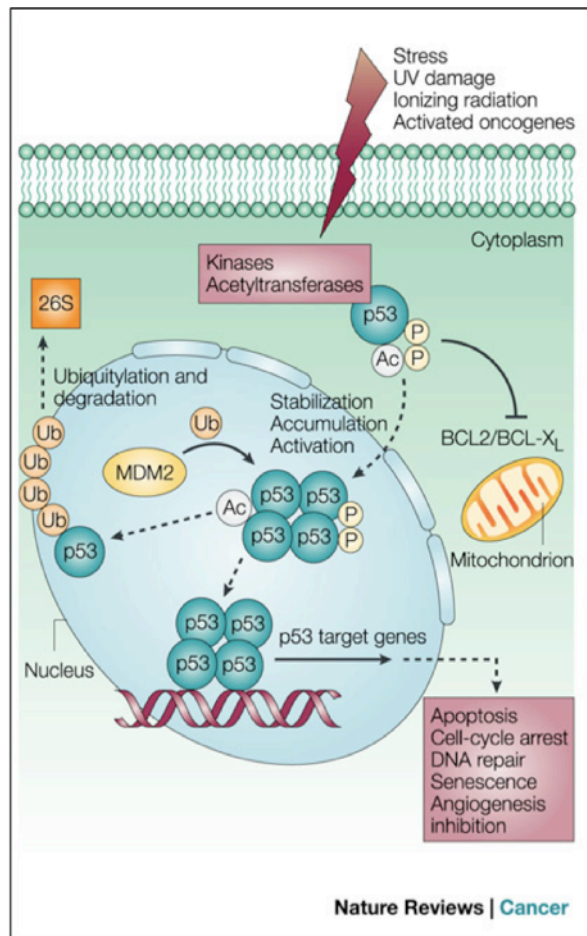
Diagram 1. Activation and outcomes of the p53-mediated transcription. Stress signals activate p53 through protein kinases and/or acetyltransferases, which phosphorylate or acetylate p53, respectively. These post-translational modifications generally result in stabilization and activation of p53 in the nucleus, where p53 (along with other members of the p53 family, p63 and p73) interacts with sequence-specific DNA binding sites of the target genes. The transcriptional activation leads to diverse cellular responses such as apoptosis, cell-cycle arrest, senescence, or DNA repair. When p53 is no longer needed, it is targeted for ubiquitylation by MDM2 and moved out of the nucleus to be degraded by the 26S proteasome. p53 can also act outside of the nucleus (e.g., in mitochondria) to induce apoptosis by binding with anti-apoptotic proteins, such as BCL2.

2.   Partial hepatectomy model of liver regeneration.

2.1.   Transcription factors control gene expression during liver regeneration.

Liver regeneration after partial hepatectomy, or after toxic injury induced by specific chemicals, offers a unique and robust model to study initiation, progression, and termination of tissue growth *in vivo*. A major goal is to understand this process in humans in order to apply this knowledge in regenerative medicine, or to oppose dysregulated proliferation and growth in tumorigenesis. Monitoring patients with liver disease, as well as healthy liver donors, provides invaluable, but limited, information about regulation of liver regeneration in humans (4, 38). To fill this gap, research turns to animal models, and several exist for the study of liver regeneration (13, 39). Among these, the mouse liver regeneration model allows more mechanistic insights, as knock-out and knock-in mutations of selected genes are more readily created in this species. Interestingly, liver regeneration in zebrafish has gained in use during the past few years; small-scale PH performed in this animal triggers a regenerative response similar to that of rodents and humans (40, 41). Here, the primary focus is on the rodent model, as it is used to study the progressive activation of transcription factors in response to inductive signals of PH. Transcription factors exert their influence through networks of targeted gene regulation that promote and precisely terminate the remarkable process of tissue regeneration.

The classical rodent model of PH requires ~65-70% removal of liver to initiate liver regeneration via hepatic cell proliferation. This initial phase of liver regeneration is often termed the priming phase, and is required for the G_0-G_1 transition of hepatocytes and non-parenchymal liver cells. The responsive phase includes proliferation (G_1-S-G_2-M

21

transitions) and cessation of growth (exit back to G_0). Unless hepatocytes are inhibited or greatly delayed in replication by extensive disease or injury, response of these differentiated cells is the primary means by which liver mass is restored.

PH-induced regeneration is initiated by hemodynamic changes due to two-thirds removal of liver mass, which triples the portal load per unit of tissue (42). Proliferation occurs in all populations of cells within the liver: mature normal hepatocytes of the liver parenchyma, as well as induction and proliferation of non-parenchymal, hepatic cell types: stellate cells, endothelial cells of sinusoid, biliary epithelium, and hepatic macrophage-like Küpffer cells (Diagram 2). Cellular proliferation begins in the periportal region, *i.e.* around the portal triad consisting of the portal vein, hepatic artery and bile duct, and proceeds toward the central vein. An increase of gut-derived factors, such as lipopolysaccharide, complement factors, and intercellular adhesion molecules in portal blood, and subsequently in liver sinusoids, activates Küpffer cells (43).

Küpffer cells come in close contact with parenchymal hepatocytes and induce replication of hepatocytes through their release of tumor necrosis factor and interleukin 6 (Il6) cytokines in a paracrine manner. Experiments performed with mice mutant for *Il6* (44) and *Tnfa receptor 1* (45) demonstrated that these factors are essential for initiation of liver regeneration (Diagram 2).

The first transcription factors are activated within approximately 4h after PH, and these include NFkB, Stat3, and AP-1 (44-46). The transcriptional activity of these factors is critical for initiation of liver regeneration, as their target genes encode many proteins, which are not expressed in quiescent hepatocytes and must be synthesized *de novo* for the G_0-G_1 and later transitions.

22

Expression of immediate-early and delayed-early response genes initiated during the priming phase of liver regeneration leads to *de novo* synthesis of transcription factors, thus starting the next cascade of transcriptional activity during the G_1-S-G_2 transition (Diagram 2). The newly synthesized transcription factors include Myc, C/EBPs, FoxM1, and others previously reviewed in (47). However, the number of transcription factors necessary for successful initiation and completion of mitosis may be significantly higher. At least 185 genes change expression during the first 4 hours after PH, as revealed by high-density microarray analysis (48).
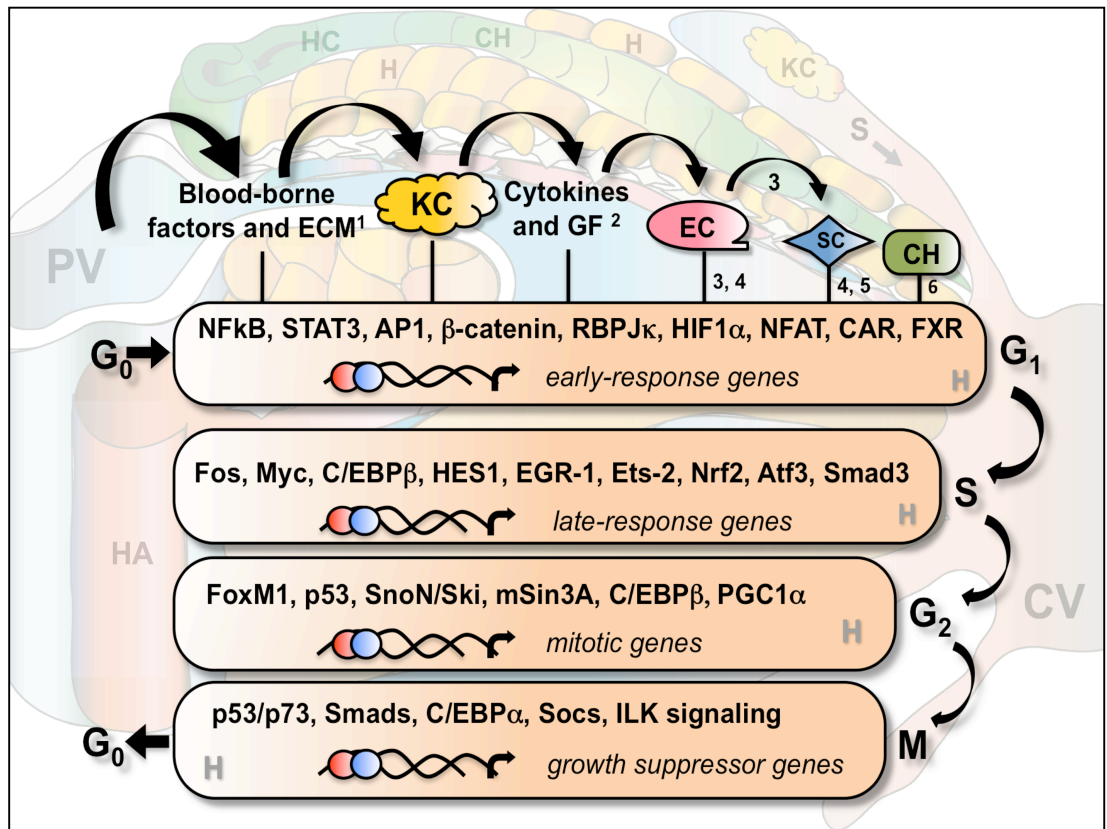
Diagram 2. Transcriptional cascades activate liver regeneration after partial hepatectomy. Blood-borne factors and components of the extracellular matrix activate non-parenchymal liver cells in response to PH surgery. Release of cytokines and growth factors activate the first transcriptional response in hepatocytes, leading to the expression of early-response genes (G_0-G_1-S transition) and consecutive activation of late-response transcription factors (S-G_2-M). Liver regeneration is complete after 1-2 mitotic cycles, and hepatocytes exit back to the quiescent state (G_0).

1 – LPS, ICAM, complement factors, insulin, acetycholine, norepinephrine, EGF, xenobiotics; 2 – Il6 and TNFα; 3 – VEGF; 4 – HGF; 5 – TGFβ; 6 – bile acids.

BD – bile duct; CV – central vein; HA – hepatic artery; HC – Hering's canal;

PV – portal vein; S – sinusoid; CH – cholangiocyte; EC – endothelial cell; H - hepatocyte; KC – Kuppfer cell; SC – stellate cell; ECM – extracellular matrix, ILK – integrin-linked kinase. *Transcription factors:* NFκB, STAT3, AP1, β-catenin, HIF1α, RBPJκ, NFAT, CAR, FXR, Fos, Myc, C/EBPβ, HES1, EGR-1, Ets-2, Nrf2, Arf3, Smad3, FoxM1, p53, SnoN, Ski, mSin3A, PGC1α, p73, C/EBPα, Socs.

Mitotic entry (G_2-M transition) occurs during the second day after PH in mice, and requires expression of *cyclin A, cyclin B*, cyclin-mediated kinases *Cdk2*, *Cdk1*, and the *Cdc25* family of protein phosphatases. FoxM1, a transcription factor activated during the early response phase of liver regeneration, induces some of these genes. Hepatocytes enter mitosis as a synchronized population at approximately 48 h after PH, followed by non-parenchymal liver cells. At approximately 72h after liver resection, a subset of hepatocytes exits cell cycle and returns back to G_0. The remainder continues to divide through one more round of mitosis prior to exit to G_0 at approximately 96 h after PH (49). Mitotic progression is controlled by Polo-like kinases Aurora A and Aurora B, which phosphorylate regulatory proteins of the mitotic machinery and mediate prophase-to-metaphase transition, chromosome alignment, mitotic spindle assembly, and cytokinesis (50, 51). Expression and post-translational modifications of the major checkpoint regulator p53 have been previously linked to Aurora A function (52, 53). Experiments demonstrated that p53 expression and/or protein stability is regulated at the G_2-M checkpoint and suggested that p53 regulates G_2-M transition during liver regeneration in liver-specific Aurora A-transgenic mice (53).

Liver regeneration after PH stops precisely when a pre-operative liver index (ratio of liver weight/body weight) is restored, within 7-14 days in mice. Liver cells therefore possess highly effective mechanisms that control mitotic exit of hepatocytes and non-parenchymal cells and return to G_0. A deeper understanding of these mechanisms will provide valuable information about regulation of proliferation and quiescence in normal cells, critical for the treatment of cancer, wound repair, and many other medical conditions associated with abnormalities of tissue growth.

2.2. Tumor suppressors p53 and TA-p73 regulate transcription of hepatic target genes *in vivo*.

Studies of cancer cell lines, mouse models, and patient samples clearly establish that loss of p53 and p73 functions is causative in tumor development (32, 54-57). However, much less is known about the status and functions of p53 and p73 in normal, quiescent tissues in the absence of cellular stress. Recent publications describe p53-mediated regulation of metabolic pathways, organ development, and stem cell renewal (58). New p53 target genes *Gfi-1* and *Necdin* were identified as regulators of quiescence of hematopoietic stem cells (59). Additionally, combined loss of p53 and p73 leads to genomic instability in mouse embryonic fibroblasts (55), further emphasizing the importance of p53 and p73 functions in normal cells.

p53 and p73 regulate gene expression by binding to a consensus DNA sequence known as a response element (RE). The canonical p53 response element (p53RE) comprises two decamer motifs, or half-sites, RRRCWWGYYY, where R is a purine, W is A or T, and Y is C or T), separated by a spacer of 0-13 base pairs (Diagram 3). Intrinsic features of the REs, such as the sequence and organization of the individual functional units in an RE, and the spacer separating the two decamer half sites, can greatly affect the transactivation potential of p53 (Diagram 3) (60). The C and G nucleotides are highly conserved, whereas WW and flanking sequences and the spacer length vary and are thought to affect transcriptional activity of p53 (61). Several studies demonstrate that p73 binds to the p53RE, either by itself, or in a complex with p53 (62, 63). However, a number of genes have been reported as unique p73-targets (64); more research is necessary to explain the differences in the specificity of p53 and p73 binding to the target genes.
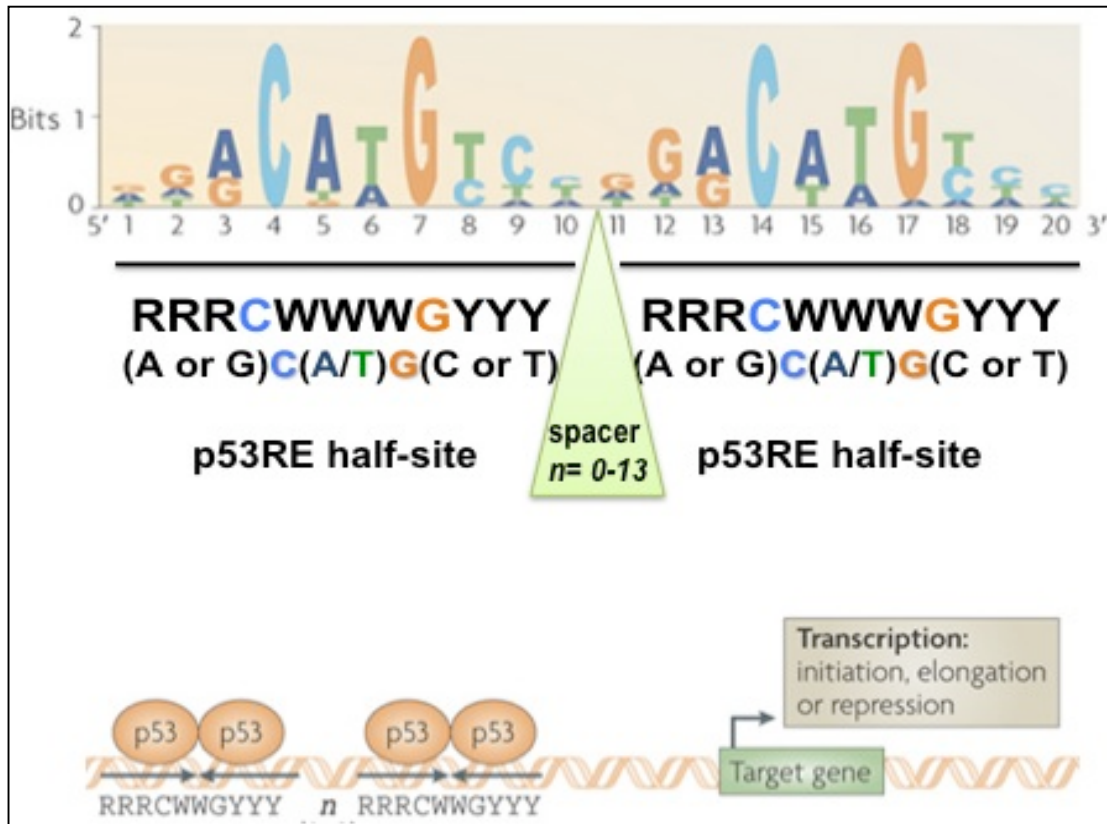
Diagram 3. The interaction of p53 protein with target response elements is essential in the transcriptional modulation of target genes. The canonical p53 response element (p53RE) comprises two decamer motifs, or half-sites, RRRCWWGYYY, where R is a purine, W is A or T, and Y is C or T) separated by a spacer of 0-13 base pairs. Logo representations of p53 REs were identified based on *in vivo* occupancy studies and show the results from a ChIP-chip study originally presented in Smeenk, L. et al. Characterization of genome-wide p53-binding sites upon stress response. Nucleic Acids Res. 36, 3639–3654 (2008). In both logos, the height of a letter at each given position of the p53 RE is proportional to the frequency of its corresponding nucleotide, at that position, among the identified p53 REs in p53-bound DNA sequences.

Modified and reproduced with permission from Nature Publishing Group (NPG): Daniel Menendez, Alberto Inga and Michael A. Resnick. The expanding universe of p53 targets. *Nature Rev Cancer* 2009.

Previous studies show that p53 protein levels are developmentally regulated in mouse liver. p53 protein is low or undetectable in newborn mice but increases within two weeks and is maintained throughout adulthood, decreasing with age (65, 66). The data presented in this work, as well as published results, suggest that TA-p63 has very low expression and little or no transcriptional activity in liver, whereas TA-p73 complements functions of p53 in adult mouse liver, *e.g.* in transcriptional repression of tumor marker gene *Afp* (32, 62). The expression of *Afp* is high in developing liver and in HCC but is undetectable in normal adult liver (67). Both p53 and TA-p73 bind to the p53RE of *Afp* in liver, targeting co-repressor proteins and repressive histone modifications to chromatin at the p53RE and *Afp* transcription start site (TSS), and repress *Afp* within 2-3 weeks of age (62, 68). In p53$^{-/-}$ mice, *Afp* repression is delayed up to 4 months, suggesting a more limited capability of chromatin-bound TA-p73 in co-repressor recruitment (62).

Several recent studies expanded on functions of p53 and TA-p73 during liver regeneration. During PH-induced liver regeneration, p53-mediated recruitment of histone demethylase LSD1 and transcriptional repression if *Afp* is decreased, suggesting a loss of p53 transcriptional activity in proliferating hepatocytes (69). Study of Aurora A transgenic mice revealed p53 activity at the G_2-M checkpoint during regeneration (53). In mice treated with the hepatocarcinogen *o*-aminoazotoluene (OAT), partial hepatectomy increased p53 protein levels and induced expression of p53 target genes, compared to the control group that underwent PH surgery without prior treatment with OAT (70). This result suggests a hepatoprotective response, mediated by transcriptional activity of p53 when regenerating hepatocytes are exposed to carcinogens. Interestingly, liver regeneration in *Trp53* KO mice proceeds without major complications (71). One possibility is that TA-p73 partially

compensates for loss of p53 transcriptional activity during liver regeneration, as it does during liver development of *Trp53* KO mice (62). *Trp73* KO mice have profound developmental abnormalities and do not live to adulthood (29); therefore, TA-p73-mediated transcription has not been studied in the context of liver regeneration.

Both p53 and TA-p73 have a potential to execute hepatoprotective functions through transcriptional activation or repression of target genes involved in cell death, cell cycle, and senescence (61, 64). p53/p73 target genes responsible for the G_1 arrest and apoptosis are activated by the recruitment of the acethyltransferase p300/CBP to p53-regulated promoters (72) and by the direct interaction of p300/CBP with TA-p73 (73). These processes, and expression of the relevant target genes, are highly regulated during regeneration of liver when quiescent, mature hepatocytes re-enter the cell cycle (74, 75). To uncover potential liver-specific targets of p53 family members, this work combines analysis of chromatin association of TA-p73 in adult mouse liver, by chromatin immunoprecipitation and hybridization to microarrays (ChIP/chip), with determinations of global expression during liver regeneration in response to partial hepatectomy. The results show that only a highly restricted number of TA-p73-target genes significantly change expression during liver regeneration. Among these are genes encoding several transcription factors, which potentially amplify the effects of tumor suppressor TA-p73 or p53 activation or repression, by establishing cascades of regulated gene expression. This study identifies the Forkhead box transcription factor Foxo3 as a new target gene of p53 and TA-p73 in normal quiescent liver.

2.3.   *Foxo3* is a tumor suppressor gene expressed in liver.

FoxO3 is a *bona fide* tumor suppressor that belongs to the Forkhead box O subfamily of a large group of Forkhead transcription factors named after the forkhead-like motif in the protein (76) (Diagram 4). *FOXO1, FOXO3,* and *FOXO4* genes are disrupted by chromosomal translocations in human cancers (77). FoxO3 is inactivated by phosphorylation, mediated by serine/threonine kinase Akt, by the inhibitor of NFκB (IκB) kinase IKKβ, or by the extracellular signal-mediated kinase ERK in response to growth factor signaling, inflammation, or mitogen activation, correspondingly (78-80). Phosphorylated FoxO3 protein loses its transcriptional activity due to nuclear exclusion and proteosomal degradation in the cytoplasm (81, 82). When active and localized to the nucleus, FoxO3 regulates expression of genes that inhibit cell cycle and activate apoptosis, sharing some of these gene targets with p53 and TA-p73 (64, 83). FoxO3 is critical for the maintenance of quiescence in hematopoetic stem cells and primordial follicles in mice and humans (84, 85). Intriguingly, recent data demonstrated that FoxO3 protein directly interacts with p53, allowing these proteins to act as transcriptional partners (86, 87). FoxO3 has been considered as a potential target for cancer treatment (88). Changes in expression of FoxO3 were implicated in several diseases including Alzheimer disease (89-91). The transcription factor E2F1 was shown to activate *FOXO3* expression, but direct regulation of endogenous *FOXO3* transcription has not been demonstrated (92). In UV-treated keratinocytes, *FOXO3* is negatively regulated by HES/HERP repressors (93). Interestingly, FoxO3 protein can directly bind to promoters of other FoxO family members and activate expression of *FOXO1* and *FOXO4*. However, despite high homology between *FOXO* genes, FoxO3 protein fails to

bind and activate *FOXO3* (94). Thus, the mechanism of transcriptional regulation of *FOXO3* remains to be identified.

At the level of protein, FoxO3 expression is detected in various organs during embryonic and post-neonatal development (95-97). However, FoxO3 expression is not ubiquitous and is controlled in a tissue-specific manner. Conditional deletion of all *Foxo* genes in liver endothelial cells (EC) enhances proliferation and survival, while FoxO-deficient lung EC cells do not have phenotypic alterations (80). FoxO3 mRNA, and not mRNAs of other FoxOs, is expressed in embryonic liver at day 13.5 (98), however, very little is known about FoxO3 mRNA and protein levels in normal adult liver.

This work demonstrates that p53 and TA-p73 bind to the p53RE of endogenous *Foxo3* in adult quiescent mouse liver. p53 and TA-p73 activate expression of the Foxo3 gene by recruiting acethyltrasferase p300 to *Foxo3 p53RE*. FoxO3 protein is also detected in normal adult liver. During liver regeneration, p53 and TA-p73-mediated transcription of the *Foxo3* gene is lost, resulting in a decrease in FoxO3 mRNA and protein levels. Binding of p53 and TA-p53, as well as expression of FoxO3, are restored after completion of post-hepatectomy liver growth. This work establishes a direct regulatory link between p53, TA-p73 and FoxO transcription factors, which are growth suppressors in normal tissues.
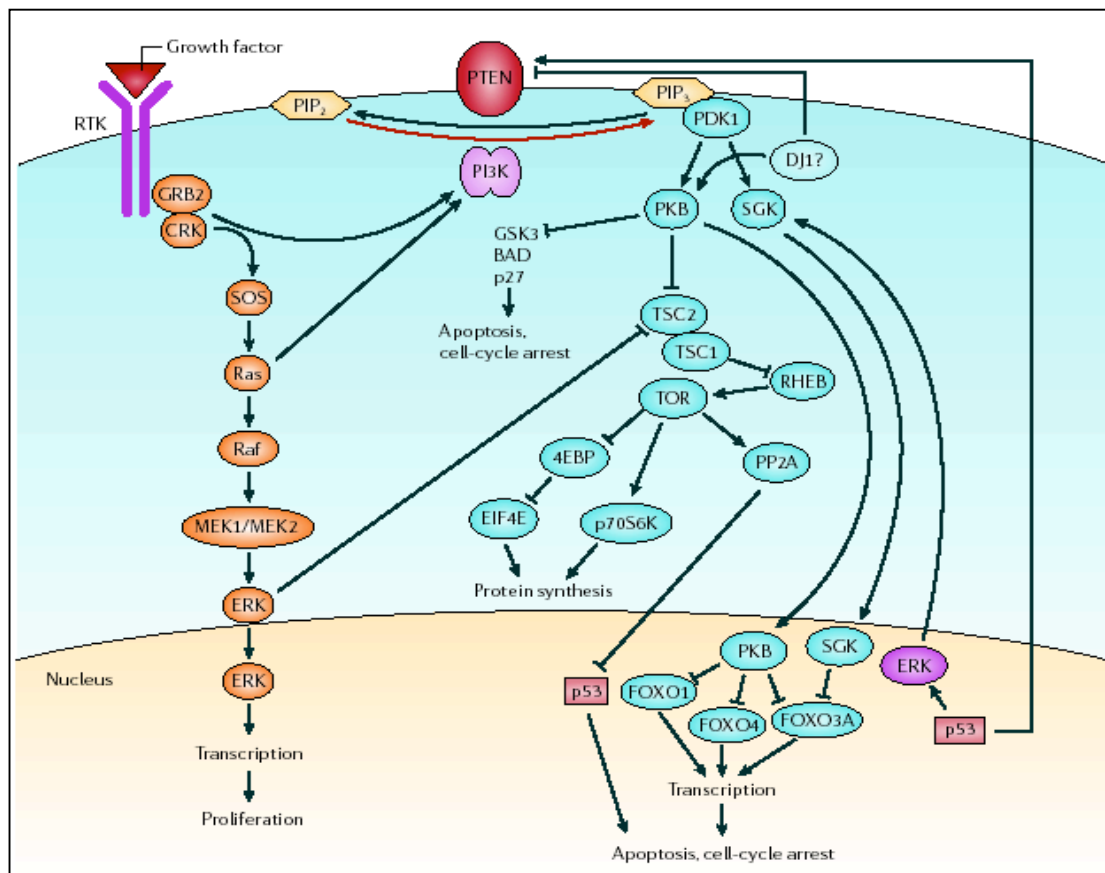
Diagram 4. Tumor suppressor pathways and growth regulation converge on FoxO transcription factors. The core phosphatidylinositol 3-kinase (PI3K) signaling pathway (blue symbols) begins with PI3K activation by receptor tyrosine kinases (RTKs). Activated protein kinase B (PKB, also known as AKT) inhibits the activities of the FOXO transcription factors, resulting in cell proliferation and survival. The Ras pathway (orange symbols) can be triggered by a set of RTKs that are activated by growth factors. The activation status of p53 can also affect the outcome of PI3K signaling by interacting with the FOXO transcription factors and with extracellular-regulated kinase 1 (ERK1) and ERK2. Other members of the PI3K and the RTK–Ras signaling pathways include SGK (serum- and glucocorticoid-induced kinase), TSC1/TSC2 (tuberous sclerosis 1 and 2), RHEB (Ras homologue enriched in brain), TOR (target of rapamycin), 4EBP (eukaryotic initiation factor 4E (EIF4E)-binding protein), p70S6K (ribosomal protein, S6 kinase 70kD), and PP2A (protein phosphatase 2A), GRB2 (growth factor receptor-bound protein 2), SOS, Ras, Raf, MEK (mitogen-activated ERK kinase) and ERK. Modified and reproduced with permission from Nature Publishing Group (NPG): Megan Cully , Han You , Arnold J. Levine and Tak W. Mak. Beyond PTEN mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis. *Nature Rev Cancer* 2009.

CHAPTER II

MATERIALS AND METHODS

1. Liver regeneration in mice

Partial hepatectomy (PH) to remove 65% of total liver tissue, or sham surgery was performed using isoflurane anesthesia according to published protocol (Cold Spring Harb. Protoc.; 2006; doi:10.1101/pdb.prot4384). Five to seven C57Bl6/Sv129 mice, 2 months of age, were used for each experimental condition according to the MD Anderson Cancer Center Institutional Animal Care and Use Committee guidelines. Mice were sacrificed 1, 2, 3, 4, or 7 days following PH and sham surgeries; remnant liver tissue was harvested, flash-frozen, and processed for RNA and ChIP analyses. A separate set of surgeries was performed to harvest and fix liver tissue in formalin for the immunohistochemistry experiments. Liver/body weight ratios were calculated to estimate the recovery of liver mass (Appendix Figure 1).

2. Cell Culture and Plasmids

Hepa1-6, mouse hepatoma cells, were obtained from ATCC and cultured under standard conditions (RPMI medium supplemented with 10% calf serum, in incubators with 5% CO_2 at 37^0C). The plasmid encoding wild type p53 has been previously described (99). Plasmids encoding p73α and p73β have been previously described and were generous gifts of G. Melino (62). The cells were transfected with 4 μg of total DNA/100-mm plate using Lipofectamine, according to the manufacturer's instructions (Invitrogen). Val5 mouse

embryonic fibroblasts stably expressing temperature-sensitive p53 R135V mutant were obtained from M. Murphy (Fox Chase Cancer Center, Philadelphia, PA) (100).

3.   ChIP/chip and microarray analyses

Chromatin immunoprecipitation (ChIP) was performed on liver tissue lysate with a p73 antibody that detects the full-length (TA) forms of p73. Liver tissue was isolated and flash-frozen from 2 month old C57Bl/129 mice, and ChIP assays were performed as described previously (62). To analyze resulting DNA, an Agilent promoter array was employed representing 60-mer oligomeric probes within -5.5kb and +2.5kb of 17,000 genes or predicted gene regulatory regions of the mouse genome. Ligation-mediated amplification was used prior to labeling and hybridization; amplified material was shown to have p73-interaction sites by analysis for *Afp* and *Cdkn1a* binding by quantitative PCR.

To analyze expression of hepatic genes in response to partial hepatectomy (PH), liver tissue was collected at T=0, 24, 38, and 48 hours following PH (n=3 for each time-point). Total RNA was isolated using the RNeasy mini columns from Qiagen and 5 μg of RNA from the PH samples was used for hybridization to an Affymetrix MOE430 high-density oligonucleotide array that contains 45,000 probe sets for mouse genes and expressed sequence tags (ESTs). Data quality control was carried out using Affymetrix Microarray Suite (MAS) 5.0 and normalized using the Robust Multichip Analysis (RMA) software. Linear modeling was carried out on the normalized data for time course analysis of gene expression using the LIMMA software available through bio conductor package (101). Gene lists were generated for the genes with a negative or positive fold change of 1.5 times or

more between 0h control and 24h, 38h or 48h time point and a significance cut-off of P-value < 0.0001 was used.

ChIP/chip and microarray data sets were annotated and analyzed using Ingenuity Pathway Analysis (IPA Vesion 7.5, Ingenuity Systems, Inc.)

4.   ChIP and RNA analyses of liver tissue and cultured cells

Liver tissue for ChIP and RNA analyses was harvested from C57Bl6/Sv129 *wt*, p53^{-/-}, and p73^{+/-} mice at 2 month of age. Chromatin lysate was precleared and incubated overnight with the following specific antibodies for ChIP: antibodies to histone H3 (Abcam), H3K4me2 (Active Motif), H3K9Ac (Active Motif), H3K14Ac (Upstate/Millipore), H4Ac (Upstate/Millipore), p53 (Novocastra), p73 (Santa Cruz) and normal sheep immunoglobulin G (IgG) (Upstate/Millipore). To analyze specific and antibody- and protein-bound DNA, real-time quantitative PCR (qPCR), and quantitative TaqMan (Applied Biosystems, ABI, Foster City, CA) real-time PCR were used. The following TaqMan real-time PCR primers and probes were used for the AFP p53RE region: forward primer, 5'-CTACGGCATTGAGGGTGAA-3'; reverse primer, 5'-TGGGCACTGACATACTTCTGA-3'; and probe, 6-carboxyfluorescein (FAM)-AGTGAAGCACTCTTA-MGB. The following real-time PCR primers were used to detect the *Foxo3* gene 5' p53 binding element: forward primer, 5'-AGTCACTGCAGTTAGAAGATTTCCA-3'; reverse primer, 5'-AACTCAGTCAGTGCTCTCATACTCA-3'; and probe, 6-carboxyfluorescein (FAM)-AGCAGGAGGAGGC-MGB. 5' region of DNA located at -2 kb from *Foxo3* TSS was

chosen as a non-specific control for ChIP experiments using following primers: forward primer, 5'-TGGGCACAGAGCCTAACAC-3'; reverse primer, 5'-AGTGGGGATTTGTGCAGAGA-3'; and probe, 6-carboxyfluorescein (FAM)-GATTCCAGGTAATAAACCCT-MGB. The percentage of the input that was bound was calculated by the dCt method and averaged over at least three experiments.

Real-time PCR primers used to test p53 and TA-p73 binding to the genes from TA-p73 ChIP/chip analysis were as follows. Jak1: forward primer: 5'-ATCACTCACGCAGGGAATAAGAAT–3', reverse primer: 5'-ACCCAGGAGAAAGCTAACACAAGA-3'; Pea15 -0.6 kb: forward primer: 5'-ATCTTGGTGAAGGGTGTTGTTG–3'; reverse primer: 5'-TCAGAATATGGCAGCAAGGA-3'; Pea15 -3.0 kb: forward primer: 5'–AACAAGCCTTTTCTGAACACATG-3', reverse primer: 5'–GGCAGACACGAGTCAGCTCAAG –3'; Tuba1a –3.3 kb: forward primer: 5'–GACAGCACATGCCTATAATCTCA-3'; reverse primer: 5'–GGCTAGCTTCCAACTTGATTCAGTATGTA – 3'.

Primers and reverse transcription-PCR determinations of RNA expression were performed as previously described (62). Briefly, total RNA from mouse liver was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. At least 3 mice were used to prepare individual RNA samples in each experiment; to avoid zonal gene expression effect, livers were homogenized prior to taking a sample for RNA isolation. cDNA was obtained by reverse transcription of 2 ug of RNA using the SuperScript first-strand synthesis system (Invitrogen). Real-time PCR was carried out using primers for indicated genes simultaneously with beta-actin (*Aktb)* primers: forward primer, 5'-

CGTGCGTGACATCAAAGAGAA-3'; reverse primer, 5'-GGCCATCTCCTGCTCGAA-3'. The following primers were used to detect expression of the Foxo3 gene: forward primer, 5'- TGGGTACCAGGCTGAAGGA-3'; and reverse primer, 5'-ACTAACCTGCTTTGCCCATT-3'. The Jak1 gene: forward primer, 5'-CTACCGCATGAGGTTCTACTTTAC-3', and reverse primer, 5'-TCGCCATACAGACTGTTCGT-3'; the Pea15 gene: forward primer, 5'-GGACACCAAGCTAACCCGTATT-3', and reverse primer, 5'-GAGGGCTGCCGGATAATGT-3'; the Tuba1 gene: forward primer, 5'-CCGGGCAGTGTTCGTAGAC-3', and reverse primer, 5'-GCCGGTGCGAACTTCA-3'. Relative amounts of RNA were determined by the ddCt method.

5.   Western Blot analysis and immunohistochemistry

Western Blot analysis of whole-cell lysates prepared from homogenized liver or from cultured cells was performed using standard SDS-polyacrylamide gel electrophoresis methodology. Whole livers were homogenized in T-PER buffer (BioRad), and cleared by centrifugation according to the manufacturer's protocol. Supernatant containing whole liver cell protein was used for WB analysis. Cultured Hepa-1-6 cells were lysed in Triton-X100 lysis buffer containing protease inhibitors (cocktail Set 1, Calbiochem) and phosphatase inhibitors (cocktail Set 1, Sigma). 25 µg of total protein was heat-denatured in SDS buffer (25mM Tris-HCl, pH 7.5, 150mM NaCl, 5 mM EDTA, 1% SDS) and separated on 10% Bis-acrylamide gel. Transferred nitrocellulose blots were blocked in 5% milk in TBST, followed by incubation with the antibody. The primary antibodies used are as follows: p53 (Santa

Cruz), TA-p73 (Santa Cruz), HA-tag (Cell Signaling), and actin (Santa Cruz). After antibody incubation and washes, WB were developed using standard ECL kit (Amersham).

For immunohistochemistry, livers harvested from mice sacrificed at indicated time points following partial hepatectomy or sham surgeries, were perfused first with ~2 ml of PBS and then with ~2 ml of 10% Neutral Buffered Formalin (Fisher) before liver dissection and fixation in formalin. Tissues were embedded in wax paraffin and sectioned by the MD Anderson Department of Veterinary Medicine and Surgery. Slides were stained with FoxO3 antibody (Cell Signaling) following the manufacturers' recommended protocols and then counterstained with hematoxylin.

6.   Isolation of primary hepatocytes and TGF-β1 treatment.

Primary hepatocytes were isolated from *wt* mice following standard liver perfusion protocol, utilizing Type IV Collagenase treatment (Sigma) to digest extracellular matrix and release liver cells into suspension. Obtained liver cell suspension was filtered through 70 μm cell strainer (BD Falcon) and centrifuged at low 50Xg speed for 10 min at 4^0C to separate hepatocytes. Supernatant containing non-parenchymal cells was removed, and pelleted hepatocytes were resuspended in cold William's Plating medium (Sigma) with added glutamine/gentamycin, insulin-transferrin-sodium selenite, glucagon, amphotericin B, and FBS (Gibco). Cell viability was measured using trypan blue exclusion method. Plated hepatocytes were allowed to attach overnight, and treated with TGF-β1 ligand (R&D Systems) at a concentration of 4 ng/ml or to vehicle control (4 mM HCl containing 0.1% bovine serum albumin [BSA]) for the indicated time points.

Similarly, Hepa 1-6 cells were cultured under standard conditions and exposed to TGF-β1 ligand at a concentration of 4 ng/ml or to vehicle control for the indicated time points.

7.   Bioinformatics analysis

Potential p53 and TA-p73 consensus binding sites (p53REs) were mapped upstream of the transcription start sites of TA-p73-bound genes using a published algorithm available online on http://rockefeller.edu (102). p53REs, identified in *Foxo3* gene, were analyzed using a motif discovery software MDScan, available at http://ai.stanford.edu/~xsliu/MDscan/ (103), and a logo was built using a sequence logo generator WebLogo software available at http://weblogo.berkeley.edu/ (104). Functional annotations of genes, comparison of ChIP/chip and microarray results, as well as identification of p53 target genes were performed using Ingenuity Pathway Analysis https://analysis.ingenuity.com.

8.   Statistical analysis

Results are expressed as means standard error of the mean (SEM). Statistical analyses were performed with t-test, and p values less than 0.05 were considered significant.

CHAPTER III

RESULTS

1.	Comparative analysis of genes bound by p73 in quiescent liver and genes that change	expression during liver regeneration.

1.2.	Analysis of potential p73-bound genes in quiescent liver.

Expression levels of p53 family members in liver remains poorly characterized; thus, we first measured relative levels of p53, p63, and p73 mRNA in mouse liver over the course of post-neonatal development (Figure 1A). During first week of post-neonatal development, p63 mRNA level was the lowest of all three family members, and was set as 1 (2d, Figure 1A). p63 mRNA remained very low through adulthood, as compared to p53 and TA-p73 mRNA levels (Figure 1A). Changes in p53 protein levels during development of mouse liver were described previously (69) and were higher at 2 weeks and 2 months of post-neonatal development, compared to newborn mice (69). TA-p73 protein levels significantly increased at weaning age, and remained unchanged throughout adulthood (Figure 1B). Thus, p53 and p73 are expressed in mouse liver during post-neonatal development, and are detected in adult mouse liver at the protein level.

To search for genes regulated by TA-p73 in normal quiescent liver, chromatin immunoprecipitation (ChIP) analysis of liver tissue from 2-month-old wild type (*wt*) mice was performed using antibody against full length TA-p73. Isolated TA-p73-bound chromatin fragments were analyzed using a custom Agilent mouse promoter array set, which presents 1-2 60-mer oligomeric probes within -5.5 and +2.5 kb of 17,000 genes or predicted gene regulatory regions of the mouse genome (ChIP/chip analysis). 158 genes were found as potential targets of TA-p73-binding activity in liver (Supplementary Table 1). Functional annotations of genes were performed using Ingenuity Pathway Analysis (IPA), which allows

comparison of several annotated datasets within a common list of functional categories (https://analysis.ingenuity.com). Annotation of 158 newly uncovered genes yielded 12 categories with cancer, cell death, and cell proliferation receiving the highest number of hits with the lowest p-value, as determined by IPA (Figure 2A). Other categories of TA-p73-bound genes in the quiescent liver included hematological disease and function, inflammatory response, cell signaling, development, and cell cycle (Figure 2A).

Of these gene targets, none were previously reported as p73-regulated and 8 were known targets of p53 (e.g, *Annexin A1* (105), *Notch1* (106), and *Bai1* (107). Thus, many of identified TA-p73 target genes are not known as p53/p73-regulated and potentially define a specific set of TA-p73-regulated genes in quiescent liver.

A
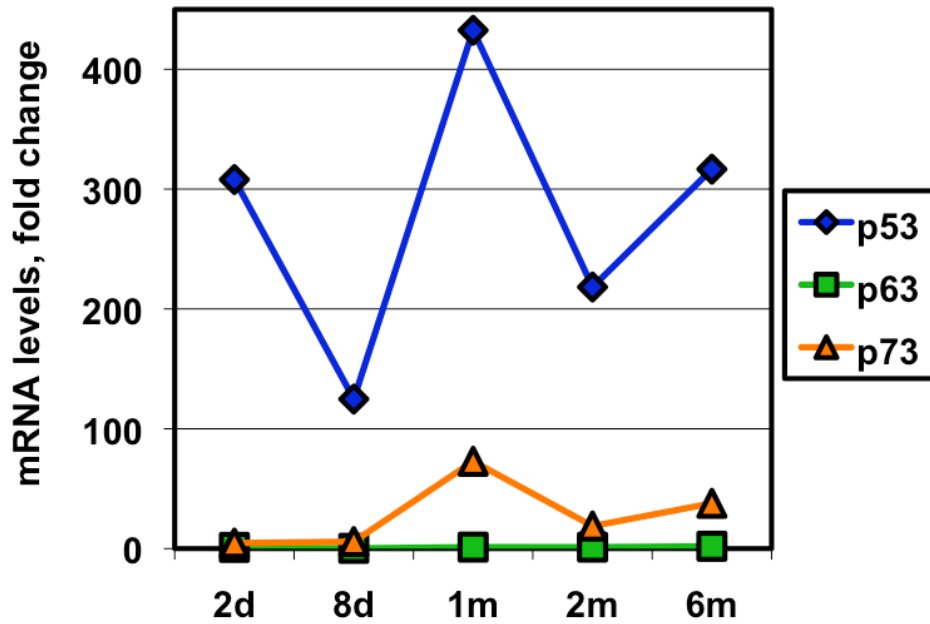
Fold	2d	8d	1m	2m	6m
p53	307.97	124.79	432.53	218.27	316.63
p63	1	0.23	1.25	1.24	1.95
p73	4.58	5.86	72.84	18.8	37.79

B
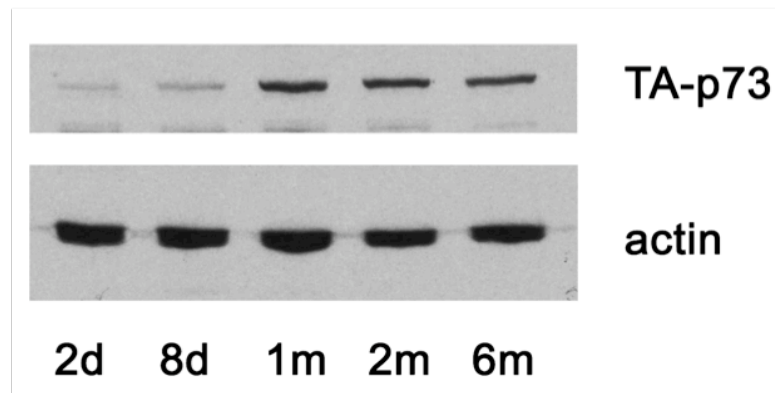
Figure 1. Expression of p53 family members p53, p63, and p73 during post-neonatal liver development. (A) Relative *Trp53, Trp63,* and *Trp73* transcript levels measured by real-time PCR in total RNA from liver samples isolated from at least 3 mice per time point (*d* indicates days, *m* – months). (B) TA-p73 protein levels measured by Western Blot analysis show increasing expression of TA-p73 in developing mouse liver.

1.2.   Analysis of genes that change expression during liver regeneration.

Two-thirds partial hepatectomy (PH) promotes proliferation of liver cells and a rapid growth of the remaining liver tissue, resulting in complete restoration of organ mass in approximately 7 days following PH in mice (75). Global analysis of mRNA levels isolated at early time points (first 4 hours) and at later time points (24, 38, 48 hours) using microarray analysis provides lists of genes that regulate the priming phase of liver regeneration vs. genes responsible for the progression of the cell cycle (108, 109). Liver tissue collected from mice at T=0, 0.5, 1, 2, 4, 24, 38, and 48 hours following PH was used to determine specific gene expression by the microarray analysis using Affymetrix 430.2 gene array platform containing 45,000 probe sets for mouse genes and expressed sequence tags. Statistical analysis yielded 434 genes that changed expression between 0.5 – 4 h after surgery (Supplementary Table 2), and 3807 genes that changed expression between 24 - 48 h following PH, as compared to T=0 (Supplementary Table 3).

Consistent with previous observations, microarray analysis of 0.5 - 4 h post-PH livers showed a significant increase in expression of 'early response' genes, upregulated during the priming phase of liver regeneration, *e.g.*, *C/EBPb*, *Jun, Myc, Tnfrsf1a, Hif1a, Atf3, Ets2* (Supplementary Table 2). Several genes not previously reported in the context of liver regeneration, included pluripotency regulator *Klf4*, transcription factors *Mxi1* and *Sin3a*, as well as anti-apoptotic Bcl2 family member *Bcl2l1*, Cbp/p300-interacting transactivator *Cited2*, and protein phosphatase 2 regulatory subunit B56α *Ppp2r5a* (Supplementary Table 2). Functional annotations of genes that changed expression in

response to partial hepatectomy at 0.5 - 4 hours yielded hematological disease as a major functional category with genes implicated in leukemia, thrombosis, apoptosis and necrosis of blood cells, as well as in hypoglycemia (Figure 2B). Hematological disease category was also present in functional annotations of TA-p73-bound genes and included *Anxa1, Casp3, Foxo3, and Notch1*. The direct comparison of early-response gene IDs from this microarray to TA-p73 bound genes showed only one known TA-p73-bound gene (RNA terminal phosphate cyclase-like 1 *Rcl1*) upregulated at 0.5 - 4 hours post-PH. Thus, the majority of identified TA-p73-regulated genes described here are novel targets of this protein.

Similar to TA-p73-bound genes in quiescent liver, the major functional categories of genes that changed expression (either increasing or decreasing) during 24 - 48 hours of liver regeneration included those associated with cancer, cell death, and cell proliferation (Figure 2C). This important finding indicates that many genes altered during liver regeneration also function in cancer development. Several genes that encode regulators of cell division and cell death, as well as transcriptional regulators, change expression during 24 – 48 h of liver regeneration: cell division protein phosphatase *Cdc25*, pro-apoptotic Bcl2 family member *Bax*, tumor suppressor *Pten*, histone acetyltransferase *Pcaf* and *Myst3* (Supplementary Table 3). Two other categories, represented among TA-p73-bound genes, were cell signaling and

A
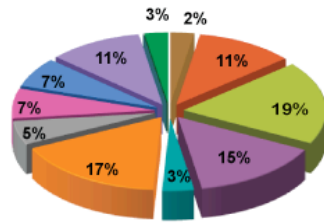
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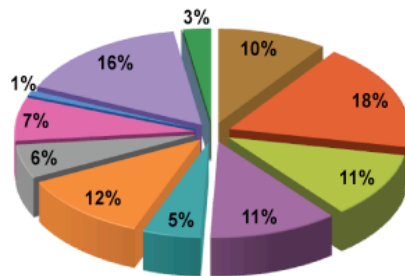
C
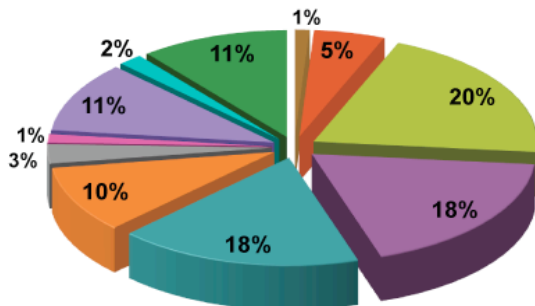
gene expression

hematological system development, function, and disease

cancer

cell death

cell cycle

cell growth and proliferation

hepatic system development, function, and disease

inflammatory response

cell signaling

development

protein degradation

DNA replication and repair

D
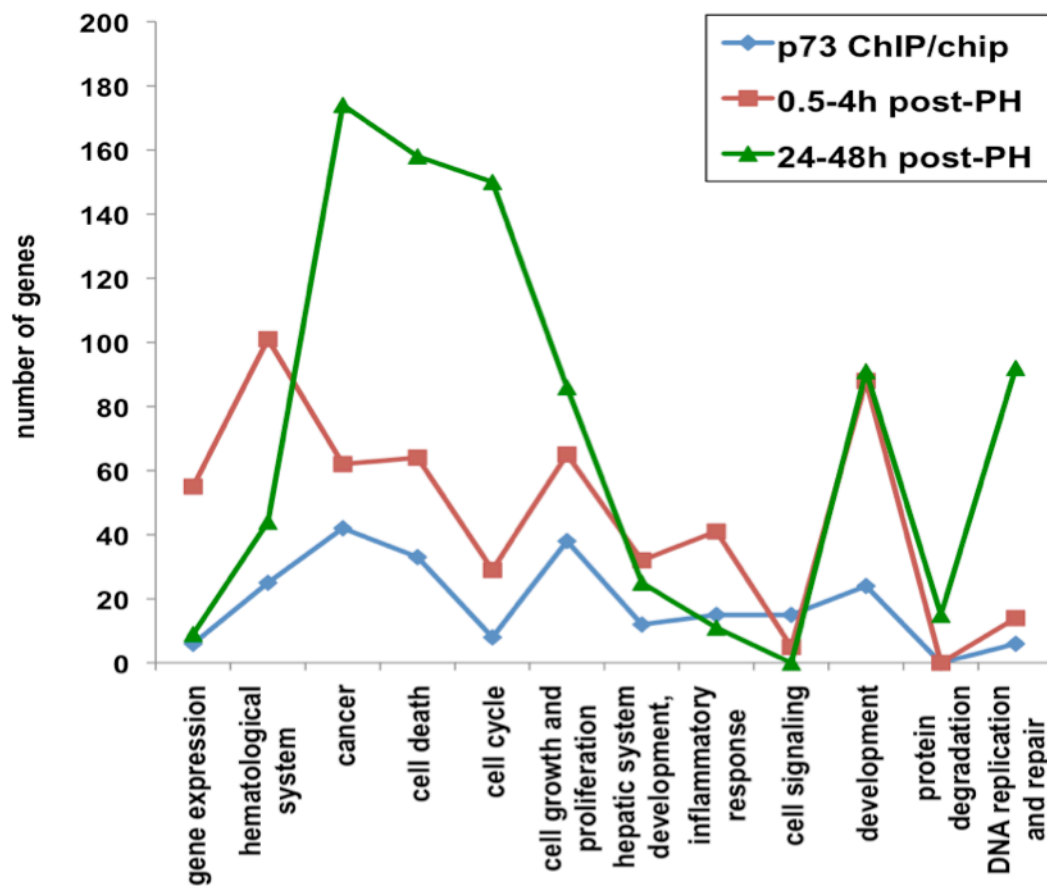
Figure 2. Functional annotations of p73-bound genes and genes that change expression during liver regeneration. Genes from p73 ChIP/chip (A), genes that change expression at 0.5 – 4 h post-PH (B), and genes that change expression at 24 - 48 h post-PH (C) were annotated and analyzed using Ingenuity Pathway Analysis application. Functional categories were selected based on the highest number of genes and lowest p-values. Pie charts were created based on the number of genes in each category. (D) Results presented in (A), (B), and (C) were combined a linear format. Functional categories are presented on the X-axis, and the number of genes in each category is presented on the Y-axis.

inflammatory response (7% each), reflecting TA-p73 functions in multiple signaling cascades described in tissue culture experiments (110) and inflammatory defects found in p73$^{-/-}$ mice (29). These categories had less than 1% hits among genes that changed expression at 24 - 48 hours following partial hepatectomy, suggesting unique functions for TA-p73 in quiescent liver that are not executed during regeneration.

Compared to earlier time points, genes that changed expression in regenerating liver at 24 - 48 hours post-PH had a significant increase in the number of targets in cell cycle and DNA replication categories (Figure 2B and 2C). TA-p73 targets were present in both of these categories and included S-phase regulator *Pea15*, metaphase protein *Smc1a*, hedgehog receptor *Ptch1*, insulin receptor *Insr,* and the Forkhead transcription factor *Foxo3* (Supplementary Table 3). Taken together, the comparison of the number of genes represented in each of the 12 functional categories in all three analyses (TA-p73 ChIP/chip, 0.5-4h post-PH, and 24-48h post-PH gene expression) suggests that TA-p73 regulates more genes that change expression at early stages of regeneration, versus cell cycle-regulated genes (24-48 h post-PH, Figure 2D).

The direct comparison of gene IDs from 24 - 48 hours microarray to TA-p73 bound genes yielded seventeen TA-p73-bound genes among the 3807 genes found to be up- or down-regulated in response to PH (Table 1). This list included heat shock proteins *Carhsp1* and *Dnajc19,* transcription factors *Dmtf1*, *Foxo3*, and *Nfatc3*, cyclin L2 *Ccnl2*, plasma membrane receptors *Insr*, *Ptch1,* xenotropic retrovirus receptor *Xtr1,* and enzymes Janus kinase 1 *(Jak1)* and polymerase epsilon *Pole* (Table 1).

To identify known p53-regulated genes that may function in quiescent and regenerating liver, the list of genes that change expression during liver regeneration

(Supplementary Table 3) was analyzed for known p53-target genes using the Ingenuity knowledge database. Based on publication records in the IPA database, a majority of the p53 target genes were discovered and confirmed in tissue culture cells, whereas few are known to be bound by p53 in liver tissue *in vivo, e.g.*, *Afp* and *Cdkn1a (p21)* (Figure 3).

Depletion of both p53 and TA-p73 in mice leads to development of HCC, which is unique to this combinatorial depletion among p53-family members. Many known TA-p73 target genes are shared with p53 (64, 111, 112). TA-p73 can bind to the same consensus site with p53 on hepatic gene *Afp* (62). Therefore, a select group of TA-p73-bound genes, which displays altered expression during liver regeneration, may offer further clues regarding liver-specific gene targets of both p53 and p73 that are either up- or down-regulated during cellular proliferation and disruption of quiescence.

Gene ID	Name	Full name	p73/input	24h	38h	48h	p value	p53RE
NM_025821	Carhsp1	Calcium regulated heat stable protein 1	37.29	1.16	1.34	1.61	1.51905E-06	
NM_033325	Loxl2	Lysyl oxidase-like 2	18.82	1.44	1.48	1.60	6.42488E-05	
NM_019740	Foxo3	Forkhead Box o3a	14.63	0.49	0.47	0.48	4.163E-10	**Y**
NM_011273	Xpr1	Xenotropic and polytropic retrovirus receptor 1	10.74	0.63	0.41	0.47	4.40E-08	
NM_133752	Opa1	Optic atrophy 1 homolog	10.38	0.59	0.51	0.58	1.21815E-07	
NM_001026211	Dnaj	Dnaj (Hsp40) homolog, subfamily c, member 19	8.7	0.80	0.64	0.67	5.35E-07	
NM_011825	Grem2	Gremlin 2 homolog, cysteine knot superfamily	7.61	0.52	0.32	0.32	1.15E-07	
NM_011063	Pea15	Mammary transforming gene 1	6.11	1.53	2.06	1.89	6.84E-06	Y
NM_011132	Pole	Polymerase epsilon	6.1	1.36	2.82	2.15	7.65336E-08	
NM_146145	Jak1	Janus kinase 1	5.21	0.83	0.41	0.51	3.0463E-05	Y
NM_010730	Anxa1	Annexin A1	5.11	2.20	2.89	2.28	8.86989E-05	
NM_011653	Tuba 1	Tubulin, alpha 1	4.98	2.33	2.64	2.55	7.78639E-07	Y
NM_008957	Ptch1	Patched homolog 1	4.52	0.39	0.33	0.40	7.80E-08	
NM_010901	Nfatc3	Nuclear factor of activated t-cells, cytoplasmic, calcineurin-dependent 3	3.79	0.73	0.49	0.59	2.51318E-05	
NM_207678	Ccnl2	Cyclin L2	3.66	0.52	0.45	0.50	3.90362E-05	
NM_011806	Dmtf1	Cyclin d binding myb-like transcription factor 1	3.25	0.61	0.50	0.68	1.85E-06	

Y - p53RE perfect match

Y - p53RE found

Table 1. p73-bound genes that change expression in response to partial hepatectomy.
List of TA-p73 bound genes (Supplementary Table 1), was compared to genes that change
expression during 24 – 48 hours of liver regeneration (Supplementary Table 3). IPA
Compare Analysis function identified 17 gene IDs that are common between two datasets.
The intensity of TA-p73 binding was determined by ChIP/chip (Supplementary Table 1),
and the expression levels of 17 genes were analyzed by microarray (Supplementary Table
3). Search for p53 response elements was performed using results of algorithm (Hoh J, Jin
S, Parrado T, Edington J, Levine AJ, Ott J. The p53MH algorithm and its application in
detecting p53-responsive gene *PNAS* 2002; 99(13): 8467-72).
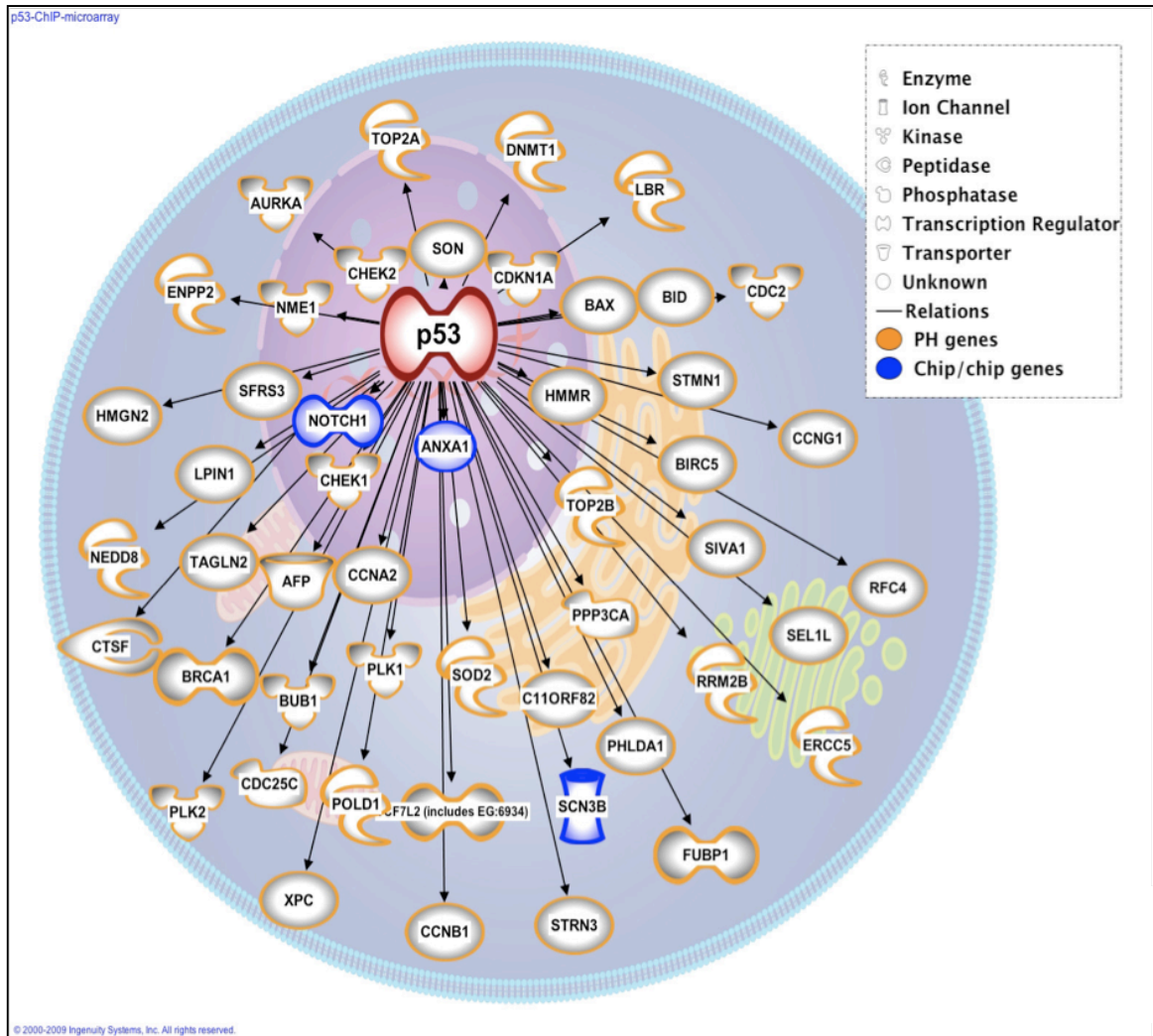
Figure 3. Diagram of putative p53 target genes in liver. List of genes that change expression during liver regeneration was analyzed for known p53-target genes using Ingenuity knowledge database. p53 target genes shown here were originally discovered and confirmed in tissue culture cells. Based on our microarray analysis, these genes are also up-regulated or down-regulated during liver regeneration. Only few of them were previously characterized as p53 targets in liver tissue *in vivo*, e.g., *AFP* and *CDKN1A (p21)*. The majority of listed here p53 target genes remain uncharacterized in liver during quiescence and regeneration.

1.3.   Identification of new target genes regulated by p53/p73 during liver regeneration.

TA-p73-bound genes, uncovered by ChIP/chip, were considered as potential p53-regulated gene-targets in normal, quiescent liver (30, 62, 113). Using a published algorithm (102), to find p53 consensus sites, potential, shared p53 and TA-p73 (p53/p73) binding sites (p53 response elements, p53RE) were mapped upstream of the transcription start site (TSS) of only four TA-p73-bound genes that changed expression during 24 - 48 hours of liver regeneration: *Forkhead Transcription Factor O3 (Foxo3), Janus kinase 1 (Jak1), phosphoprotein enriched in astrocytes 15A (Pea15),* and *tubulin alpha 1A (Tuba1)* (Table 1). p53 REs were found within intronic regions of *Jak1*, at -3.7 kb of *Foxo3* TSS, at -0.6 kb and -3.0 kb of *Pea15* TSS, and at -3.3 kb of *Tuba1*. Binding of p53 and TA-p73 to p53REs of *Foxo3, Pea15, Jak1,* and *Tuba1* were verified by ChIP using antibody against p53 and TA-p73 (Figure 4). Since no p53REs were found in the region covered by the Agilent promoter probe for *Jak1* (within -5.5kb and +2.5kb of TSS), ChIP primers for the probe region located at -1.5 kb from *Jak1* TSS were used to test p53/p73 binding (Figure 4). *Afp* p53RE served as a positive control for p53/p73 binding in quiescent liver, whereas upstream regions of *Alb* and *Brn3B* genes served as negative controls for p53 and TA-p73 binding (62, 68). Binding of p53 and TA-p73 was observed for all examined genes at identified or potential p53REs, thus confirming that putative targets uncovered by TA-p73 ChIP/chip may be bound by both p53 and TA-p73 in quiescent liver *in vivo*. By microarray analysis, expression of *Foxo3* was downregulated at 24 – 48 hours after partial hepatectomy, *Jak1* expression was decreased at 38 - 48 h post-PH, whereas other two genes, *Pea15* and *Tuba1*, showed a significant increase in mRNA levels between 24 – 48 hours of liver regeneration

(Supplementary Table 3). To test whether *Foxo3*, *Jak1*, *Pea15*, and *Tuba1* are regulated by p53 at the level of basal expression in normal quiescent liver, mRNA levels for these genes were determined in livers collected from p53$^{-/-}$ mice, and compared to *wt* littermates. By real-time RT-PCR, a 2-fold decrease of *Foxo3*, and a 1.5 fold increase of *Pea15* and *Tuba1* expression was observed in p53$^{-/-}$ livers, compared to *wt* (Figure 5). *Jak1* expression did not change significantly in p53$^{-/-}$ mice, suggesting p53-independent regulation of this gene during liver regeneration.

Taken together, these results suggest that p53 and TA-p73 act to activate or repress target genes in quiescent liver, and that the transcriptional activity of p53 and TA-p73 changes during liver regeneration. *Foxo3*, *Pea15*, and *Tuba1* are regulated by p53 in quiescent liver, and bound by TA-p73 at the p53RE located at the 5' regulatory region of a gene. Since TA-73 compensates for p53 in regulating expression of the *Afp* gene in p53$^{-/-}$, we suggest that expression of *Jak1*, *Foxo3*, *Pea15*, and *Tuba1* is regulated by TA-p73.
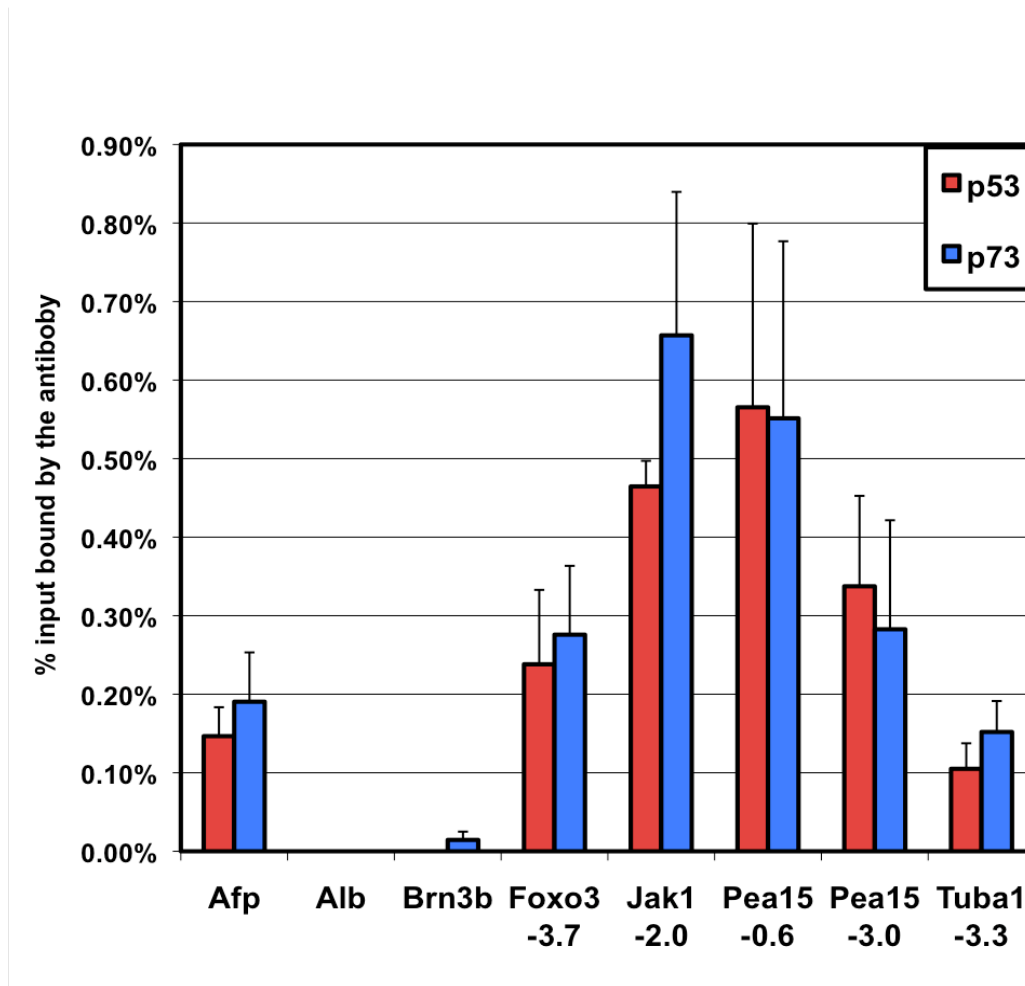
Figure 4. p53 and TA-p73 binding to genes from TA-p73 ChIP/chip analysis. ChIP from adult mouse liver tissue was performed using antibodies against each p53 and TA-p73. Binding of p53/p73 to the previously identified p53RE upstream of *AFP* served as a positive control; primers to Alb and Brn3B genes were used as a negative control. Average of at least three independent ChIPs for each p53 and p73 is shown as percentage of input bound. Differences between p53/p73 binding to *AFP*, *Foxo3*, *Jak1*, *Pea15*, and *Tuba1a* genes as compared to Alb and Brn3B, are statistically significant (p<0.05).
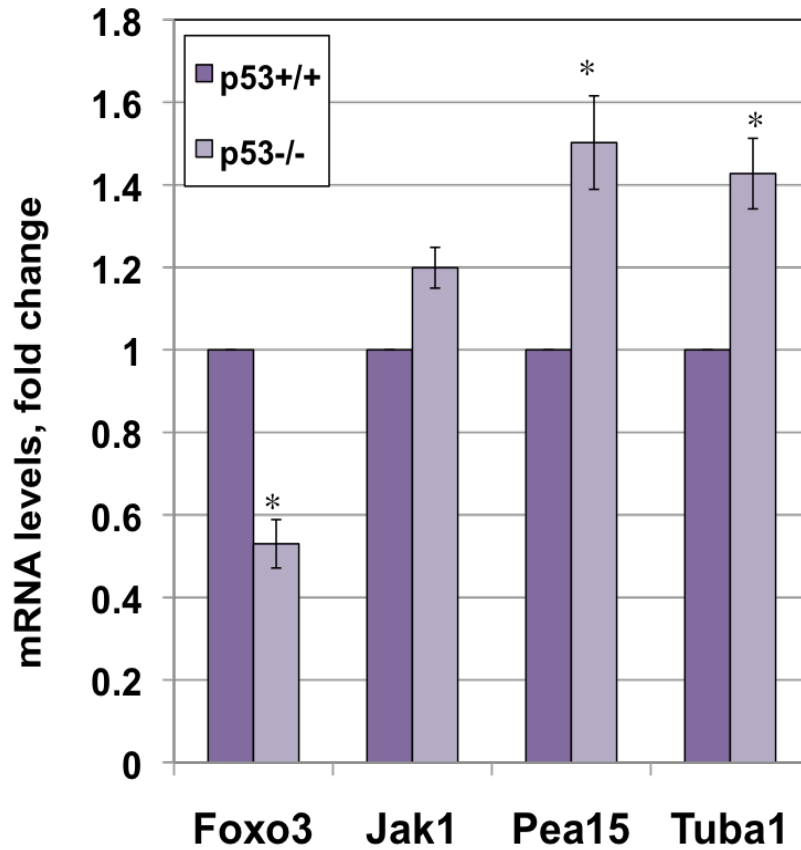
Figure 5. Expression of potential p53/p73 target genes in quiescent mouse liver.
RNA levels of 4 genes bound by p53 and TA-p73 in quiescent WT mouse liver was compared to the expression in p53$^{-/-}$ mouse liver. Differences in basal transcript levels expression of *Foxo3, Pea15,* and *Tuba1* are statistically significant and marked with asterisks (p<0.05).

2.   Identification of Foxo3 gene as a new transcriptional target of p53/p73.

2.2.   p53 and p73 directly bind and activate expression of Foxo3 gene in mouse liver and in cultured cells.

Among the seventeen TA-p73 gene-targets revealed by ChIP/chip, the gene encoding the Forkhead box transcription factor O3 *(Foxo3)* had the most significant change in expression in response to partial hepatectomy (>2 fold decrease, p value $4.163*10^{-10}$) and a high p73-binding ratio (14.63 fold over input, Table 1). A p53 consensus site was found 3.7 kb upstream of the TSS of *Foxo3* gene, as well as at several other locations within the second and third introns of the gene (Figure 6A). The p53REs identified in the *Foxo3* gene were analyzed using a motif discovery software MDScan (103), and a logo for the *Foxo3* p53 half-site was built using WebLogo software (104) (Figure 6B). Seven p53REs found in *Foxo3* gene correspond to the canonical p53RE (61, 114), having two half-sites separated by a 1-10 bp spacer (Figure 6A). As discussed in more detail later, the structure of *Foxo3* p53REs suggest that p53 binding to these consensus sequences activates expression of the Foxo3 gene. Only one p53RE was found upstream (at -3.7 kb) of TSS; the other six were located within the 2nd and the 3^{d} intron of *Foxo3* (Figure 6A). To access the specificity of p53/p73 binding to the *Foxo3* p53RE during ChIP analysis of *wt* mouse liver tissue, primers for the region located at -2.0 kb upstream of *Foxo3* TSS was used as a negative control (non-specific region, n.s). This region contains no p53REs and shows background levels of interaction as compared to the binding of p53 and TA-p73 to the *Afp* p53RE and *Foxo3* p53RE (Figure 6C and 7A). Both p53 and TA-p73 showed significant levels of binding to the   upstream   p53RE   of   the   Foxo3   gene   in   *wt*   liver   (Figure   7A).

A

Gene	Position	Sequence
Foxo3	-7316 bp	GAGCAAGGTCGAGGGCAGGACATGCCA
Foxo3	**-3703 bp**	**GGGCATGTTAACAGGGCATGCCT**
Foxo3	2nd intron	GGGCAAGTTTTGGGCATGCCC
Foxo3	3^{d} intron	TGACAAGTTCAAGGCTAGCCT
Foxo3	3^{d} intron	AAACTTGCCTGGGACCTTTCAGACTAGATT
Foxo3	3^{d} intron	CAGCATGTTCAAGTAGTAGCATGTTC
Foxo3	3^{d} intron	GAGCAAGCCATGAAAAGCAAGCCA

B

C
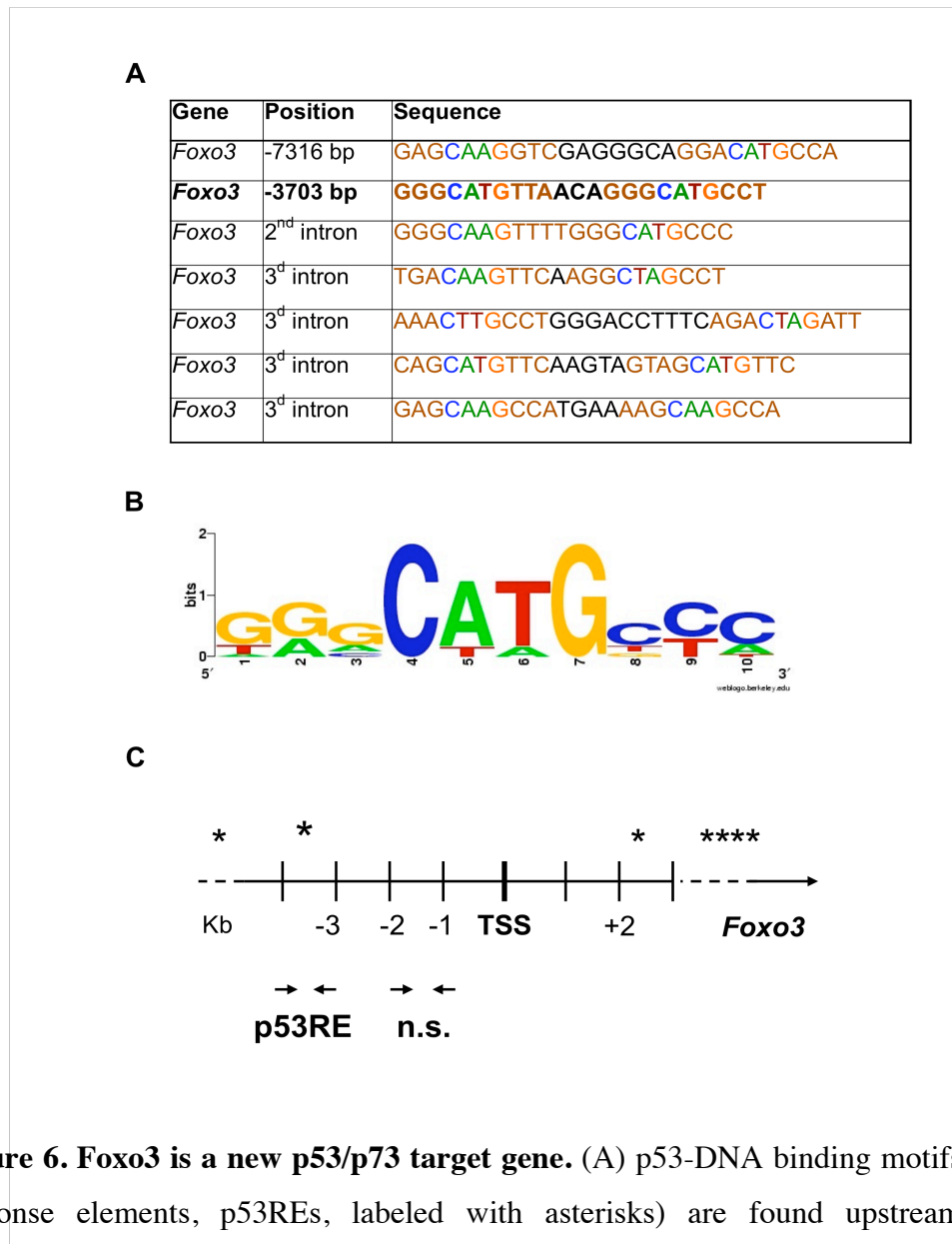
Figure 6. Foxo3 is a new p53/p73 target gene. (A) p53-DNA binding motifs (p53 response elements, p53REs, labeled with asterisks) are found upstream and downstream of the *Foxo3* transcription start site (TSS). Half-sites nucleotides have colors that correspond to the structure of the p53 half-site on Figure 5B. Spacer region is in black. (B) p53REs, identified in *Foxo3* gene, were analyzed using a motif discovery software, and a logo was build using a sequence logo generator. The height of a letter at each given position of the p53RE is proportional to the frequency of its corresponding nucleotide at that position among the identified p53REs in p53-bound DNA sequences (C) Primers used for consequent ChIP experiments for the p53RE site (marked in the table in bold) and to the non-specific site (n.s.) are shown with pairs of arrows below. Position of *Foxo3* p53REs are marked with asterisks.
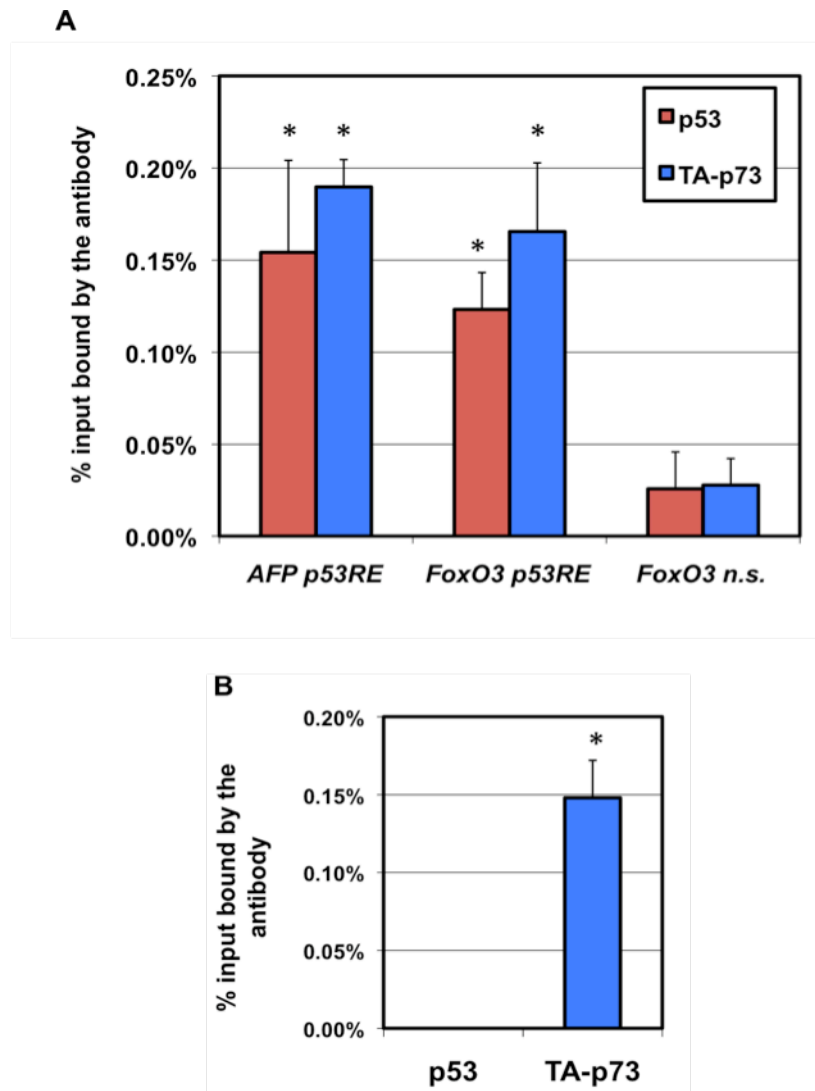
Figure 7. p53 and TA-p73 bind to the p53 RE located at -3.7 kb 5' *Foxo3* region.
(A) ChIP from adult mouse liver tissue was performed using antibodies against each p53
and p73. Binding of p53/p73 to the previously identified p53RE upstream of *AFP*
served as a positive control. Primers to the -2 kb region of *Foxo3* (non-specific site, n.s.)
was used as a negative control. Average of at least three independent ChIPs for each p53
and p73 is shown as percentage of input bound. The difference between p53/p73 binding
to *AFP* and *Foxo3* p53REs as compared to n.s. site is statistically significant and marked
with asterisks (p<0.05). (B) p73 binds Foxo3 p53RE in the absence of p53. ChIP using
p53^{-/-} adult liver tissue was done with antibodies against p53 and p73. Average of three
independent ChIPs is shown; p73 binding to *Foxo3* p53REs is statistically significant
and marked with an asterisk (p < 0.05).

Previous work showed that binding of p53 to *Afp* in adult mouse liver is essential for repression of *Afp* transcription within 2-3 weeks of age (68). However, TA-p73 compensates for loss of p53, by binding to the *Afp* p53RE in the absence of p53, to promote a delayed but significant reduction of *Afp* expression in liver by 4-months of age in p53$^{-/-}$ mice (62). ChIP analysis performed using liver tissue collected from p53$^{-/-}$ mice at 2 months of age demonstrated that TA-p73 binds the p53RE of *Foxo3* in the absence of p53 (Figure 7B). Thus, both p53 and TA-p73 may regulate transcription of *Foxo3* in adult mouse liver.

FoxO3 functions as a tumor suppressor, activating pro-apoptotic target genes, and suppressing proliferation. Based on known functions of p53 and TA-p73 as tumor suppressors and positive transcriptional regulators of pro-apoptotic and anti-proliferative genes, the original hypothesis was expanded to suggest that p53 and TA-p73 act as positive regulators of *Foxo3* at the level of transcription. First, levels of *Foxo3* mRNA, isolated from liver tissue collected from p53$^{+/-}$, p53$^{-/-}$, and p73$^{+/-}$ mice, were determined in comparison to *wt* littermates. By real-time RT-PCR, a significant decrease of *Foxo3* expression was observed in p53$^{-/-}$ and p73$^{+/-}$ mice (Figure 8). Next, expression of *Foxo3* was determined in liver tissue isolated on day 2, 8, and at 1, 2, 6 months of post-neonatal development in *wt* mice (Figure 9). Expression of *Afp* is dramatically repressed in mouse liver after birth (115), and p53 and TA-p73 mediate *Afp* repression in adult mouse liver (62, 68). In contrast to *Afp* repression, Foxo3 expression was activated in 1 - 6 month old mouse liver (Figure 9), suggesting that p53 and TA-p73 activate expression of Foxo3 in adult liver when the organ is fully developed and liver cells become quiescent.
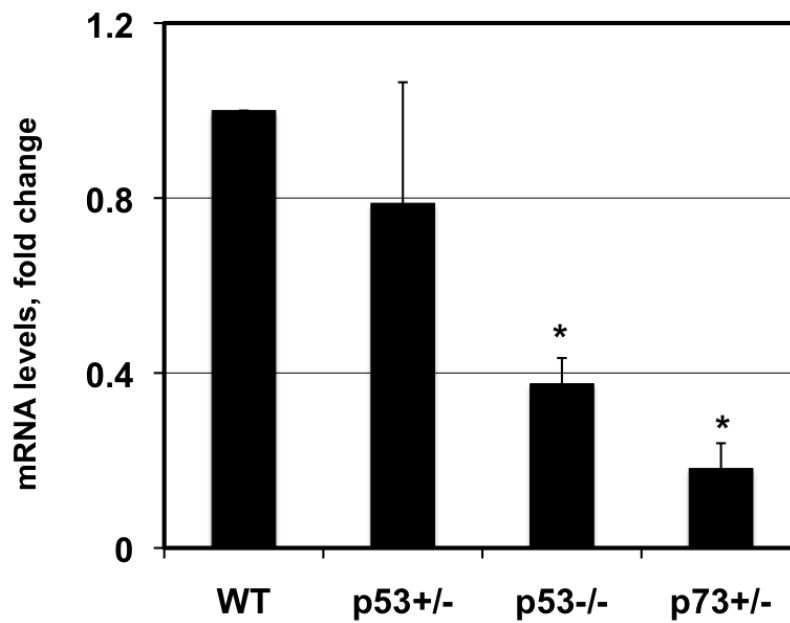
Figure 8. p53 and p73 activate expression of *Foxo3* gene in quiescent adult mouse liver. Foxo3 mRNA levels in p53^{+/-}, p53^{-/-}, and p73^{+/-} mice were compared to the Foxo3 expression in WT mice by real-time PCR. The difference between Foxo3 mRNA levels between WT and p53^{-/-} and between WT and p73^{+/-} statistically significant and marked with asterisks ($p < 0.05$).
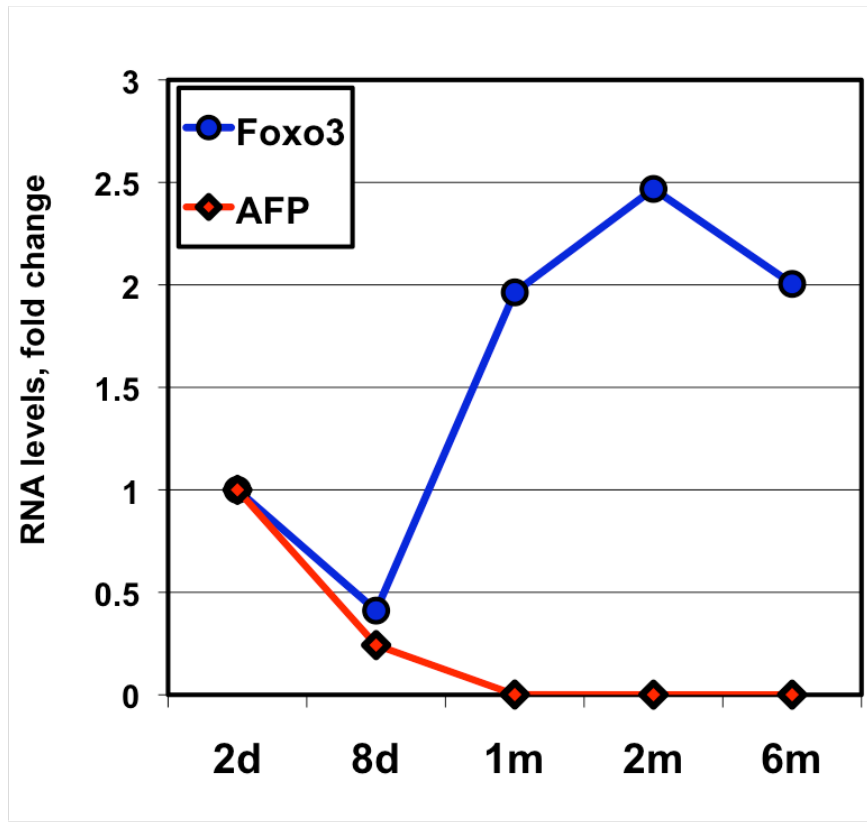
Fold	2d	8d	1m	2m	6m
Foxo3	1	0.41	1.96	2.47	2
Afp	1	0.24	0.001	0.001	0.001

Figure 9. Foxo3 expression during post-neonatal liver development.
Relative *Foxo3* and *Afp* transcript levels measured by real-time PCR in total
RNA from liver samples isolated from at least 3 mice per time point during
post-neonatal development. Expression levels of Afp and Foxo3 in liver at two
days after birth are set as 1; the other developmental time points are shown as
fold change compared to day 2. The difference in expression between all time
points are statistically significant, as compared to day 2 separately for each
gene.

Transcription of *Trp73* from alternative promoters, together with the alternative mRNA splicing at the C'-terminus, results in approximately 28 isoforms of p73 (116). To test if ectopically overexpressed p53 and TA-p73 isoforms activate *Foxo3* expression in liver cancer cells, transient transfection of a mouse hepatoma-derived cell line Hepa1-6 (117) was done with plasmids expressing HA-tagged p53, HA-TA-p73α, and HA-TA-p73β. A significant increase in endogenous *Foxo3* mRNA levels is observed in cells over expressing p53, TA-p73α, and TA-p73β, as compared to an empty vector transfection (Figure 10). Immunoblotting with antibodies against p53 and all TA-p73 isoforms (Figure 10, lower panel) shows that, despite expression of HA-TA-p73β at a lower level than HA-TA-p73α, the induction of *Foxo3* expression is comparable. This is consistent with increased transcriptional activity previously reported for TA-p73β versus other TA-p73 isoforms (63, 113, 118); however, more experiments are required to elucidate a mechanism behind this difference in TA-p73-mediated transcriptional regulation.

To establish cause-and-effect in direct transcriptional regulation of *Foxo3* by p53, immortalized MEFs that express a temperature-sensitive p53 conformational mutant: p53^{val135} (Val5MEFs) were used for *Foxo3* mRNA analysis. In this model system, Val5MEFs incubated at a restrictive temperature (37°C) have only cytoplasmic-localized p53, p53^{val135}, which is unable to regulate target gene expression (100). At the permissive temperature of 32°C, p53^{val135} assumes a *wt* conformation and moves to the nucleus to activate or repress its target genes, including endogenous *Foxo3* (Figure 11). Together, these results demonstrate that endogenous *Foxo3* is activated by p53 and TA-p73 in mouse liver, and by nuclear translocation of p53 in Val5 MEFs or ectopic expression of p53 or TA-p73 in hepatoma cells.
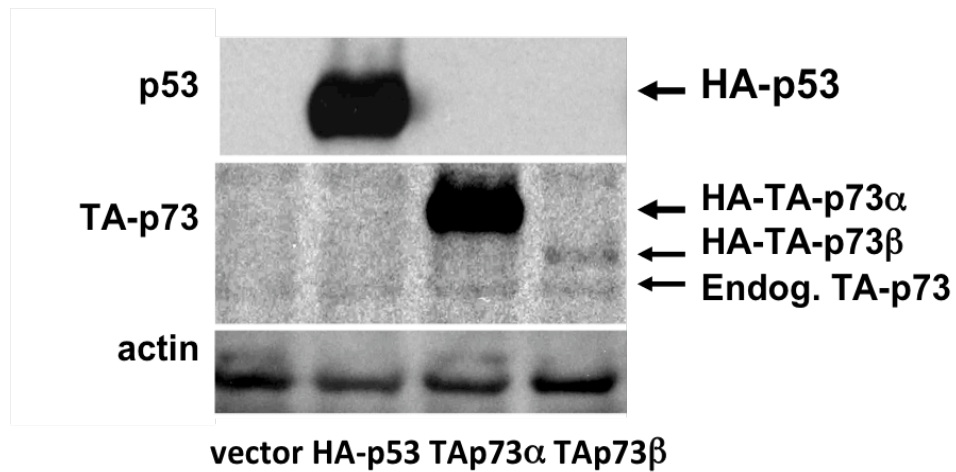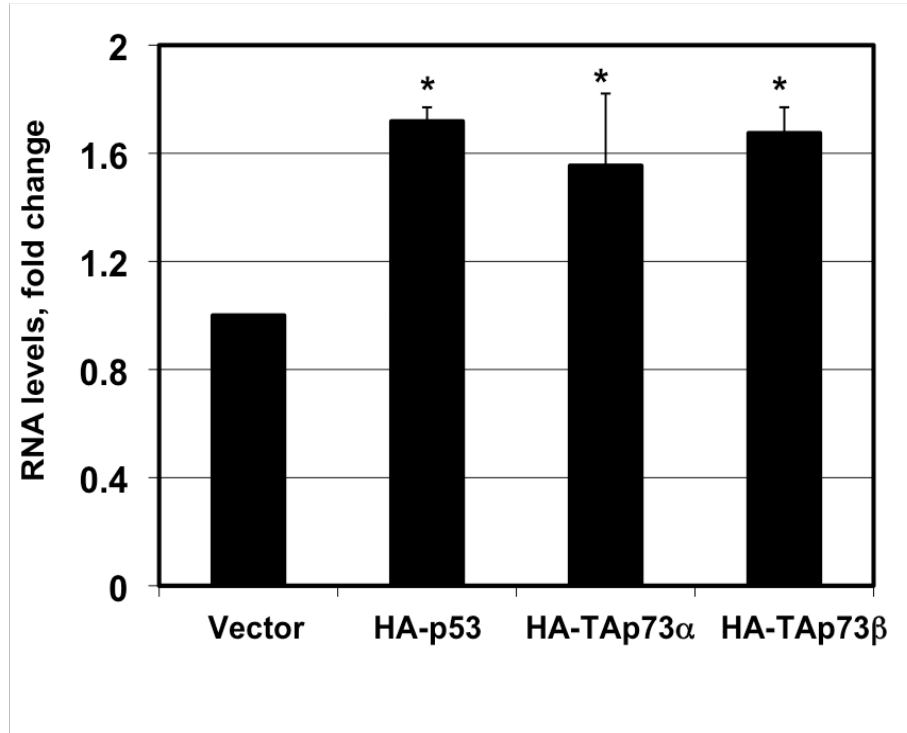
Figure 10. p53 and TA-p73 activate expression of *Foxo3* gene in liver cancer cells. Hepa1-6 mouse hepatoma cells were transiently transfected with HA-p53, HA-TAp73α and TA-p73β; mRNA levels were measured by real-time PCR. Average of three independent trasfection experiments is shown; the difference between Foxo3 expression in vector-transfected cells and cells overexpressing p53 and TA-p73 is statistically significant ($p < 0.05$).
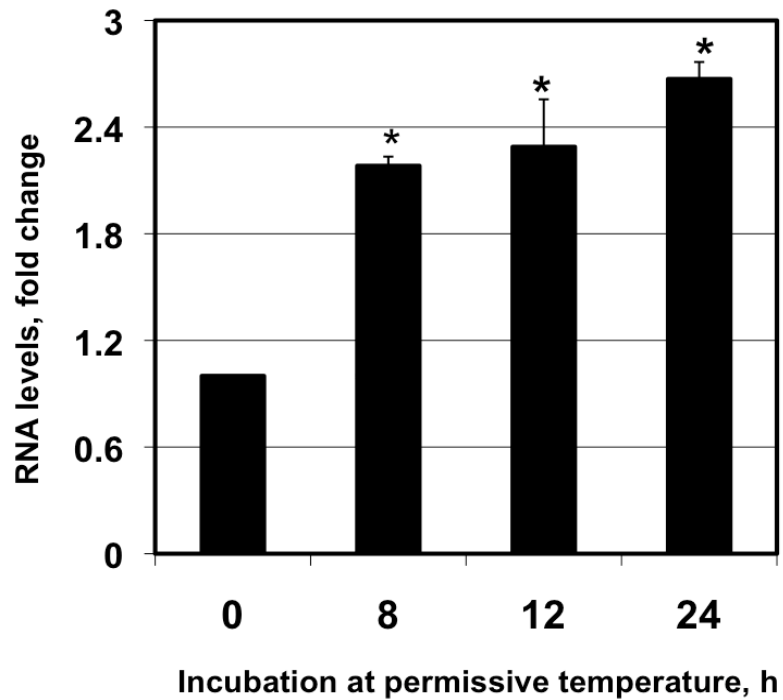
Figure 11. Temperature-sensitive p53 activates expression of *Foxo3* gene in immortalized mouse embryonic fibroblasts. *Foxo3* expression was measured in immortalized MEFs overexpressing temperature-sensitive p53 R135V mutant. RNA was isolated from cells incubated at permissive temperature (32°C) for 0, 8, 12, and 24h. Average of three independent experiments is shown; the difference between each time point and T=0 is statistically significant and marked with an asterisk ($p < 0.05$).

2.3.   Binding of p53 and p73 to Foxo3 gene is lost during liver regeneration.

Analysis of global gene expression levels (Supplementary Table 2 and 3) suggested that *Foxo3* expression is dramatically decreased during a time period following partial hepatectomy when hepatocytes undergo DNA replication and enter mitosis, 24 - 48 hours after surgery. To test if *Foxo3* expression decreases in regenerating liver due to loss of p53 and p73 binding to chromatin at the p53RE of *Foxo3*, ChIP analysis of liver tissue, collected at 1, 2 and 7 days after PH and sham surgeries, was performed using antibodies that recognize p53 and TA-p73. Chromatin interaction of p53 at *Foxo3* p53RE was dramatically reduced at 1 day and 2 days after PH, accompanied by an equally significant reduction in TA-p73 binding (Figure 12). Binding of both p53 and TA-p73 is partially restored after 7 days of liver regeneration (Figure 12), but is not equivalent to the level of binding observed in quiescent liver (T=0), suggesting that complete restoration of p53 and TA-p73 binding to Foxo3 gene occurs at later time points following PH.
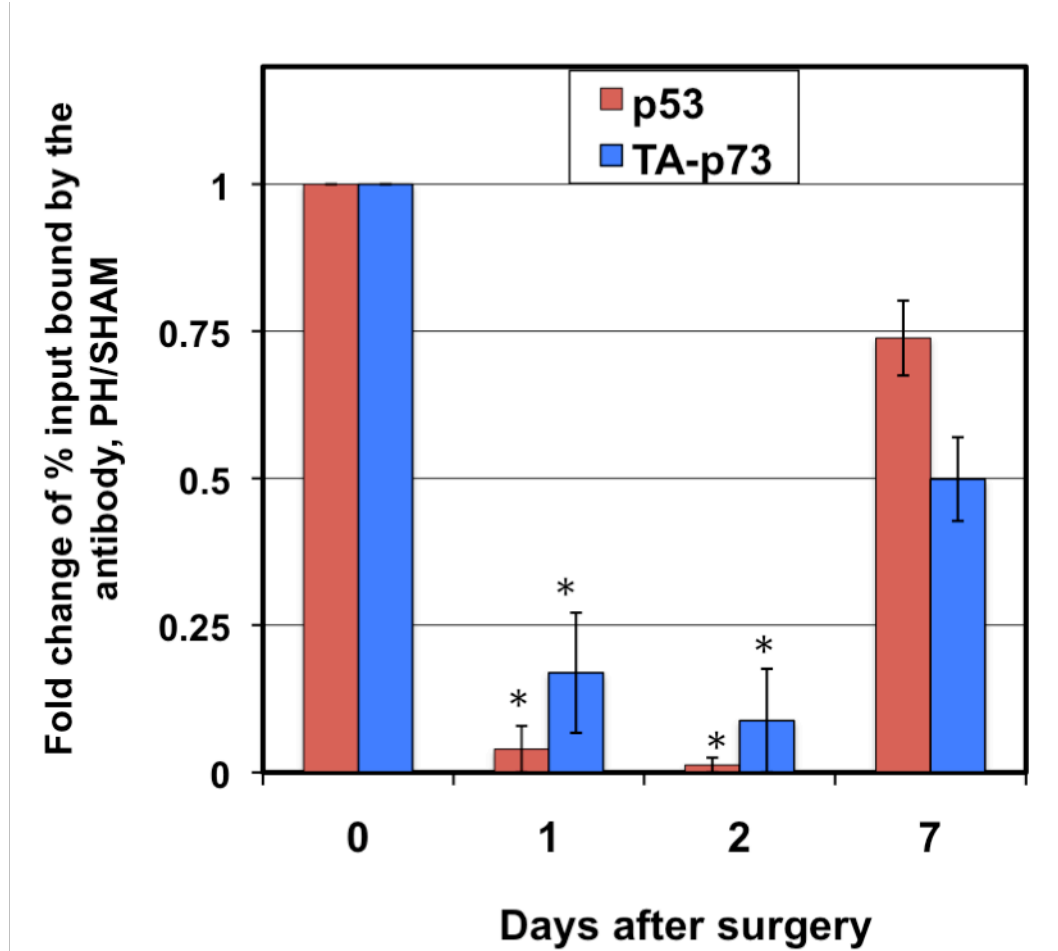
Figure 12. p53/p73 binding to *Foxo3* 5' p53RE changes during liver regeneration. ChIP from mouse liver tissue collected at 1, 2, and 7 days following sham and PH surgeries were performed using antibody against p53 and p73. Average of at least three independent ChIPs for each time point is shown as a fold ratio of % input bound for PH over % input bound in sham. The difference between p53/p73 binding in sham and at each time point following PH is statistically significant and marked with an asterisk ($p < 0.05$).

2.4.   Expression of FoxO3 is decreased during proliferative stage of liver regeneration, and is restored during termination of liver growth.

FoxO3 is a critical regulator of cell proliferation and tissue growth (119), but our understanding of FoxO3 regulation during cell cycle and tissue growth *in vivo* is limited. Liver regeneration in mice is a unique model allowing study of cell cycle *in vivo* during the first three days of regeneration, in comparison to growth cessation, 4-7 days following partial hepatectomy. The initial microarray data showed a significant decrease in *Foxo3* expression in liver collected at 24, 38, and 48 hours following PH, as compared to T=0 (Supplementary Table 3). It is important to note that microarray analysis of earlier time points (0.5 – 4 h) showed an insignificant decrease in *Foxo3* expression, suggesting that loss of FoxO3 occurs during the G_1-S-G_2 transition versus the priming phase (0 - 4 hours) of liver regeneration. Sets of PH and sham surgeries were performed on 2-month-old *wt* mice, and livers were collected at 1, 2, 3, 4, and 7 days after surgeries. Levels of FoxO3 mRNA were measured separately for each mouse in the PH or sham group, and compared to FoxO3 mRNA at T=0. Consistent with the global expression data, a significant decrease in FoxO3 mRNA levels was observed between 1-3 days after PH, with the lowest *Foxo3* expression on day 2 (Figure 13A). *Foxo3* expression was gradually restored back to T=0 level on day 4 following PH (Figure 13A), when final adjustments of regenerating liver tissue restores a normal liver/body weight index by 7 days in mice (Appendix Figure 1). No significant difference in *Foxo3* expression is observed following sham surgeries, compared to T=0 (Figure 13B), supporting a regenerative-specific response in regulation of *Foxo3*.

FoxO3 protein levels were determined by immunohistochemistry analysis of liver tissue collected at 24 hours and 7 days following PH and sham surgeries. FoxO3 protein was detected in sham-operated liver, and in the fully regenerated liver collected at 7 days following PH (Figure 14). There was a reduction in FoxO3 protein staining at 24 hours after PH, indicating that a decrease in FoxO3 mRNA levels, observed at this time point (Figure 13A), is followed by the loss of FoxO3 protein in hepatic nuclei (Figure 14).

A
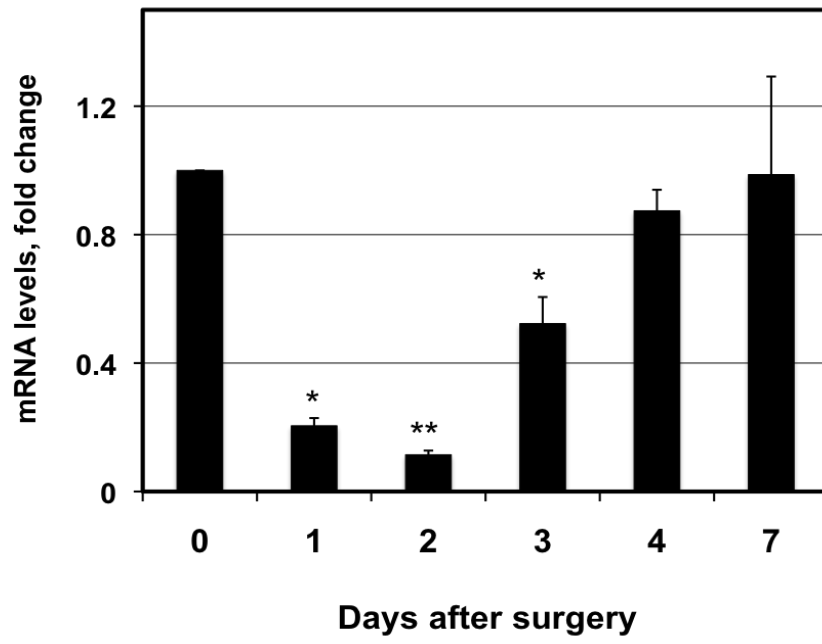
B
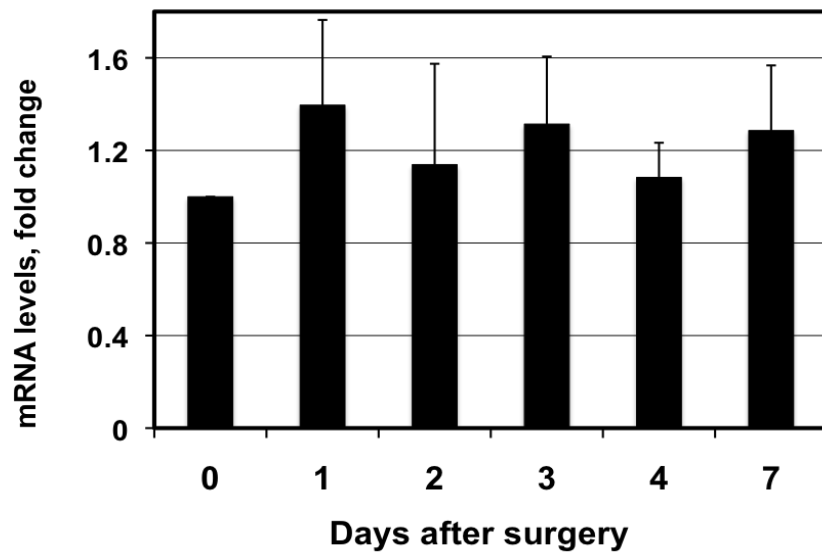
Figure 13. Foxo3 expression is decreased at 1-3 days following PHx and increased when regeneration is complete. Foxo3 mRNA was isolated from mouse liver tissue at indicated time points following the surgery. Relative expression levels were measured by real-time PCR using primers to *Foxo3*. (A) Foxo3 expression in response to 65% PH. Time points following PH with statistically significant difference in Foxo3 expression as compared to sham are marked with asterisks * $p<0.05$; ** $p<0.01$. (B) Foxo3 expression in response to sham surgery. There is no statistically significant difference in Foxo3 mRNA levels between T=0 and all time points following sham surgeries.
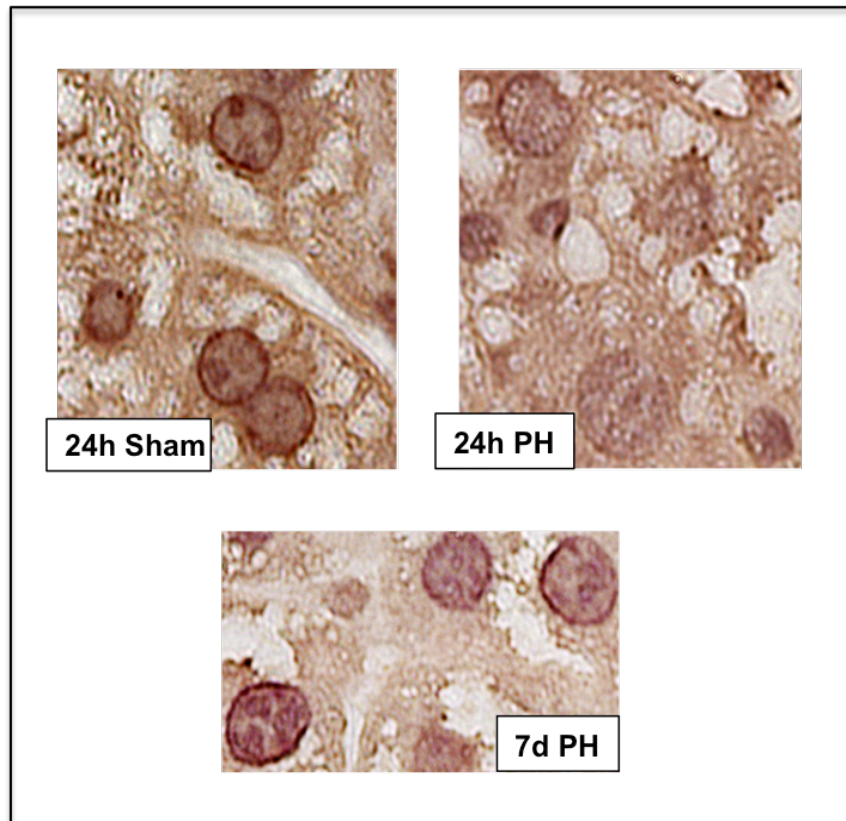
Figure 14. FoxO3 protein levels decrease in nuclei of regenerating hepatocytes. Livers were collected from WT mice at 24 hours and 7 days following sham and PH surgery. Paraffin-embedded sections were stained with FoxO3-specific antibody and counterstained with hematoxylin.

2.5.   TGFβ-signaling does not regulate expression of Foxo3 gene in liver.

Termination of liver regeneration is a poorly understood process that requires hepatocytes to exit the cell cycle back into a quiescent G_0 state. Several molecules were suggested in the previous studies as potential terminators of hepatocyte proliferation, among them TGFβ-ligand and plasminogen activator inhibitor PAI-1 protein (reviewed in (13, 49). TGFβ activates PAI-1 expression and suppresses DNA synthesis in hepatocytes (13, 120). Anti-proliferative functions of TGFβ are suppressed during liver regeneration (120), suggesting that TGFβ–mediated activation of anti-proliferative genes occurs in quiescent hepatocytes and during termination of liver growth.

Mouse hepatoma cells (Hepa1-6) respond to TGFβ1 treatment within hours of ligand application by the recruitment of p53 and Smad2/4 to the *Afp* p53RE, resulting in transcriptional repression of *Afp* (121). Another TGFβ–responsive gene, *Pai1*, has also been identified as a p53 target gene during replicative senescence in primary mouse embryonic fibroblasts (122). Transfection with small-interfering RNA specific for Foxo3 significantly inhibited caspase activation and apoptosis in rat hepatoma cells treated with TGFβ1 (123). Therefore, we tested whether TGFβ–mediated signaling regulates p53-mediated activation of *Foxo3* expression in Hepa1-6 cells and in primary hepatocytes. No significant change in *Foxo3* expression was observed in Hepa1-6 cells (Figure 15A) or in primary hepatocytes in response to TGFβ1 treatment (Figure 16), despite strong activation of *Pai1* and repression of *Afp* (Figure 15B, and Figure 16). Thus, signaling events, other than TGFβ1 induction, account for the p53-mediated transcriptional activation of *Foxo3* in liver-derived cells.

73
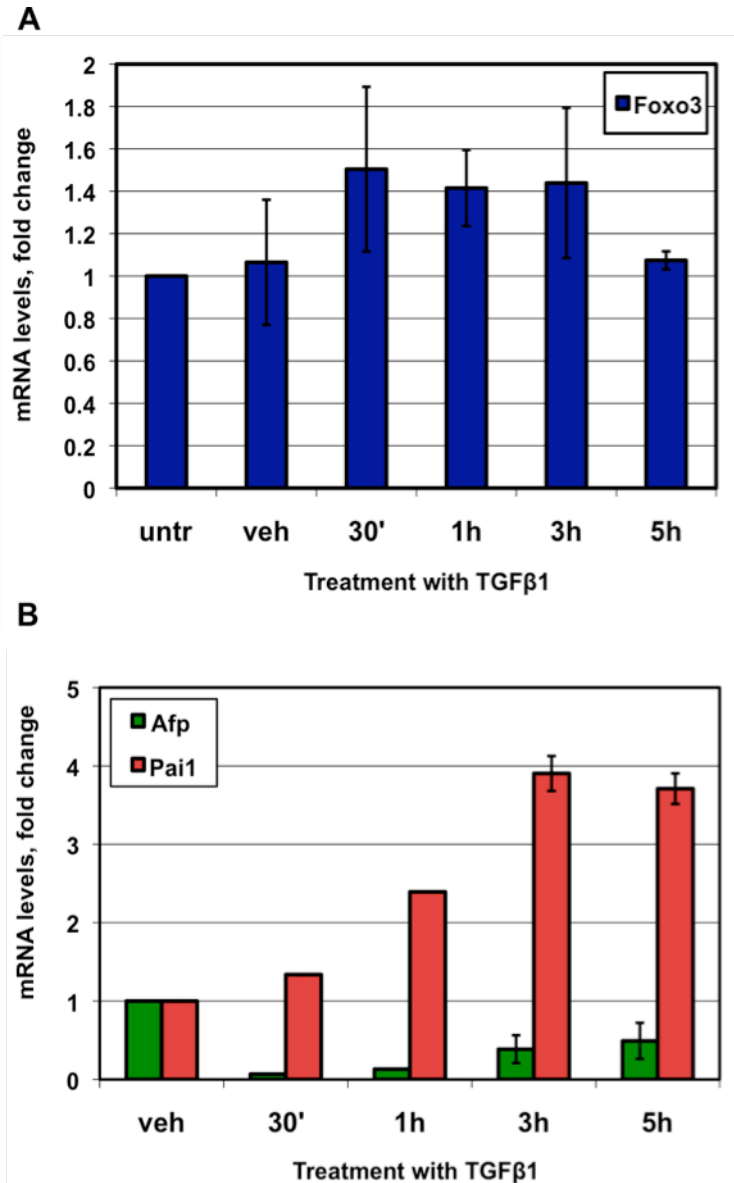
Figure 15. Expression Foxo3 does not change in response to TGFβ treatment in mouse hepatoma cells. Hepa1-6 cells were treated with TGFβ1 ligand and vehicle control for the indicated time points. *Afp, Pai1*, and *Foxo3* mRNA levels were measured by real-time PCR. Average of three independent treatment experiments is shown. There is no statistical difference in *Foxo3* expression between untreated cells, vehicle-treated, and TGFβ1−treated cells. *Afp* was significantly repressed, and *Pai1* expression was significantly increased by TGFβ1 treatment, and served as controls for TGFβ1 treatment.
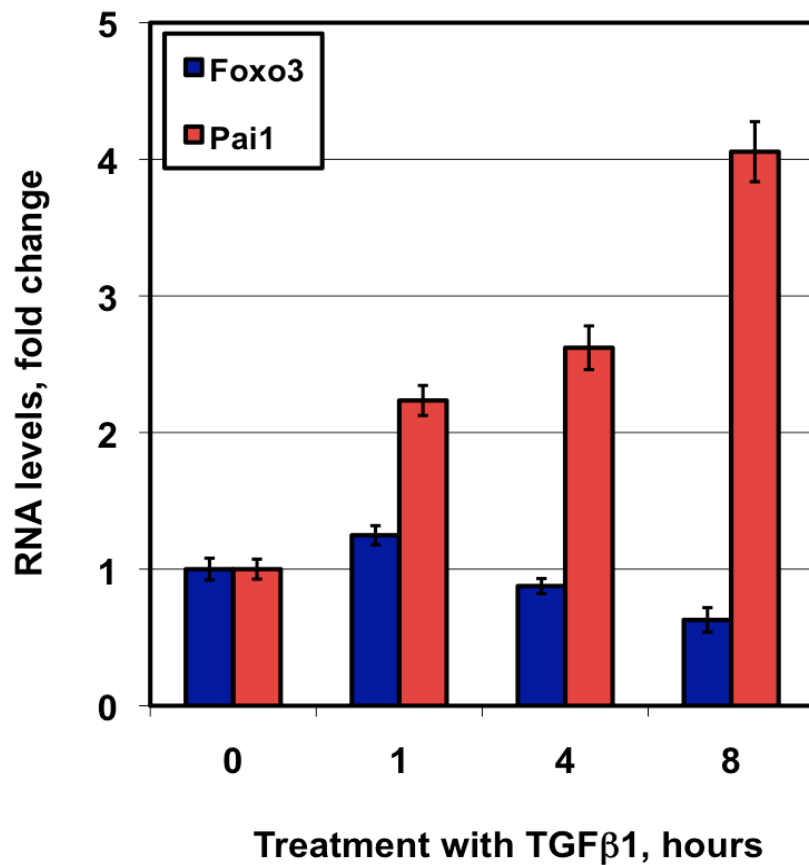
Figure 16. Expression Foxo3 is does not change in response to TGFβ treatment in primary hepatocytes. Primary hepatocytes were isolated from WT mice, cultured and treated with TGFβ1 ligand vs. vehicle control for the indicated time points. *Foxo3* and *Pai1* expression was measured by real-time PCR in triplicates. Average of three independent treatment experiments is shown. There is no statistical difference in *Foxo3* expression between vehicle-treated and TGFβ1−treated cells. *Pai1* expression was significantly increased by TGFβ1 treatment and served as a positive control.

3.	p53 and TA-p73 recruit histone acetyltransferase p300/CBP to activate expression of Foxo3 gene in mouse liver.

3.2.	Loss of FoxO3 expression during liver regeneration is associated with decreases in histone H3 and H4 modifications observed in transcriptional activation.

Regulated gene expression is associated with modifications in chromatin structure; loss of histone post-translational modifications associated with activation of transcription, *e.g.*, dimethylation of histone H3 at lysine 4 (H3K4me2) and acetylation of H3K9, H3K14, and several lysines of histone H4 are associated with repression of transcription (124). We performed ChIP analysis of liver tissue collected 1 day after PH and sham surgeries to determine histone modifications, associated with active chromatin. We observed no change in histone H3 levels at the *Foxo3* p53RE in livers collected from sham and PH mice at day 1 after surgeries (Figure 17A). Decreases in H3K4me2, H3K14Ac, and H4Ac, without a significant change in H3K9 acetylation, were observed at the *Foxo3* p53RE in regenerating liver compared to sham (Figure 17A). These results suggest that decreases in H3K4me2, H3K14Ac, and H4Ac levels, but not changes in histone occupancy manifested by H3 levels, account for the loss of Foxo3 expression during liver regeneration. Importantly, these decreases occur concomitantly with loss of p53 and TA-p73 binding at the *Foxo3* p53RE region (Figure 12), and suggest that p53 and TA-p73 recruit histone modifying enzymes to activate expression of *Foxo3* at T=0.

3.3.   Recruitment of p300 to the p53/p73 binding site of Foxo3 gene decreases in regenerating liver.

p53 and TA-p73 recruit acetyltransferase CBP/p300 (KAT3A/KAT3B) to activate transcription of target genes (72, 73). Using antibodies against p300, a significant decrease in p300 binding to *Foxo3* p53RE was observed in quiescent liver (T=0) of p53$^{-/-}$ mice (Figure 17B). A more significant loss of p300 binding to *Foxo3* p53RE was observed in regenerating liver of *wt* mice at 24 hours post-PH (Figure 17C), suggesting that both p53 and TA-p73 contribute to the recruitment of p300 to activate expression of *Foxo3* in normal quiescent liver. It is important to note that recruitment of p300 by p53 and TA-p73 may activate Foxo3 transcription not only by acetylating histone H3K14 and H4 residues, but also by acetylating C'-termini of p53 and TA-p73 – a post-translational modification known to enhance p53/p73-mediated activation of target genes (125-127).
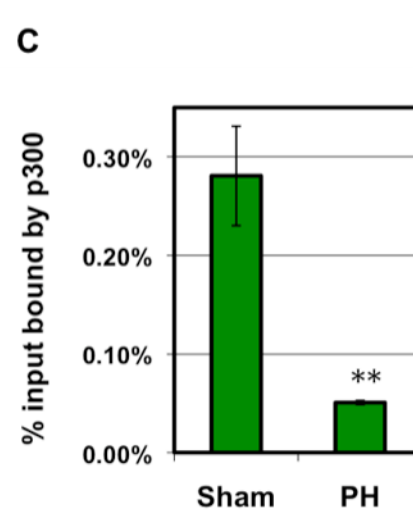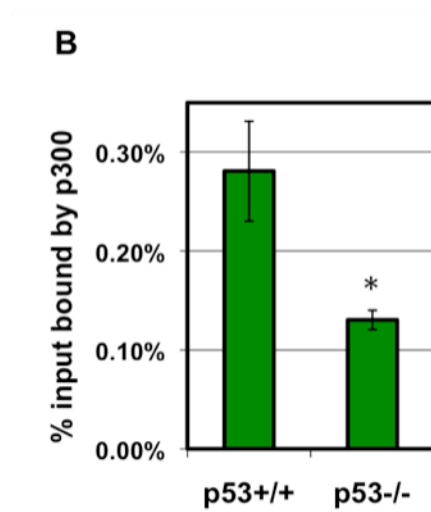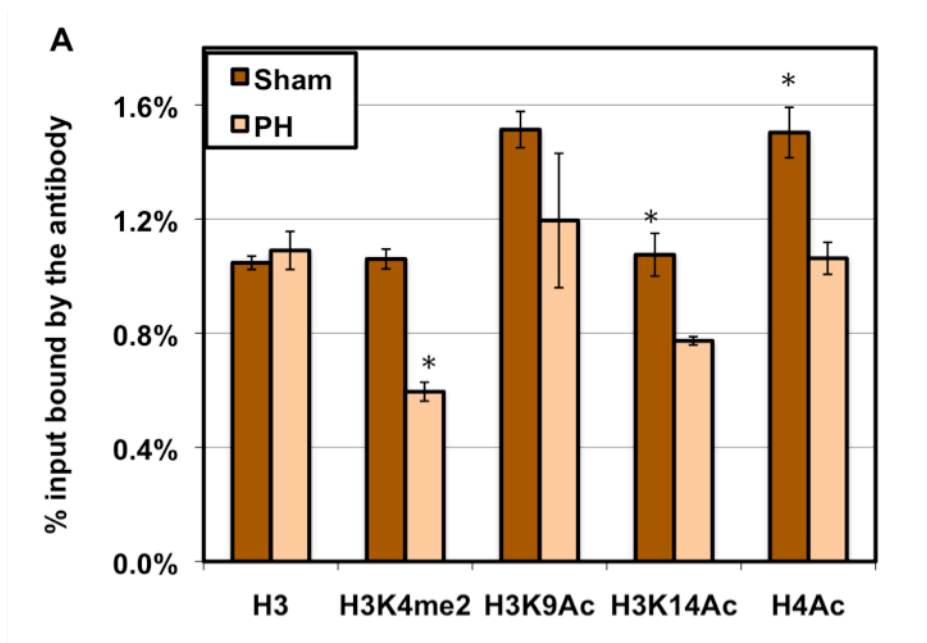
Figure 17. Acetyltransferase p300 binds p53RE. (A) Histone modifications at *Foxo3* p53RE region during liver regeneration. ChIP from mouse liver tissue collected at 24 hours following sham and PH surgeries were performed using antibodies against modified histones H3 and H4. An average of at least three independent ChIPs for each time point is shown as percentage of input bound for PH and sham. The difference between sham and PH is statistically significant when marked with ** asterisks ($p<0.01$). (B) p53 and p73 recruit p300 to the p53RE of 5' *Foxo3* region. ChIP was performed using antibodies against p300. Average of at least three independent ChIPs for is shown as percentage of input bound. The difference between p300 binding to *Foxo3* p53REs in WT as compared to p53$^{-/-}$ mouse liver is statistically significant and marked with an asterisk ($p<0.05$). (C) p300 binding to *Foxo3* p53RE is lost during liver regeneration. ChIP experiments using liver tissue collected at 24h post-PH and sham surgeries were done with antibodies against p300. Average of three independent ChIPs is shown; decrease in p300 binding to *Foxo3* p53REs is statistically significant and marked with ** asterisks ($p<0.01$).

CHAPTER IV

DISCUSSION

SIGNIFICANCE OF FINDINGS

1.   Global analysis of p53/p73 target genes in normal liver tissue.

Definition of regulatory mechanisms employed by p53 family members in specific tissues or biological responses requires evaluation of their chromatin interactions and functional consequences in recruitment of co-factors, histone modifications and target gene expression. Experimental approaches using chromatin immunoprecipitation (ChIP) in conjunction with microarrays (ChIP/chip) or ChIP-paired-end (ChIP-PET) sequencing identified multiple p53 target genes responsive to DNA damage, irradiation, and actinomycin D treatment of cancer cell lines (60, 128-130). More recent p53 ChIP/chip analyses, utilizing an array with 540 p53-PET sites, 62 additional p53 target regions, and 846 random promoter regions, revealed profound differences in p53 DNA binding activity between primary cells (fibroblasts and periferal blood cells) and cancer cell lines after exposure to genotoxic stress (131). In tumor-derived cell lines, there is little correlation between DNA binding by p53 and active expression of several, known p53-regulated genes, in the absence of genotoxic stress, suggesting that "latent" or inactive p53 has limited binding activity at its target genes (131). However, ChIP/chip analysis and comparison of p53-binding in nontransformed, primary cells (fibroblasts and blood cells) and cancer cell lines, revealed profound difference in p53 functions in normal vs. continuously cultured, tumor-derived cell lines (131). This result further emphasizes the importance of characterization of p53-mediated transcription in normal tissues.

Genome-wide binding of p63 and p73 yielded additional sets of targeted genes, many of which overlap with p53 target genes, which regulate DNA damage response, cell cycle arrest, and apoptosis (60, 64, 132, 133). These results are not surprising, since p53

81

family members have highly homologous DNA-binding domains, and can bind each other to form heterotetramers (54, 134). p53, p63, and p73 bind to a very similar DNA consensus sequence (60), referred here as a p53RE (112). However, despite these similarities, p53 family members also have distinct functions *in vivo*, demonstrated by differences in phenotypes of p53$^{-/-}$, p63$^{-/-}$, and p73$^{-/-}$ mice (27-29), as well as distinct tumor profiles of p53$^{+/-}$, p63$^{+/-}$, p73$^{+/-}$ compound mice (32). In order to explain both overlapping and unique functions of p53 family members *in vivo*, it is necessary, therefore, to analyze the transcriptional activity of these proteins in normal tissues during different stages of cell cycle and stress response.

In our work, mouse liver was used to perform ChIP/chip analysis and identify potential p53/p73 target genes for the following reasons. Two types of cancer, hepatocellular carcinoma and pancreatic carcinoma, are found in p53$^{+/-}$p73$^{+/-}$ mice (32). Since hepatocytes and pancreatic cells share a common developmental progenitor (135), tumor cells in these mice may originate from hepatic progenitor cells. Genome-wide evaluation of p53- and p73-bound sequences by ChIP/chip analysis, using cultured cells, demonstrated that 72% of p53-bound sites were also bound by p73 *in vivo* (60). Previously published work provided a detailed mechanism for p53/p73-mediated repression of transcription of the *Afp* gene (62, 136), demonstrating that both p53 and p73 can bind p53RE within the distal promoter of *Afp* in adult mouse liver. Transcriptional targets of p53 and p73 have been extensively studied in a variety of cell types both by *in vitro* assays and by ChIP (64); however, very few of these genes have been confirmed as direct p53/p73 transcriptional targets *in vivo*, and there is no global analysis of p73 target genes in normal tissues. The lack of data could be explained by the difficulties of analyzing the very low level of p53 and p73 protein in nuclei of normal

cells. Chromatin isolated from mouse liver can be used for ChIP/chip experiments to identify genes bound by TA-p73 (and, potentially, p53) in normal quiescent liver cells. Using this approach, 158 genes bound by TA-p73 in normal mouse liver were uncovered, and considered as potential p53-bound genes as well (Supplement Table 1). Among these, only ten targets were previously reported p53-regulated genes, whereas most of the identified genes had not been previously connected to p53/p73-mediated transcriptional regulation. In a recent review, Riley *et al*. identified criteria for *bona fide* p53 target genes, and generated a list of 129 genes containing at least one p53RE per gene. These 129 genes also satisfied two other criteria: changing expression upon *wt* p53 activation and bound by p53 at a p53RE site (61). In our ChIP/chip experiments, we identified a similar number of TA-p73 target genes (158, Supplementary Table 1). The liver regeneration model allowed the discovery of genes that change expression during activation of cell cycle in normal cells responding to signaling induced by partial hepatectomy. Seventeen TA-p73-target genes significantly change expression during the G_1-S transition that occurs at 24-48 hours following PH (Table 2). A search for p53REs within these genes, revealed four genes with potential p53/p73 binding sites that also changed expression during liver regeneration: Forkhead transcription factor *Foxo3*, mammary transforming gene *Pea15*, *Janus kinase 1*, and *tubulin a1*. Using ChIP with primers to p53RE-containing regions of these genes, both p53 and p73 were found to bind to the upstream p53REs of *Foxo3*, *Pea15*, *Jak1*, and *Tuba1* (Figure 4). Foxo3 gene was chosen to study the mechanism on p53/p73-mediated regulation of gene during liver regeneration for the reasons discussed in more detail below.

2.   p53/p73-mediated activation of Foxo3 expression as a defense line in tumor suppression

Studies of tumor suppressor properties of FoxO proteins began with the characterization of chromosomal translocations in alveolar rhabdomyosarcoma, a tumor of skeletal muscle prevalent in children (76). Typical chromosomal translocations found in this tumor, t(2;13)(q35;q14) and t(1;13)(q36;q14), result in an in-frame fusion of a paired-box gene *PAX7* to the forkhead in rhabdomyosarcoma (*FKHR,* or *FOXO1*) gene, located on chromosome 13 (137). A full-length FKHR-like gene (*FKHRL1*, or *FOXO3*) was cloned from an acute leukemia patient with a chromosomal translocation t(6;11)(q21;q23) (138). Later, a *FOXO4* gene was identified and characterized from acute leukemia patients carrying a chromosomal translocation t(X;11)(q13;q23) (139). Unlike other Forkhead box family members predominantly expressed in adipose cells (FoxO1) and muscle (FoxO4), FoxO3 is expressed primarily in liver during embryogenesis in mice (98). While expression in adult tissues, responsiveness to growth factors, and transcriptional activity of FoxO3 is similar to other members of the subfamily, FoxO1 and FoxO4 (98), FoxO3 KO mice display developmental abnormalities and early onset of sterility of female mice (85, 140).

Overexpression of *Drosophila dFoxO* induced cell death and organ size reduction in flies (141). The most closely related mammalian ortholog of *dFoxO* is *Foxo3*. Unlike other FoxOs, FoxO3 controls cell proliferation and suppresses tumorigenesis by activating genes that control inflammation, cell cycle and apoptosis in mammalian cells (142). The transcriptional activity of FoxO3 is inactivated by protein kinases Akt, IKKβ, and ERK, all of which phosphorylate FoxO3 and promote its nuclear exclusion and protein degradation in

response to growth factor signaling, inflammation, or mitogen activation (78, 79, 142). Frequent inactivation of PTEN in human cancers leads to the constitutive activation of Akt and inactivation of FoxO3-mediated transcription (143). Aberrant activation of the Ras-ERK pathway leads to a rapid Mdm2-mediated degradation of FoxO3 and promotes cell proliferation and tumorigenesis *in vivo* (142).

Inhibition of FoxO3 significantly augments tumor growth in mice, whereas restored expression of FoxO3 inhibits colony formation of breast cancer cells *in vitro* (79). Most importantly, injection of FoxO3-siRNA transfected breast cancer cells into fat pads of nude mice significantly increased tumor formation, suggesting that downregulation of FoxO3 expression can enhance tumorigenesis *in vivo*. In contrast, the ability to form tumors was dramatically decreased in breast cancer cells when expression of FoxO3 was restored (79). However, the expression of FoxO3 in normal adult tissues remains poorly characterized, and the transcription factors that activate expression of Foxo3 gene *in vivo* have not been described. Our work identified p53 and p73 as activators of endogenous Foxo3 gene expression *in vivo*. It also demonstrated expression of FoxO3 in quiescent adult mouse liver at the level of mRNA and protein.

FoxO3 protein levels and nuclear localization are negatively regulated by Akt signaling. Studies of *Pten*$^{+/-}$ and Promyelocytic leukemia *Pml*$^{-/-}$ mice with constitutively active Akt, demonstrated that Akt-mediated phosphorylation of FoxO3 results in a complete loss of FoxO3 nuclear localization and loss of FoxO3-mediated activation of pro-apoptotic and cell cycle arrest genes *Bim* and *p27* (143). Importantly, colon and prostate tissues isolated from *Pten*$^{+/-}$ *Pml*$^{-/-}$ mice showed increased proliferation and neoplastic formations, suggesting that loss of FoxO3 protein from the nucleus may lead to tumor development *in*

vivo (143). In our experiments, FoxO3 protein was present in nuclei of quiescent hepatocytes (in livers from sham-operated mice, as well as in fully regenerated livers collected at 7 days post-PH, Figure 14), suggesting that Akt-mediated inactivation of nuclear FoxO3 is not present in quiescent liver cells.

Foxo3 KO mice show a reduction of the quiescent HSC pool and a deficient repopulating capacity in serial transplantation assays, similar to *Pml*^{-/-} mice (84). Loss of *Pten* or *Trp53* in the mouse prostate does not favor a tumor phenotype, whereas combined inactivation of *Pten* and *Trp53* elicits invasive prostate cancer and is invariably lethal by 7 months of age (144). Therefore, combined inactivation of *Trp53* and *Pten* is required for maximal tumor growth in some tissues. Intriguingly, FoxO3, a downstream target positively regulated by Pten, as well as p53 and p73, all interact with PML in the nucleus, promoting apoptosis and cell cycle arrest (143, 145, 146). PML is involved in the early stage of hepatocarcinogenesis (147). Loss of interaction of p53 with PML protein also leads to development of HCC as a result of a decrease in p53 transcriptional activity toward pro-apoptotic genes (148). Surprisingly little is known about functions of PML in normal liver cells, despite several reports demonstrating loss of PML functions in development of liver cancer (148-150). The Pml/Pten/Akt/FoxO signaling pathway has critical functions in regulating the sensitivity of cancer stem cells to chemotherapy, and can offer new targets for more efficient cancer stem cell-specific therapy (151). These results suggest hepatoprotective functions for p53, p73, FoxO3, and PML in normal quiescent liver. It would be interesting to determine if interactions and transcriptional activities of these tumor suppressors play any roles in normal liver during regeneration.

One of the most comprehensive reviews on liver regeneration, written by Rebecca Taub (49), establishes that three patterns or waves of gene expression occur during liver regeneration. The first two waves are marked by expression of growth-regulated genes, activated immediately after PH, and cell cycle regulated genes, activated at 24 – 48 hours following PH. The third wave of expression (after 48 hours PH) includes anti-proliferative genes that are downregulated during the period of maximal growth and are re-expressed after the growth phase has occurred (Diagram 5). This third wave includes genes that are least studied in liver regeneration, but might function as tumor suppressors, preventing excessive proliferation of liver cells. Reactivation of *Foxo3* expression at 3-7 days after PH follows the pattern of gene expression in the after-growth phase of liver regeneration (Figure 13A).

As mentioned previously, common molecular mechanisms may regulate liver carcinogenesis and regeneration (Chapter I). Several intracellular signaling pathways, including those mediated by p53, have been studied in association with liver regeneration and liver cancer (11-14). A recently published clinical report provides direct evidence that emphasizes the importance of restoration of p53 functions for successful treatment of liver cancer (19). A 62-year-old patient with a massive 16x13.5 cm tumor in the right hepatic lobe had blood levels of AFP equal to 12947 µg/l (normal value, <8.1 µg/l). The patient was treated with adenovirus-packaged *wt* p53 (Ad-p53), followed by 5-fluorouracil (5-FU) chemotherapy to disrupt DNA synthesis. Injection of Ad-p53 into the tumor feeding artery decreased tumor size 2-fold, within 16 days of the treatment; after prolonged injection of Ad-p53 + 5-FU the tumor became a 3x2 cm hypovascular lesion, and AFP levels decreased to 4.2 µg/l (19). *Afp* expression in normal mouse liver and mouse hepatoma cells is

repressed by p53 and TA-p73 (62, 68). During liver regeneration, p53 binding to the *Afp* p53RE decreases, and expression of the Afp gene increases (69), suggesting similar mechanisms regulating transcriptional activity of p53 in liver cells during regeneration and HCC development. This case demonstrates that restoration of p53 functions is useful for patients with progressive liver cancer, and suggests that further studies of p53 functions in regenerating hepatocytes will provide new strategies for the treatment of liver cancer.
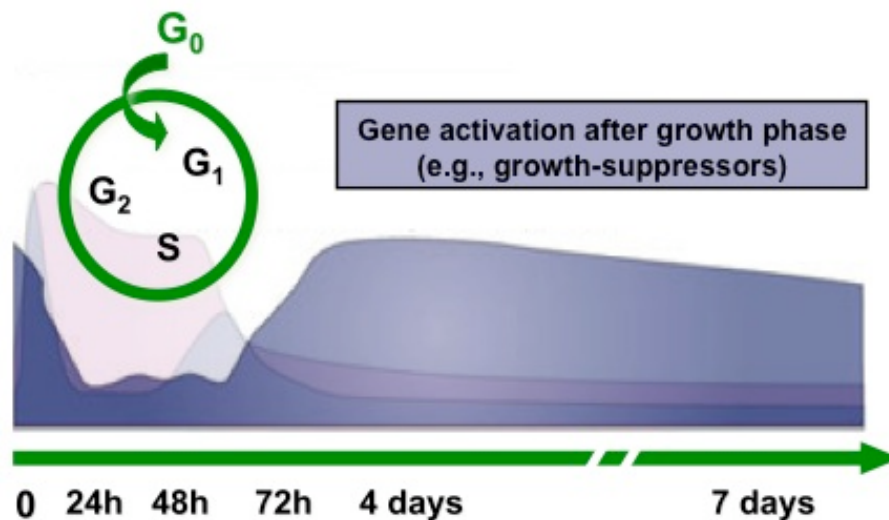
Diagram 5. Activation of growth suppressor genes during liver generation. Liver cells enter mitosis (24 – 48 h post-PH), and hepatocytes proliferate 1-2 times before liver restores its original mass by 7 days. The majority of hepatocytes exit mitosis at 72 - 96 h (4 days) following PH. During this time, reactivation of growth suppressor genes reestablishes quiescent state of liver cells, leading to a cessation of liver growth.

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3.   p53, TA-p73, and FoxO3 protect quiescence of normal cells.

Many studies have shown that p53/p73-mediated growth suppression relies on transcriptional regulation of target genes that promote senescence an and anti-proliferative state. Recently, new p53-mediated functions came to light that do not involve acute stress response, but are based on a basal constitutive presence and activity of p53 in the nucleus. Recent publications describe p53-mediated regulation of metabolic pathways, organ development, and stem cell renewal (58, 152). p53 can limit the self-renewal of adult neuronal stem cells and regulate quiescence of HSC, pointing to distinct roles of p53 in resting versus cycling cells (59, 153). Studies of MEFs from p53$^{-/-}$ embryos, and primordial (quiescent) ovarian follicles of TA-p73$^{-/-}$ mice demonstrated that TA-p73 is important for the maintenance of genomic stability in normal cells, protecting them in the G_0 state (55, 154).

Several studies suggest a function for p53 in the maintenance of cellular quiescence. Stable transfection of cancer cells with a temperature-sensitive mutant of p53 retained cells in G_0-like quiescent state at the permissive temperature, when p53 has *wt* confirmation and translocates to the nucleus to bind target genes (155). p53-mediated repression of *Myc* at the permissive temperature has been directly linked to cell cycle arrest and the accumulation of cells in the G_0 state, but not to pro-apoptotic functions of p53 (155, 156). A p53 ChIP carried out at the permissive temperature, in cancer cell lines overexpressing a temperature-sensitive p53 mutant protein, showed that p53 binds to the p53RE located at -1.7 kb from the TSS of the mouse *Myc*. Binding of p53, loss of acetylated histone H4 and recruitment of co-repressor mSin3A were observed at the +48 to +236 promoter region at permissive

temperature; however, the mechanism of p53 binding at the promoter region and p53-dependent recruitment of co-repressors have not been fully characterized (156). This study suggested that mouse *Myc* expression was repressed by p53 in spleen, thymus and bone marrow upon irradiation, but the mechanism of endogenous p53 activity as a transcriptional regulator was not characterized. More recently, the gene encoding leukemia inhibitory factor LIF was identified as an endogenous p53 target in normal mouse ovaries (157). p53 interacted with p53REs in intron 1 of both mouse and human LIF genes and activated transcription of LIF *in vivo* (157). However, the mechanism of this activation has not been described. In the present study, p53-dependent recruitment of acetyltransferase p300 and acetylation of histone H3K14 and histone H4 at *Foxo3* p53RE activated expression of Foxo3 gene in quiescent liver cells.

In striking concordance with p53/p73 functions, characterization of FoxO3^{-/-} mice revealed a pivotal role of FoxO3 in maintaining the HSC pool (84) and in suppressing activation of primordial ovarian follicles before the onset of sexual maturity (85). FoxO3 may protect quiescent cells from oxidative stress by increasing expression of ROS scavenger *SOD2* (158) and by inducing a G_2/M checkpoint through activation of *GADD45* in response to oxidative stress (159). Despite similar outcomes of p53, p73, and FoxO3 activity *in vivo*, little is known about specific mechanisms underlying the functions of these transcription factors in normal quiescent cells. The results described here, demonstrate that p53 and p73 bind and activate *Foxo3* in normal quiescent hepatic cells, suggesting a new mechanism to amplify the effects of p53 and TA-p73 by establishing cascades of tumor suppressor-regulated expression of genes encoding additional tumor suppressors.

Cross-talk between p53 and FoxO3 also exists at the level of protein-protein interaction. Activation of p53 by DNA damage leads to a change in FoxO3 subcellular localization in MEFs (160). In return, FoxO3 protein regulates p53 proapoptotic activity in MEFs (161). p53, TA-p73, and FoxO3 function along the same axis in a tumor suppressor network (Diagram 4), as many FoxO3 target genes are also well-known p53/p73 targets (*p21, p27, GADD45, cyclin G*). The results of our study demonstrate that p53 and TA-p73 activate expression of Foxo3 gene in normal, quiescent liver, emphasizing an additional cross-talk between p53 and FoxO transcription factors at the level of transcription.

4.   Transcriptional activity of p53 and TA-p73 changes during liver regeneration.

Proliferation of liver cells in response to 2/3 hepatectomy is initiated by signaling cascades that further engage additional transcription factors, allowing cells to exit quiescence and enter mitosis. Pre-existing transcription factors (NFkB, Stat3, and Jun/AP-1), induced in hepatic nuclei during the first 4 hours following PH, activate early response genes necessary for G_1-S-G_2-M transitions. At the same time, pre-existing transcription factors that regulate genes in G_0, to protect quiescent state of hepatic cells, lose their transcriptional activity in favor of activators of proliferation, as illustrated by the reciprocal activity of CCAAT enhancer-binding proteins C/EBPα and C/EBPβ during liver regeneration (162). C/EBPα is expressed in hepatocytes of quiescent livers and is down-regulated through the G_1-S-G_2-M phases of liver regeneration, whereas the transcriptional activity of C/EBPβ is maintained throughout the G_1-S-G_2-M transition (162-164). Based on previous findings, it was hypothesized that transcriptional activity of p53 and TA-p73 is lost

during proliferative stage of liver regeneration. Thus, liver regeneration is impaired in mice with liver-specific knock-out (KO) of *Jun* (165). Regeneration of Jun KO livers is restored on a p53$^{-/-}$ or p21$^{-/-}$ background, suggesting that inactivation of p53-mediated transcription is critical for initiation of liver regeneration (165). In our work, the results show that loss of p53 binding to the Foxo3 gene during the first two days of liver regeneration, results in a significant decrease of *Foxo3* expression. TA-p73 binding to *Foxo3* is independent of p53 but is also dramatically decreased during the first two days of liver regeneration, concomitantly with loss of p53 binding and decrease in *Foxo3* expression. Thus, both p53 and TA-p73 lose the ability to bind and regulate expression of hepatic genes during the G_0-G_1-S transition.

Importantly, p53 and p73 binding to the *Foxo3* p53RE is restored when liver regeneration is complete, leading to the activation of FoxO3 expression as hepatocytes re-enter G_0. Similarly, p53 binding to *Afp* is restored at 7 days following PH (65). However, restoration of transcriptional activity of p53 is not required for termination of liver regeneration, as livers from p53$^{-/-}$ mice complete regeneration within the same time as their *wt* littermates (71). It is possible that TA-p73 compensates for the lack of p53 binding, to terminate proliferation of hepatocytes in p53$^{-/-}$ mice, since p73 binds the *Afp* gene and represses its expression during liver development in p53$^{-/-}$ mice (62). More studies are required to define functions of these transcription factors in termination of hepatocyte proliferation. Liver regeneration studies in p73$^{-/-}$ mice could not be performed due to developmental and inflammatory defects these mice develop at weaning age (29).

In addition to the role of p53 in G_0-G_1-S transition during liver regeneration, recent studies revealed p53 activity at the G_2-M checkpoint in regenerating livers of Aurora A

liver-specific KO mice (53). PH surgery in these mice results in premitotic arrest and impaired G_2-M transition of hepatocytes during regeneration. This phenotype is rescued by crossing Aurora A transgenic mice into a p53^{-/-} background (53). This finding suggests that p53 protein is present in regenerating liver in a transcriptionally inactive state, or that it is rapidly synthesized *de novo* in response to the activation of cell cycle checkpoints. Taken together, these results demonstrate that transcriptional activity of p53 and p73 change in normal resting versus cycling cells and point to a critical role of p53 and p73 in a mechanism that regulates cell proliferation and tissue growth in a precise temporal manner.

Previous studies, as well as our results, demonstrate that p53 and TA-p73 mediate repression of the Afp gene (68, 69), but activate expression of the Foxo3 gene in mouse liver. Several potential mechanisms can determine whether p53 and p73 act to activate or repress transcription of their target genes. A recent study by Wang and colleagues demonstrates that the WW core elements in p53RE (RRRCWWGYYY, where R is a purine, W is A or T, and Y is C or T) help to define whether p53 activates or represses transcription of a target gene (114). If the core is AT, AA or TT, the p53RE is activating, whereas CC, GG, CG, GC, TG and CA all result in repression of the target gene. The Weblogo motif of *Foxo3* p53REs shows that activating A and T core nucleotides are present in all p53REs found in Foxo3 gene (Figure 6B), suggesting that the binding of p53 to any Foxo3 p53RE activates transcription of *Foxo3*. In addition, activation sites tend to have shorter spacer lengths between half-sites (1-13 bp), compared with repressor sites (61) (Diagram 3). Seven p53REs, found in *Foxo3* gene, have spacer length ranging from 1-10 bp (Figure 6).

The position of the p53RE relative to the TSS is also important for p53-mediated transactivation, as more than 50% of known p53REs are located at the 5' enhancer regions

of a gene, and ~25% are found within the intronic regions close to TSS (166). However, ChIP experiments coupled with sequencing of immunoprecipitated DNA fragments (ChIP-Seq analysis) indicate that p53 binding sites also exist at large distances from a TSS (our unpublished observations). Interestingly, identified *Foxo3* p53REs are found not only at the 5' enhancer region, where the p53/p73 binding was confirmed by ChIP (Figure 7), but also within the intronic regions of Foxo3 gene, suggesting that the expression of *Foxo3* might be modulated by the binding of p53 to other *Foxo3* p53REs. Additional studies are needed to verify this and determine if all sites contribute equally to regulation of *Foxo3* under specific conditions.

The p53 homologues p63 and p73 also have complex interactions with p53, and can form complexes that positively or negatively affect p53's affinity to bind specific target genes. A few reports suggest that the expression of p73 promoter and splice variants affect formation of HCC (30, 167). The relative expression of p53 and p73 isoforms in liver may alter the expression of their target genes. More studies are necessary to describe the expression and transcriptional activity of p53 and p73 isoforms in normal proliferating liver cells, and in liver cancer.

The recruitment of other transcription factors to DNA sites adjacent to p53REs might alter p53/p73 binding to Foxo3. In our experiments, binding of both p53 and TA-p73 to *Foxo3* p53RE at 7 days after PH was not equivalent to the level of p53/p73 binding observed at T=0 expression (Figure 12), while Foxo3 mRNA levels at 7 days after PH were comparable with T=0 (Figure 13A), suggesting that regulatory mechanisms in addition to those mediated by p53 and TA-p73 may activate transcription of *Foxo3*. Genome-wide analysis of transcriptional binding motifs overrepresented in the vicinity of known p53REs

suggest that independent binding of other transcription factors (*e.g.*, bHLH, Klf4, Ets2) might affect p53 binding to adjacent p53REs and the final outcome of transcriptional activation or repression of the target gene (Diagram 6) (166). Interestingly, several potential binding sites for bHLH HES/HERP transcriptional repressors, as well as consensus sequence for the Notch-docking protein RBPJ were found in the vicinity of p53RE of *Foxo3* gene (not shown here). Intriguingly, Notch, RBPJ and HES have been implicated in regulation of liver regeneration (168, 169). An independent study of primary human keratinocytes showed HES/HERP binding to the upstream region of *FOXO3* gene and repression or *FOXO3* transcription after UV-triggered activation of Notch signaling (93). It has been suggested that p53 regulates expression of *Notch1* (106), and this study identified *Notch1* as a potential p73 target gene by ChIP/chip (Supplementary Table 1). It is possible therefore that Notch-mediated transcriptional repression is linked to p53/p73 transcriptional activity during proliferation or stress response.

Another transcriptional regulator shown to cooperate with p53 in promoter-specific binding is E2F/Sp1 (170) (Diagram 6). E2F factors are downstream effectors of the RB tumor suppressor that have a pivotal role in controlling cell cycle progression. A member of the E2F family, E2F1, can induce apoptosis by both p53-dependent and p53-independent pathways (171). Importantly, E2F1 was shown to activate *FOXO3* gene expression in human neuroblastoma cells, and two E2F1 binding sites were identified on the *FOXO3* promoter (92). E2F1, p53, and p73 are known to cooperate in regulation of cell cycle and apoptosis (172, 173); however, it is not known whether recruitment of E2F1 can affect p53/p73 binding to target genes. Intriguingly, E2F1, like p53 and p73, is acetylated by p300, and directed to activate pro-apoptotic genes (174, 175). Further studies are necessary to

describe a cross-talk between two major tumor suppressor pathways, mediated by RB/E2F and p53, as they converge on activation of common target genes. *Foxo3* gene provides a good model to further investigate mechanisms of cooperative recruitment of p53/p73, E2F1, and RBPJ/Hes/Herp to endogenous target genes *in vivo*.
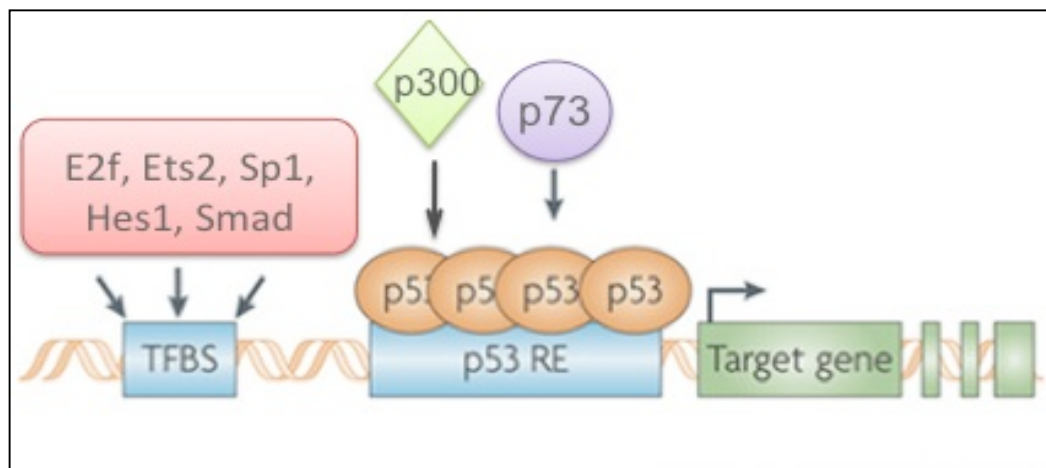
Diagram 6. Modulators of the p53-mediated transcription. Partially overlapping or closely located REs of other transcription factors can modulate p53 transcriptional activity. Other transcription factors and histone modifying enzymes can bind to p53 on p53RE and regulate p53-mediated transcription of target genes. In the case of p63 and p73, interactions may occur through shared REs; the relative affinity may dictate the expression of the associated gene. *Transcrption factors:* E2f, Ets2, Sp1, Hes1, Smad, p73; p300 acetyltransferase; TFBS, transcription factor binding site.

Modified and reproduced with permission from Nature Publishing Group (NPG): Daniel Menendez, Alberto Inga and Michael A. Resnick. The expanding universe of p53 targets. *Nature Rev Cancer* 2009.

5.   The mechanism of p53- and TA-p73 -mediated activation of *Foxo3* expression in the quiescent mouse liver.

Post-translational and gene-specific chromatin modifications can strongly influence transactivation at specific promoters (166). An increasing number of p53 cofactors influence promoter-selective p53 transcriptional activity, altering the balance between cell life and death (58). Both p53 and p73 regulate transcription of target genes via recruitment of histone-modifying enzymes that control methylation of lysine residues of histone H3, or acetylate lysine residues on histone H3 and H4 (62, 65, 72, 73, 124, 176). In present study, several histone marks associated with activation of transcription (H3K4me2, H3K14Ac, and H4Ac) were detected by ChIP analysis at *Foxo3* p53RE prior to liver regeneration. This result suggests that p53/p73-mediated activation of target genes in normal quiescent cells occurs via recruitment of histone acetylases and methyltransferases. Recent genome-wide mapping of histone acetytransferases using ChIP sequencing analysis demonstrated that transient binding of histone acetyltransferases and deacetylases, together with prior H3K4 methylation, may prime genes for activation (177). Particularly, histone acetyltransferase p300 was highly enriched in promoter and enhancer regions identified by intergenic DNase I hypersensitive sites. This is consistent with another recent report that p300 binding sites can be used to predict functional enhancers (178). A highly homologous and functionally redundant binding partner of p300 acetyltransferase, CBP demonstrated, a global distribution pattern very similar to p300 (177).

Several studies showed direct interaction of p53/p73 with p300/CBP, followed by transcriptional activation of target genes (72, 73, 179). However, p53/p73-mediated changes

in histone modifications associated with activation of endogenous genes in the context of cell proliferation have not been reported previously. While SET domain-containing methyltrasferases could be implicated in p53/p73-mediated transcription, p300/CBP is the most likely p53/p73-binding complex to be involved in activation of anti-proliferative and pro-apoptotic genes (72, 73). Results of described ChIP experiments in this work demonstrate that p53/p73-dependent recruitment of histone acetyltransferase p300 to *Foxo3* p53RE. p300 binding to *Foxo3* p53RE is decreased but not lost in livers of p53$^{-/-}$ mice. These results suggest that transcriptional activity of p73 is similar, but independent of p53, providing a compensatory mechanism that protects normal cells when p53 is lost.

As mentioned earlier, remarkable similarities of function exist between p53 and FoxO proteins, as they share the ability to promote apoptosis, induce cell cycle arrest, and support the repair of damaged DNA (180). The commonality between p53 and FoxO extends to include nuclear/cytoplasmic shuttling, interaction with 14-3-3 proteins, proteosomal degradation following ubiquitylation, and extensive posttranslational modification including multiple site-specific phosphorylations. Both proteins also interact with p300 and are acetylated, and this seems important for their activation in response to stress (181). The discovery that SIRT1 is able to complex with, deacetylate, and, hence, down-regulate both p53 and FoxO is exciting because it identifies SIRT1 as an integrator of cellular pathways activated in response to diverse types of stress (*e.g.*, nutritional starvation/FoxO, reactive oxygen species/FoxO/p53, and UV-induced DNA damage/p53) (72, 182, 183).

Acetylation levels of H3K14 and H4, as well as p300 binding and activation of *Foxo3* expression significantly decrease during first 24h of liver regeneration when both p53

and TA-p73 lose binding to *Foxo3* p53RE, providing additional evidence for p53/p73-mediated recruitment of p300 as a critical mechanism to activate expression of Foxo3 gene in quiescent liver. However, the upstream signaling events leading to formation of p53/p73 complex with p300 in quiescent vs. proliferating cells requires further investigation.

The only upstream pathway described to control expression of FoxO3 gene is prolactin (PRL)-mediated signaling, which results in a strong repression of *Foxo3* expression and severe ovarian defect (184). Interestingly, prolactin has recently been shown to accelerate liver regeneration in rats and to increase DNA binding activity of Jun/AP-1, C/EBPa, HNF and STAT-3 in liver tissue within first 5-12 hours of regeneration (185). It is tempting to speculate therefore on a possibility of an additional, prolactin-mediated, regulation of FoxO3 expression during liver regeneration, and its possible link to p53/p73-mediated signaling.

CONCLUSIONS AND FUTURE DIRECTIONS

Over the past several years, mounting evidence points to a unique function of p53 family members p53 and TA-p73 in preventing liver overgrowth and tumor development. However, the mechanism of p53/p73-mediated tumor suppression in normal liver remains unknown. Relatively low levels of p53 and TA-p73 proteins in adult hepatocytes require new approached to the analysis of these proteins. Recently developed ChIP, followed by deep sequencing of precipitated DNA (ChIP-Seq), together with the liver-specific conditional KO of p53 and p73 genes would provide better tools to answer vital questions. Which molecular mechanisms make liver a source of 'poor seed' in cancer development? Perhaps, liver-specific transcriptional targets of p53 and p73 contribute to a low tumorigenicity of hepatic cells. Amazing ability of liver cells to quickly repair damaged parts of the organ require a flexible, responsive to the upstream signaling events, on/off switch for a fast G_0-G_1 transition. In addition to its flexible upstream control, such molecular switch must be tightly regulated to prevent oncogenic transformation and abnormal proliferation of hepatocytes when damage is introduced. Previously described functions of p53 and p73 in cell cycle arrest, response to DNA damage, senescence and apoptosis make these proteins good candidates for further investigation of hepatoprotective network of transcription factors. In addition to studies of p53/p73 functions in quiescent vs. actively proliferating hepatocytes, liver regeneration model can be used to investigate role of p53 and p73-mediated transcription in inflammatory response, which occurs during first hours following PH. Inflammation processes have been previously linked to the development of HCC; however, molecular events that connect inflammatory response to transformation and

100

uncontrolled proliferation of hepatic cells remain elusive (186). The data presented in our work, as well as published results, suggest that p53/p73-medaited transcription regulates both inflammatory and proliferative response in hepatocytes (29). p73 may be compensating for p53-mediated transcription in livers of p53 KO mice, since liver regeneration in these mice is similar to the *wt* (71), and p53 is not required for binding of p73 to *Afp* (62) and *Foxo3* p53RE. Using conditional, liver specific p53 and p73 KO mice for liver regeneration experiments will allow to dissect functions of these transcription factors in regulating quiescence, inflammation, cell cycle, and tissue growth in normal liver.

Our work identified tumor suppressor and transcription factor *Foxo3* as a new target gene of p53 and TA-p73. The Foxo3 gene is actively transcribed in quiescent hepatocytes, and decreased in actively proliferating cells. The upstream signaling events that activate p53/p73-dependent transcription of Foxo3 remain unknown and should be an important area of future studies.

Seventeen TA-p73-bound genes in quiescent liver were found to be up- or down regulated at the level of mRNA in response to partial hepatectomy; however, more experiments are necessary to demonstrate the mechanism of p53 and TA-p73 transcriptional activity during liver regeneration. ChIP with antibodies detecting histone H3 and H4 modifications, implemented in regulation of gene expression, as well as ChIP with antibodies against specific protein modifications of p53 and TA-p73, can be used to identify the mechanism of p53/p73-mediated transcription. For example, loss of histone H3 dimethylation on Lys4 (H3K4me2), observed at the *Foxo3* p53RE after PH, suggests that p53/p73 may recruit histone modifying enzymes (methyltransferases) to activate expression of hepatic target genes in quiescent liver. Very little is known about *in vivo* interaction of

p53 family members with histone methyltransferases in transcriptional regulation of tissue-specific target genes. Methylation of p53 by Set7/9 (KMT7) methyltransferase is required for the binding of the acetyltransferase Tip60 to p53 and for the subsequent acetylation of p53 (187). Importantly, methylation of Lys369 (Lys372 in humans) of p53 by Set7/9 is required for the transcriptional activation of p53 target genes (188). Set7/9 also methylates Lys4 of histone H3 (H3K4) (189, 190); however, H3K4 is methylated by at least seven other methyltransferases (Set1, Ash1, MLL 1-5), none of which is known to bind p53/p73. No similar studies were performed in liver, to identify histone H3 methyltransferases as binding partners of p53 in regulation of gene expression. Only one study identified p53- dependent recruitment of H3K4 demethylase LSD1 to repress transcription of p53/p73 target gene *Afp* in liver (69). Sequential ChIP and co-immunoprecipitation experiments are necessary to identify other transcriptional partners of p53 and TA-p73.

ChIP analysis of p53/p73-bound chromatin at different time points following PH is necessary to identify other target genes, whose change in expression may be affected by the loss of p53 and TA-p73 binding to DNA in proliferating hepatocytes. These genes may provide important clues for p53/p73 functions in normal cells during different stages of cell cycle, particularly, at the point of mitotic exit and the maintenance of the quiescent G_0 state.

Binding of p53 and TA-p73 to *Foxo3* was detected at the end of regeneration (7 days post-PH), however not to the level observed in quiescent liver. At 4-7 days following PH, expression of *Foxo3* is completely restored to the T=0 levels, thus suggesting that binding of other transcriptional activators, or loss of a repressor, activates transcription of this growth suppressor gene during termination of liver regeneration. Search for DNA-binding sites of other transcription factors near *Foxo3* p53RE, followed by the ChIP analysis of *wt* vs. p53^{-/-}

102

mouse liver, may identify other transcription factors that regulate expression of *Foxo3* in p53-dependent manner in hepatocytes during termination of proliferation. These findings may provide us with the mechanism of cell cycle termination that is dysfunctional or absent in cancer cells.

Our results also demonstrate that p53 and TA-p73 bind other novel target genes in quiescent liver (*Jak1, Pea15,* and *Tuba1*), which may function during liver regeneration. Global expression analysis of genes that change expression during liver regeneration, coupled with the identification of p53 and TA-p73 bound genes in quiescent liver, suggests unique functions for p53 and TA-p73 in quiescent liver that are not executed during regeneration. Quiescence in other cell types, *e.g.*, hematopoietic stem cells and ovarian follicles, is supported by p53 and TA-p73, respectively. However, more research using tissue-specific conditional p53 and TA-p73 KO mice is necessary to characterize functions of these tumor suppressors in normal tissues *in vivo*.

Several regulated genes, not previously reported in the context of liver regeneration, included pluripotency regulator *Klf4*, transcription factors *Mxi1* and *Sin3a*, as well as anti-apoptotic Bcl2 family member *Bcl2l1*, Cbp/p300-interacting transactivator *Cited2*, and protein phosphatase 2 regulatory subunit B56α *Ppp2r5a*. Forty-nine genes that change expression during 24 - 48 hours following PH are known p53 target genes (Figure 3); however, mechanisms of p53-mediated transcriptional regulation of these genes during liver regeneration remain to be determined.

Post-translational modifications (PTMs) of p53 and p73 are among the mechanisms that can alter DNA binding and the recruitment of co-factors by these transcription factors. A recent study demonstrated that fully acetylated endogenous p53 has significantly higher

binding to endogenous *Cdkn1a (p21)* chromatin, and that p53 acetylation levels correlate with p53-mediated transcriptional activation *in vivo* (125). Overall, post-translational modifications controlling tissue-specific transcriptional activity of p53 and p73 in normal cells *in vivo* remain unidentified. Since we showed that both p53 and TA-p73 recruit acetyltransferase p300 in quiescent (G_0) but not proliferating hepatocytes, it would be interesting to determine whether DNA-binding by p53 and TA-p73 to target genes in G_0 normal hepatocytes is controlled by p300-mediated acetylation. This modification may be lost during liver regeneration, preventing p53 from activating G_1-S checkpoint and allowing cell cycle entry. Other PTMs altering p53 ability to bind DNA include methylation, phosphorylation, sumoylation, and ubiquitination (53, 187, 191-193). It would be important therefore to determine post-translational modifications of p53 and TA-p73 in hepatocytes at different stages of the cell cycle during liver regeneration. Mass-spectrometry analysis and antibodies specifically recognizing modified p53 and TA-p73 can be used to determine PTMs of p53 and TA-p73 in such studies. PTM-specific antibodies can also be used for ChIP analysis to identify modifications of p53 and TA-p73, required for their binding to anti-proliferative target genes, *e.g., Foxo3* (Diagram 7).

Finally, tumor profiles of p53[+/-]p73^{+/-} compound mice indicate that p73 has functions in liver both shared with p53 and unique to p73 (32). Consistent with this, p73 has been shown to regulate a set of target genes independently of p53 (64). However, it remains unknown whether p73 always depends on p53REs to bind DNA, or if p73 has a specific DNA consensus site. Results of p73 ChIP/chip or ChIP/Seq analysis coupled with bioinformatics tools described here (MDScan, WebLogo) can be used to identify potential, new response elements in genes regulated by p73.
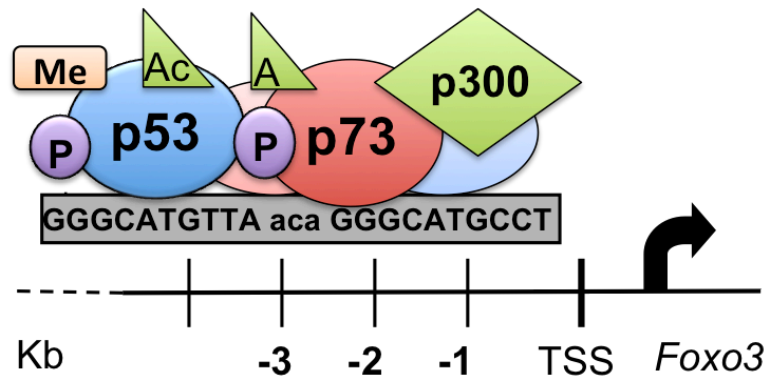
Diagram 7. Post-translational modifications regulate transcriptional activity of p53 and p73. p53 and p73 recruit acetyltransferase p300 and activate transcription of target gene Foxo3. Acetylation, methylation, and phosphorylation of p53/p73 heterotetramer may regulate its DNA binding and recruitment of cofactors to the promoter region of target genes.

APPENDIX

NOTES ON SURGICAL PROCEDURES

The procedure used to remove ~2/3 (65%) of liver in a mouse model of partial hepatectomy (PH) was performed using standard protocol, described in detail by Andreas Nagy and colleagues in CSH Protocols 2006; doi:10.1101/pdb.prot4384 http://cshprotocols.cshlp.org/cgi/content/full/2006/18/pdb.prot4384. To remove 2/3 of liver tissue, incision is made in skin and in the abdominal wall (sham surgery stops at this point). After separate ligatures are applied at the base of the left lateral and median lobes to constrict the blood vessels and reduce bleeding, the left lateral and median lobes are resected (Appendix Figure 2). Various models, different techniques and limitations of hepatic resections in rodents is described in a review by Martins and colleagues in (39).

More details on the procedure of 2/3 PH in mice with on-line supplementary video was published by Claudia Mitchell and Holger Willenbring in (194). In addition to a very comprehensive protocol, this publication provides kinetics of BrdU incorporation after 2/3 PH in adult *wt* C57Bl6 mice. Importantly, it also compares the extent of liver injury after 2/3 PH in animals anesthetized with 3 different types of anesthetics: avertin, ketamine/xylazine, and isoflurane. The authors measured serum alanine aminotransferase (ALT) levels in mice after PH and found that increase in ALT levels in blood serum at 16-42 hours post-PH is minimal when isoflurane anesthesia is used during the surgery (194). Release of ALT to blood increases when liver is damaged or diseased. However, an increase in ALT does not indicate a non-specific inflammatory response, which may be observed in shams after the invasive surgery (an incision made in skin and the abdomen over the same area as done for PH). In our work, we observed a change in expression of tested genes in response to the sham surgery, performed using avertin (data not shown) and ketamine/xylazine, compared to

the T=0 (quiescent liver harvested without prior surgical procedures). Appendix Figure 2 shows a significant change in Foxo3 mRNA levels in response to sham surgery at 24 hours and 7 days after the procedure, indicating adverse effects of ketamine/xylazine on normal, non-resected liver. No significant change in Foxo3 expression was observed in response to sham surgery using isoflurane anesthesia (Appendix Figure 3).

Avertin, however commonly used for 2/3 PH, is highly hepatotoxic and is associated with higher postoperative morbidity, an outcome that is not usually seen when other types of anesthetics are used (194). Ketamine/xylazine is less problematic than avertin but still it has more side effects (in addition to a change in gene expression, we observed longer awakening times and higher mortality rates, compared to isoflurane). Isoflurane anesthesia is thus the least toxic among the commonly used anesthetics. Isoflurane is an inhalant anesthetic that is widely used in veterinary medicine because of its safety and rapid recovery of the animal after surgery (194). However, its use requires a vaporizer, connected to the oxygen supply.
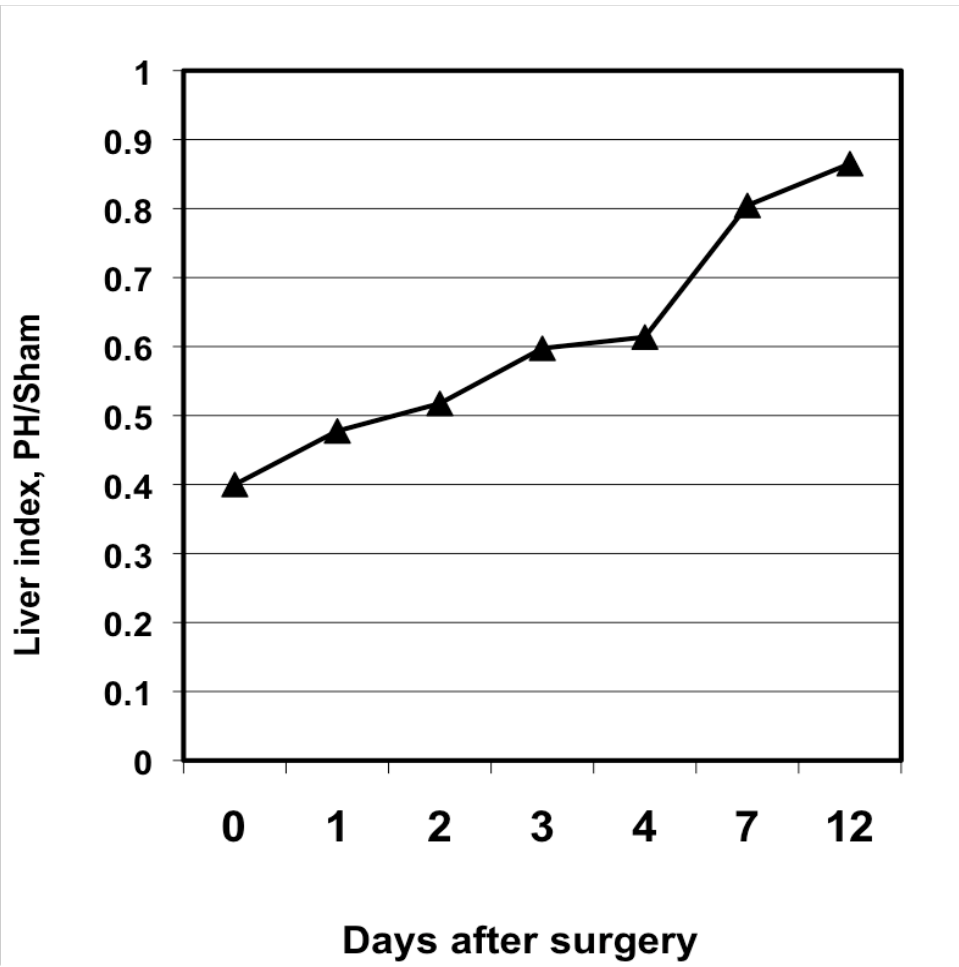
Appendix Figure 1. Liver regeneration in C57Bl6/129 WT mice 2 months of age. Liver mass recovery was measured as a ratio of liver/body weight (liver index) in mice after PH and Sham surgeries at indicated time points.

Appendix Figure 2. 2/3 partial hepatectomy model of mouse liver regeneration. Photographs of WT C57Bl6/129 2-month-old mouse liver before PH surgery (T=0) and after 2/3 of liver resection. The left lateral and median lobes are resected and shown separately (the median lobe is ligated and resected in two parts to avoid damage to the superior vena cava and the gall bladder). Fully regenerated liver collected at 7 days post-PH is shown in the lowest panel.

Photographs courtesy of Sabrina Stratton.
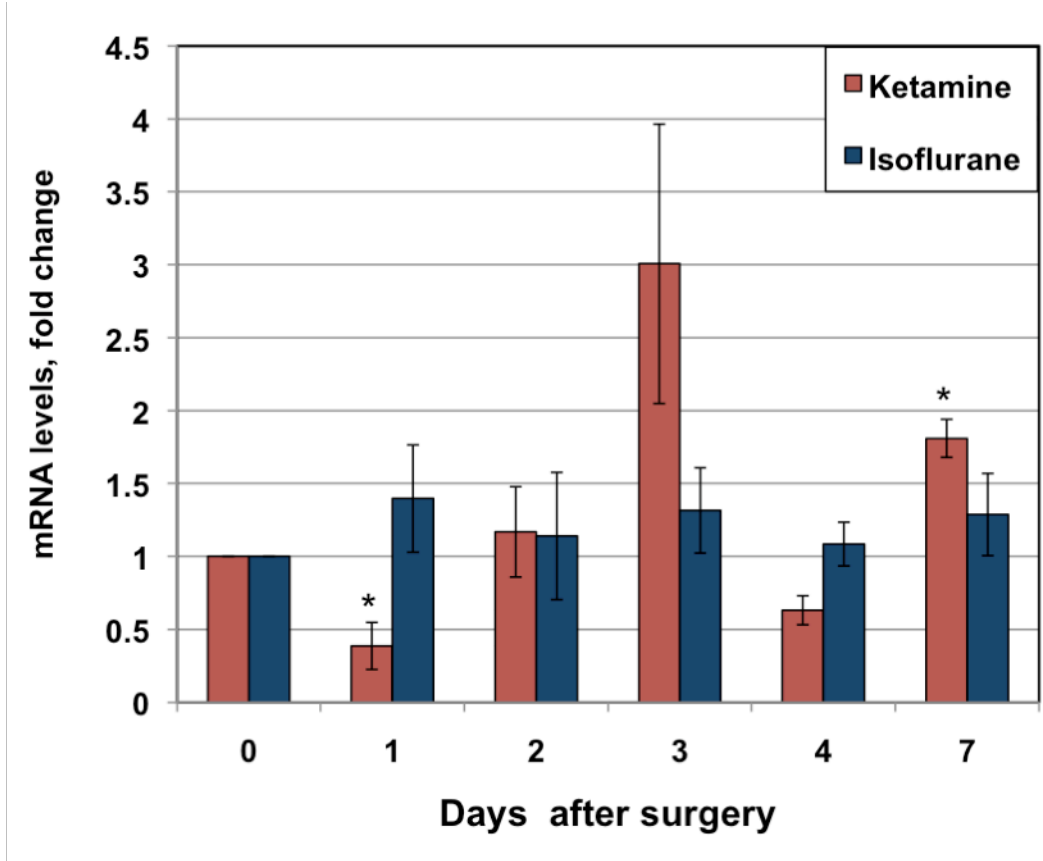
Appendix Figure 3. Expression of Foxo3 changes in response to Sham surgery under ketamine anesthesia. Foxo3 mRNA was isolated from mouse liver tissue at indicated time points following the Sham surgery under ketamine or isoflurane anasthesia. Relative expression levels were measured by real-time PCR. Foxo3 expression was significantly changed in livers harvested after ketamine Sham surgeries (*$p<0.05$). No statistically significant difference in Foxo3 expression was observed in livers from isoflurane-operated Sham mice.

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VITA

Svitlana Mikhailivna Kurinna was born in Kyiv, Ukraine on July 16, 1979, the Daughter of Natalia Mykolaivna Kurinna and Mikhailo Evgenovych Kurinnyy. After completing her work at the Technical High School, Kyiv, Ukraine in 1996, she entered Taras Shevchenko University in Kyiv. She received the degree of Bachelor of Biology in 2000, and the degree of Master of Science from Taras Shevchenko University in 2001. For the next three years, she worked as a research assistant, first at the National Academy of Sciences in Kyiv, Ukraine, and later at the UT Health Science Center in Houston, Texas. In August of 2004 she entered The University of Texas M. D. Anderson Cancer Center Graduate School of Biomedical Sciences.