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p53 MAINTAINS HEPATIC CELL IDENTITY DURING LIVER REGENERATION

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A

DISSERTATION

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by

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Houston, Texas

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DEDICATION

To my parents and my beloved husband, who love me and support me endlessly

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Above all, I would like to express my special gratitude to the Creator, for giving me this wonderful opportunity to grow as a scientist. As I observe and contemplate in science, I have a better perception of how intricate the processes of nature are and how human body is by far the most complex system in the universe.

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ABSTRACT

p53 MAINTAINS HEPATIC CELL IDENTITY DURING LIVER REGENERATION

Zeynep Hande Coban Akdemir, B.S.,M.A.

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p53 is a tumor suppressor that has been well studied in tumor-derived, cultured cells. However, its functions in normal proliferating cells and tissues are generally overlooked. We propose that p53 functions during the G1-S transition can be studied in normal, differentiated cells during surgery-induced liver regeneration. Two-thirds partial hepatectomy (PH) of mouse liver offers a unique model to compare p53 functions in regenerating versus sham (control) cells. My **hypothesis** is that intersection of global expression analyses (microarray and RNA sequencing) and profiling of p53 interactions with chromatin (ChIP sequencing) at the G1-S transition of normal cell cycle, corresponding to 24h post-PH in mice liver regeneration, will reveal p53 functions during cell cycle regulation in normal cells and during tissue regeneration.

Combining chromatin immunoprecipitation with next generation sequencing technology (ChIP-Seq) allowed detection of genome-wide binding of p53 to target genes in liver. We found 5074 *de novo* p53 target genes, 92% of which participate in non-canonical p53 functions, mainly developmental processes. Integration of ChIP-Seq findings with global expression profiling (RNA-Seq) of both normal and p53-null liver allowed us to identify functional p53 target genes. Intriguingly, our data analysis revealed that a specific subset of p53-activated target genes is involved in liver-enriched functions such as lipid biosynthetic process, steroid metabolic process, circadian rhythm, and drug detoxification. These findings suggested that the loss of p53-chromatin interactions in regenerating liver may result in a decreased activity of differentiation-specific cellular processes and in attenuation of hepatic cell identity. Remarkably, p53 cooperates with the master regulator of hepatocyte differentiation, HNF4α, to induce 78% of these genes, including a number of liver-enriched transcription factors such as CCAAT/enhancer binding protein beta (CEBPβ), hepatocyte nuclear factor 6 alpha (HNF6α), hepatocyte nuclear factor 6 beta (HNF6β). Thus, p53 acts in concert with HNF4α to promote the maintenance of liver functions during the G1→S transition of the cell cycle of normal proliferating livers cells.

TABLE OF CONTENTS

APPROVAL SHEET i

TITLE PAGE ii

DEDICATION iii

ACKNOWLEDGMENTS iv

ABSTRACT vii

TABLE OF CONTENTS ix

TABLE OF ILLUSTRATIONS xiii

LIST OF TABLES xv

CHAPTER 1 INTRODUCTION 1

1.1 Liver regeneration 1

1.1.1 Partial hepatectomy-induced liver regeneration 1

1.1.2 Gene expression alterations during PH-induced liver regeneration 4

1.2 p53: a new player in tissue regeneration 7

1.2.1 p53 as a tumor suppressor 7

1.2.2 The role of p53 in liver 9

1.2.3 p53 regulation during tissue regeneration 10

1.2.4 The role of p53 in lipid metabolism 11

1.3 Liver-enriched transcription factors 14

RATIONALE 15

DESCRIPTION OF THE OBJECTIVES 16

CHAPTER 2 MATERIAL AND METHODS 17

2.1 Partial hepatectomy 17

2.2 Chromatin immunoprecipitation (ChIP) 17

2.3 RNA analysis 18

2.4 ChIP-Seq analysis 18

2.5 Generation of ChIP-Seq signal density files for browser data visualization 19

2.6 Heatmap generation for ChIP-Seq signal 20

2.7 Distance plot calculations 20

2.8 Average gene profile calculations 21

2.9 Conservation plot calculation 21

2.10 Motif analysis 21

2.11 Gene ontology analysis 22

2.12 RNA-Seq analysis. 22

2.13 Principal component analysis 23

2.14 Binomial test 23

CHAPTER 3 RESULTS 24

3.1 p53 binds to a significant number of genomic sites in regenerating liver 24

3.1.2 p53 binds novel genes in regenerating liver 30

3.1.3 A novel role of p53 in regulating lipogenesis 39

3.1.4 p53 and HNF4α shares a significant portion of their target genes 50

3.1.5 p53 binds to tissue-specific enhancers in regenerating liver 54

3.1.6 p53 is required for HNF4α-mediated maintenance of hepatic cell identity during liver regeneration 57

CHAPTER 4 DISCUSSION AND FUTURE DIRECTIONS 61

4.1 DISCUSSION 61

4.1.2 FUTURE DIRECTIONS 67

REFERENCES 72

VITA **96**

TABLE OF ILLUSTRATIONS

Figure 1. Liver architecture....4
Figure 2. p53 enhances lipid catabolism and inhibits lipid anabolism....13
Figure 3. HNF4α is the master regulator of hepatic-linage specification in differentiation of human pluripotent stem cells to hepatocyte-like cells. Reprinted by permission from Company of Biologists: Development, copyright (2011) (25). 15
Figure 4. p53 gains a significant number of binding sites in regenerating liver....26
Figure 5. p53 exhibits binding at 13,662 sites in regenerating liver 27
Figure 6. The centers of p53-bound peaks are well-conserved 28
Figure 7. The centers of p53-bound peaks are significantly enriched with p53 consensus motif 29
Figure 8. 38% of p53 binding sites are located within 10 kb of TSS 33
Figure 9. p53 binding is mostly enriched within 1 kb of TSS....34
Figure 10. p53 binding signal peaks at TSS of genes....35
Figure 11. Individual examples of p53 binding at TSS of genes 36
Figure 12. A significant number of non-canonical p53-bound genes 37
Figure 13. Non-canonical p53-bound genes has a significant overlap with p53-bound genes in mESCs 38
Figure 14. Principal component analysis of RNA-Seq samples....42
Figure 15. Hierarchical clustering of p53-responsive genes 43
Figure 16. p53-responsive genes are involved in liver-specific functions and regulation of immune response....44
Figure 17. p53-activated genes are enriched in oxidation reduction, steroid metabolism, and fatty acid metabolic process....45
Figure 18. p53-dependent lipid accumulation at the G1-S transition of the cell cycle in regenerating liver 46
Figure 19. p53 direct target genes are involved in liver-specific functions and regulation of immune response....47
Figure 20. There is no major difference between spacer lengths of p53 response elements at activator and repressor sites 48
Figure 21. There is no major difference between p53 DNA-binding motifs at activator and repressor sites....49
Figure 22. p53-bound peak centers are enriched with HNF4α DNA-binding motif 51
Figure 23. p53 and HNF4α shares a significant portion of their target genes....52
Figure 24. A significant portion of p53 direct target genes coincide with HNF4α target genes 53
Figure 25. p53 binds to tissue-specific enhancers in regenerating liver 55

Figure 26. An individual example of p53 and HNF4α co-bound enhancer regions 56
Figure 27. p53 promotes HNF4α binding to the *Plg* proximal promoter during liver regeneration 58
Figure 28. p53 promotes HNF4α binding both at the *Fga* proximal promoter and the distal *Fga* enhancer 59
Figure 29. p53 regulation of HNF4α binding during liver regeneration 60
Figure 30. Tissue-specific p53 deletion induces liver carcinoma 70

LIST OF TABLES

Table 1: HNF4α ChIP primer sites 71

CHAPTER 1 INTRODUCTION

1.1 Liver regeneration

1.1.1 Partial hepatectomy-induced liver regeneration

The liver is an important organ in the body that performs many essential functions such as carbohydrate metabolism, lipid and protein synthesis, bile acid production, and biodegradation of harmful compounds. The liver is a heterogeneous tissue, consisting of various cell types, including hepatocytes, hepatic sinusoidal endothelial cells, blood-resident macrophages (Kuppfer cells) and hepatic stellate cells (Fig.1) (119). As an essential compartment for detoxification, the liver is exposed to hepatic injuries induced by toxic substances, infectious agents and immune disorders. However, the liver has remarkable regenerative ability to respond to injury. Liver regeneration after partial hepatectomy (PH) offers a unique experimental model to study liver response to injury (51). In this model, two-thirds of the liver is surgically removed, and then the remaining liver proliferates until the liver index (the ratio of liver weight to body weight) is fully restored. In the normal adult liver, merely 0.005% of hepatocytes are in cell cycle and the rest of them are usually in a quiescent G0 cell cycle state (56, 82). After PH, the hepatocytes, which form approximately 80% of liver

cells, take a leading role in the proliferative process by re-entering the cell cycle in a synchronized wave for 1-2 rounds of mitosis. 90% of the remaining hepatocytes engage in the first round, whereas only a third undergo the second round of cell cycle (35, 88). During this proliferation period, hepatocytes secrete growth factors in a paracrine manner to trigger the non-parenchymal liver cells to proliferate in the following sequential order: biliary ductular cells, Kuppfer cells (hepatic macrophages) and hepatic stellate cells, and sinusoidal endothelial cells to restore the liver index within approximately 1 week (43, 87, 89). The liver regeneration terminates at a certain ratio when newly proliferating cells are sufficient to meet the metabolic demands of the body. In this light, the regenerative response after partial hepatectomy is governed mainly by hepatocytes.

Misregulation of the hepatic regenerative process has been shown in several mouse models. Upon severe liver damage in transgenic mice overexpressing the serine protease urokinase plasminogen activator (uPA), transplanted hepatocytes require approximately 12-18 rounds of cell cycle to reestablish the liver mass (105). In addition, the knockout mice deficient for the tyrosine catabolic enzyme fumarylacetoacetate hydrolase (FAH) exhibit liver failure, as the liver is no longer able to regenerate. The transplantation of hepatocytes into Fah-null liver prompts a successful restoration of liver cell population (92).

In contrast to hepatocytes dividing to restore the liver index, oval cells, sometimes called liver precursor "stem" cells, are the main source of new hepatocytes in the liver regeneration process after toxic injury. In these cases, an oval cell compartment, consisting of various kinds of liver precursor cell populations that have the ability to differentiate into both hepatocytes and biliary ductular cells, is activated and differentiates into hepatocytes that then regenerate the liver (30). In a specific subset of animal models recapitulating toxic injury-induced liver regeneration in humans, hepatocytes are unable to proliferate after severe damage and undergo necrosis in response to treatment by chemical compounds such as D-Galactosamine (GalN) and 2-acetylaminofluorene (2-AAF) treatment (21, 34, 119). The transformation process of hepatocytes from oval cells is still controversial and may be associated with transformation of liver cells (31, 33).

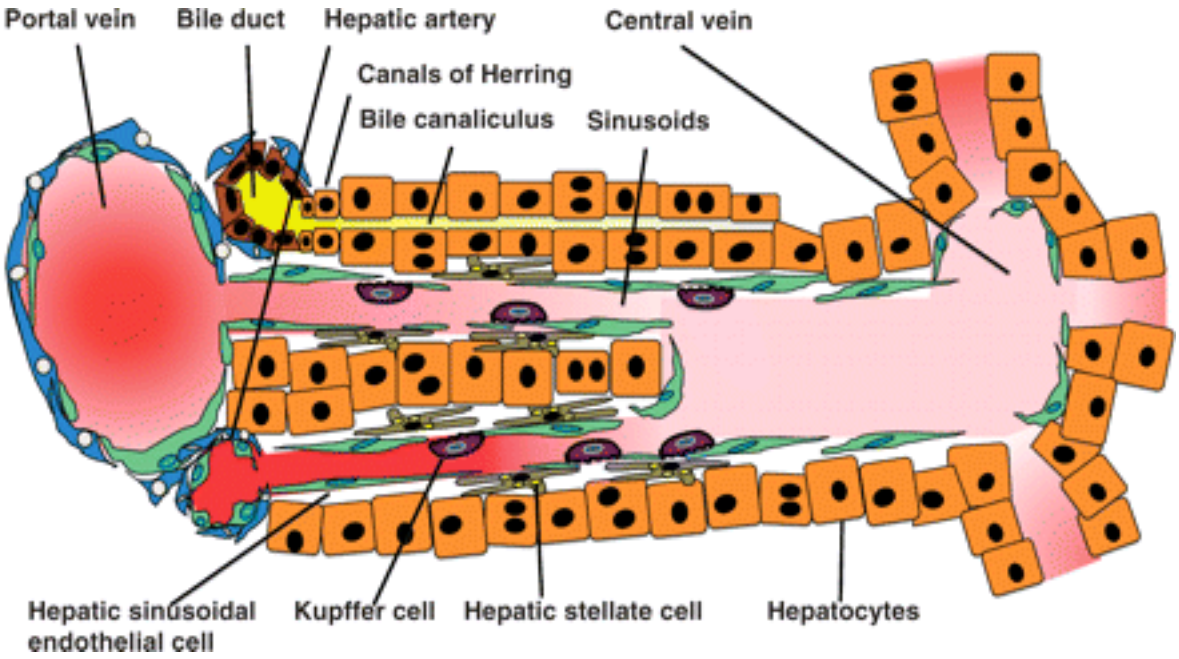

Figure 1. Liver architecture. Reprinted by permission from Japanese Biochemical Society: The Journal of Biochemistry, copyright (2011) (119).

The liver mainly consists of hepatocytes, which are highly polarized epithelial cells, stacked next to each other with sinusoid capillaries. Hepatocytes secrete bile acids to bile canalicus that is linked to bile duct via canals of Herring. The other major cell types in the liver include hepatic sinusoidal endothelial cells, blood-resident macrophages (Kuppfer cells), and hepatic stellate cells.

1.1.2 Gene expression alterations during PH-induced liver regeneration

Profiling of gene expression alterations during PH-induced liver regeneration sheds light on the molecular mechanisms regulating this process. Rodent liver regeneration consists of mainly two phases: the priming phase and the responsive phase (the phase of cell progression) (30). In the priming phase, which lasts approximately 4 hours after partial hepatectomy in mice, post-mitotic hepatocytes and

non-parenchymal liver cells exit from their G0 state and re-enter into the cell cycle (G0-G1 transition). Although hepatocytes are stimulated by various mitogens to proliferate in culture, several studies suggested that hepatocytes must initially undergo the priming phase in order to respond to growth factors during liver regeneration (133, 134). The priming phase in liver regeneration after PH exhibits rapid gene expression alterations in a coordinated fashion. This phase is initiated by the immediate activation of transcription factors, nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), signal transducer and activator 3 (STAT3), the activator protein 1 (AP1), and CCAAT/enhancer binding protein beta (CEBPβ) (18, 36, 42). Their binding on DNA leads to transactivation of a wide variety of genes (immediate early genes). Examples include proto-oncogenes encoding c-Fos and c-Jun (45, 122). Global gene expression profiling by high-density oligonucleotide arrays in the priming phase revealed that 185 genes have altered expression levels including other transcription factors such as the gene encoding early growth response protein 1 (EGR1) (5, 115). Mice with deletion of immediate-early genes such as *Egr1* demonstrated impaired liver regeneration due to deranged cellular proliferation (75).

Cytokines secreted by Kuppfer cells act upstream of transcription factors NFκB, STAT3, AP-1 and C/EBPβ. Binding of Interleukin 6 (IL6) secreted by Kuppfer cells to its receptor, Interleukin 6 receptor (IL6R), on hepatocytes leads to rapid activation of

both STAT3 and mitogen-activated protein kinase (MAPK) signaling (121). Mice deficient for IL6 exhibited a delay in liver regeneration, accompanied with a loss of STAT3 activation and impaired mitogenesis (19). Tumor necrosis factor alpha (TNFα), a cytokine secreted from Kuppfer cells, binds the receptors tumor necrosis factor receptor 1 and 2 (TNFR1, TNFR2) on hepatocytes to induce their replication (121). It was also hypothesized that TNFα is an upstream regulator of IL6 with the finding that TNFR1 knockout mice restored liver regeneration deficit by the induction of IL6 (121, 139).

Later, during the responsive phase of liver regeneration, cell cycle genes are activated. Cell cycle genes promote hepatocytes to undergo the G1-S transition in cell cycle. Included in genes participating in cell cycle regulation, cell cycle stimulators, cyclins and cyclin dependent kinases (CDKs) have elevated expression levels. Cyclin D1/CDK4 and Cyclin E/CDK2 complexes phosphorylate the retinoblastoma protein (Rb), thereby diminishing its inhibitory effect on E2F proteins. This allows E2F proteins to induce their target genes involved in the promotion of S phase (98).

In addition to cell cycle stimulators, cell cycle inhibitors such as p53 and p21 are induced during liver regeneration (32, 66). This is extremely surprising, based on the prevailing knowledge, since it is known that their activity must be attenuated in order to allow cells to exit from cell cycle arrest and proceed to cell proliferation. However, their

induction during cell cycle progression of normal proliferating cells as safeguards may enable liver regeneration to be a tightly regulated cell cycle process. For instance, p21 gene expression levels are quite low in quiescent liver and become markedly augmented, due to mRNA stability, during G1 and S phases of cell cycle after PH in mice and rat (2). The use of genetic manipulations revealed that p21-null mice exhibited accelerated entry into S phase by an earlier induction of cyclin D1 compared to wild type (WT) mice (3). On the other hand, p21 overexpression prevents hepatocyte replication (137). Taken together, these noted studies all suggest that p21 must be maintained at a certain level, thus allowing liver regeneration to proceed in a timely and coordinated fashion.

1.2 p53: a new player in tissue regeneration

1.2.1 p53 as a tumor suppressor

The tumor suppressor protein p53, with mutations in 50% of all human cancers, has well-established important roles as a transcription factor (7, 27, 52, 91). The DNA-binding domain of p53, which is a site of frequent mutations in tumors, is pivotal for p53 tumor suppressor activities. Subsequent studies identified a significant number of p53-responsive genes that respond to various forms of cellular stress, including DNA

damage, oncogene activation and hypoxia, as determined by luciferase reporter assays, chromatin immunoprecipitation (ChIP) and quantitative PCR (99). These genes were classified into major subgroups according to their biological function in the cells, such as regulation of cell cycle control, DNA-replication and repair, cell proliferation, apoptosis, and angiogenesis inhibition (125).

However, identification of a more extensive p53-binding repertoire is essential to obtain a more complete picture of its transcriptional function in normal cells. At this point, high-throughput technologies such as ChIP-on-chip (ChIP-chip), which combines ChIP with microarray technology, ChIP followed by next generation sequencing (ChIP-Seq), and ChIP followed by paired-end tag sequencing (ChIP-PET) and overlap with microarrays and RNA-Sequencing (RNA-Seq) have been performed. These studies offer a genome-wide profile of p53 interactions with chromatin, as well as p53-mediated changes in gene expression levels, in cancer cell lines (11, 86, 90, 113, 135). The substantial work conducted to elucidate the functions of p53 mostly has been completed with cultured cancer cell lines. Although there is compelling evidence that p53 has other roles beyond tumor suppression in normal cells, e.g. its involvement in development, aging, and senescence (128), its functions in normal proliferating cells and tissues remain relatively unknown.

1.2.2 The role of p53 in liver

The loss of p53 function is associated with 35% of hepatocellular carcinoma (HCC) cases. Dysfunction of p53 may be caused by mutation of the p53 gene or its repression by hypermethylation of the promoter region (61, 83). HCC samples lacking p53 activity are likely less differentiated, more proliferative, progressive, and more invasive (95, 116). Sugo et al. reported that p53 mutation in primary lesions are associated with a shorter post-recurrence survival in HCC patients (P-value < 0.01) (116). Although there is a highly established correlation between p53 status and clinical outcome of HCC, less is known about tumor-suppressive roles of p53 in liver. Using mice to examine p53 functions in liver cancer offers some promising results, for example, 15% of double heterozygous p53/p73 mice develop HCC at 5-7 months of age (37). Recently, Xue et al. observed that WT p53 restoration caused tumor regression in murine liver carcinomas (138). As a whole, these studies suggest a protective function of p53 in liver. However, there are only a few studies investigating the function of p53 in normal proliferating liver cells after PH, including our previous studies (67, 68). To understand the roles of p53 better in the liver and loss of its protective function in liver diseases including HCC, we need to acquire a global view of p53 regulation in normal and proliferating liver.

1.2.3 p53 regulation during tissue regeneration

Whether p53 acts as a tumor suppressor to suppress tissue regeneration or if it is essential for compensatory regeneration remains unknown (93). Recently, several studies underscore the significance of p53 regulation during tissue regeneration. It was elucidated that *dp53* is crucial for the regenerative response in Drosophila imaginal discs (136). Another study demonstrates that p53 expression is maintained at different levels at different phases of salamander limb regeneration. It was shown that p53 is expressed at relatively low levels when differentiated muscle cells dedifferentiate and proliferate during the first phase of muscle regeneration. In contrast, during the second phase, it was induced to promote differentiation of dedifferentiated muscle cells (141). This study suggests that the maintenance of differentiated cell identity by p53 has a significantly important role in tissue regeneration. My studies address whether this is the case in liver regeneration. In liver regeneration, after two-thirds PH, hepatocytes are the main cell type that proliferates to restore the liver mass when the environment is conducive for their proliferation (31). While undergoing proliferation, there is an enormously increased metabolic load imposed on hepatocytes in the remnant liver. As the liver still needs to perform normal liver functions, hepatocytes likely need to preserve a differentiated cell identity. *In vivo* fate-tracing studies by Malato et al. (2011) confirm that hepatocytes do not display a loss of their hepatic cell identity after acute

injury during liver regeneration (81). However, there is still a gap of knowledge in the p53 field in terms of any role for p53 during cell cycle control of normal proliferating liver cells.

1.2.4 The role of p53 in lipid metabolism

Mounting evidence suggests that p53 mediates a vast array of functions in cellular metabolism interlinked with its tumor suppressor activity. Numerous studies emphasize the significance of p53 and its family members in the regulation of a specific subset of genes involved in glycolysis, oxidative phosphorylation and amino acid metabolism. Associated with its tumor suppressor function, p53-mediated alterations in cellular metabolic pathways are utilized as a means to counteract the metabolic changes in cancer cells (9). One of the main metabolic alterations in cancer cells, as a results of an increased demand for free energy and biosynthesis, is the elevated rate of glycolysis, a phenomenon called as “the Warburg effect” (132). The intermediary substances produced by glycolysis are utilized for nucleotide and lipid synthesis to satisfy the increased biosynthetic demands of cancer cells (24, 126). In this context, the prevention of lipid anabolism and the augmentation of lipid catabolism by p53 are used as a means to inhibit cancer cell proliferation illustrated in Fig.2 (41).

However, the regulation of lipid metabolism by p53 is not only associated with its tumor suppressor activity. In addition, it serves as another means to promote the maintenance of metabolic homeostasis in normal cells (41). In response to glucose deprivation, AMP-activated protein kinase (AMPK) and p53 act in a feedback loop. The induction of AMPK leads to serine 15 phosphorylation on p53. Thus, the stabilized and activated p53 push the cells into p53-dependent cell cycle arrest at G1-S transition of cell cycle (58). Furthermore, the activation of p53 in human embryonic kidney cell line HEK293 induces its downstream target genes Sestrin1 and Sestrin2 in order to promote elevated AMPK levels (13). Overall, this feedback loop results in a reduction in lipid biosynthesis through AMPK-mediated inhibition of two key enzymes, acetyl-CoA carboxylase 1 (ACC1) and fatty acid synthase (FASN), responsible for fatty acid synthesis. In addition, the AMPK-p53 feedback loop enhances lipid catabolism by means of p53 direct and indirect target genes in rat liver (23, 38, 46). The p53-mediated induction of guanidinoacetate N-methyltransferase (GAMT) and lipin 1 (LPIN1) both lead to an increased rate of lipid catabolism in human cell lines upon glucose starvation (6, 55). A specific subset of mitochondrial enzymes, carnitine palmitoyltransferase 1C (CPT1C) and 1 (CPT1) were identified as p53 direct and indirect target genes, in brain cells and hematopoietic cells, respectively (25, 104). The induction of CPT1C and CTP1 in the p53-AMPK pathway facilitates fatty acid import

into mitochondria, and thereby increases fatty acid oxidation. Taken together, these studies suggest that p53 promotes fatty acid oxidation as a metabolic switch in normal cells under glucose-deprived conditions (59, 94).

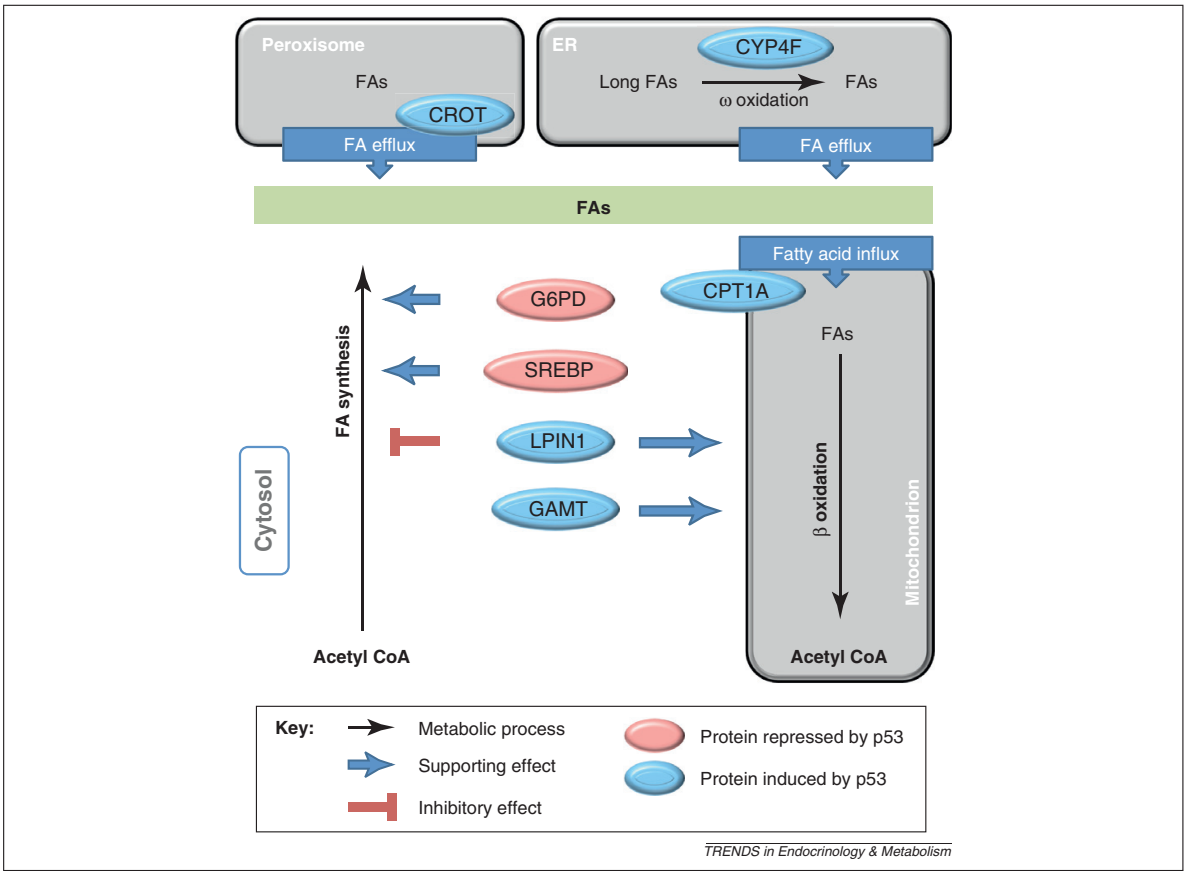

Figure 2. p53 enhances lipid catabolism and inhibits lipid anabolism. Reprinted by permission from Elsevier: Trends in Endocrinology & Metabolism, copyright (2012) (41).

p53 induces the expression of proteins involved in fatty acid (FA) metabolism and FA transport into mitochondria to be broken down through β-oxidation. In addition, among p53-activated genes, there are genes encoding proteins that play roles in mitochondrial β-oxidation while p53 represses the expression of proteins that support FA synthesis.

1.3 Liver-enriched transcription factors

In organ development and in progenitor cells, a specific group of transcription factors must be regulated in a timely fashion to activate liver-specific genes. As one example, HNF4α is a member of the steroid hormone nuclear superfamily and a hepatocyte-enriched transcription factor that binds to the DNA as a homodimer (112). HNF4α homozygous knockout mice exhibit embryonic lethality and impaired gastrulation due to defects in visceral endoderm. The rescue of knockout embryos by implantation of functional visceral endoderm enabled the study of HNF4α functions in fetal liver. HNF4α-null fetal liver failed to express a multitude of liver-specific genes required for functioning liver, including liver-enriched transcription factors hepatocyte nuclear factor 1 alpha (HNF1α) and xenobiotic nuclear receptor pregnane X receptor (PXR) (14, 73). Adult liver-specific HNF4α knockout mice exhibit loss of liver functions and develop steatosis as a result of disrupted regulation of a number of genes involved in the control of lipid homeostasis (47). In sum, these studies suggest that HNF4α-mediated transcription is required for liver development. Furthermore, HNF4α plays a critical role in differentiation of human pluripotent stem cells to hepatocyte-cell like cells. It acts as a master regulator and induces several key hepatic transcription factors, which promote hepatic-lineage specification (Fig.3) (26).

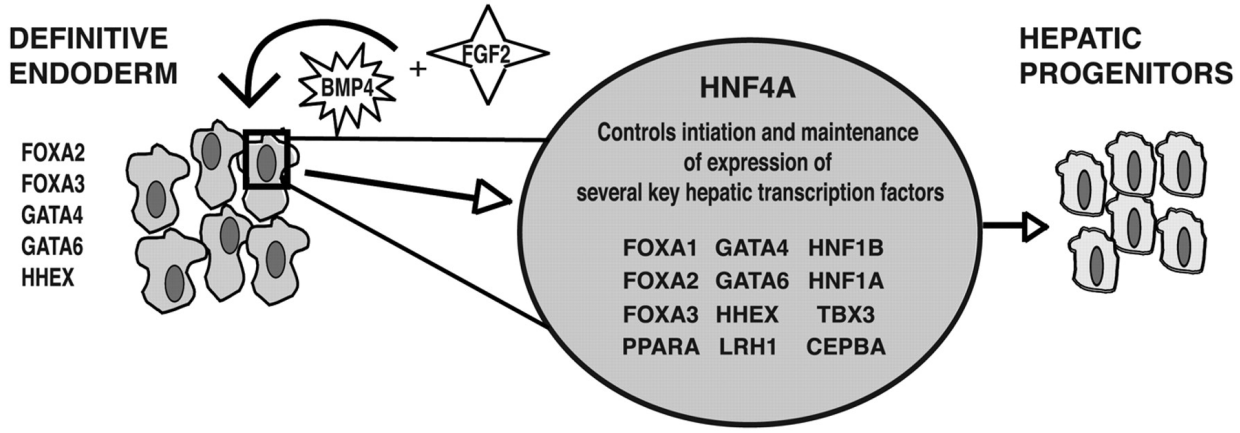

Figure 3. HNF4α is the master regulator of hepatic-linage specification in differentiation of human pluripotent stem cells to hepatocyte-like cells. Reprinted by permission from Company of Biologists: Development, copyright (2011) (26).
In differentiation of human pluripotent cells, definite endoderm transforms into nascent hepatic progenitor cells that express HNF4α in response to bone morphogenetic protein 4 (BMP4) and fibroblast growth factor (FGF). Hnf4α, in turn, establishes the hepatic transcriptional factor network that is essential for the differentiation of hepatic progenitor cells towards to functional hepatocytes.

RATIONALE

Combining next generation sequencing technology with chromatin immunoprecipitation (ChIP-Seq), at specific time points of regeneration, allows detection of genome-wide binding of p53 to *de novo* target genes in liver during cell cycle. p53 has been implicated in cell cycle arrest both at G1-S and G2-M checkpoints in various cell types (72). We use liver regeneration after two-thirds PH, as a model to assess p53 response in regenerating compared to sham liver (surgical control). Analysis of ChIP-Seq data, at specific time points of regeneration, will provide *de novo*

targets of p53 in regenerating liver. My **hypothesis** is that intersection of global expression analyses (microarray and RNA sequencing) and profiling of p53 interactions with chromatin (ChIP-Seq) at the G1-S transition of normal cell cycle, corresponding to 24h post-PH in mice liver regeneration, will reveal p53 functions during cell cycle regulation in normal proliferating cells and during tissue regeneration.

DESCRIPTION OF THE OBJECTIVES

Objective 1: To identify and annotate p53 *de novo* target genes at the G1-S transition of cell cycle of normal proliferating cells, corresponding to 24h post-PH in mice liver regeneration

Objective 2: To establish p53 *de novo* target genes that have altered regenerative response at the G1-S transition of cell cycle of normal proliferating cells, corresponding to 24h post-PH in mice liver regeneration

Objective 3: To elucidate the mechanism of p53-mediated maintenance of hepatic cell identity during liver regeneration

CHAPTER 2 MATERIAL AND METHODS

2.1 Partial hepatectomy

WT and p53-null mice were anesthetized using isoflurane and two-thirds PH or sham surgery was performed as described in CSH Protocols; 2006; doi:10.1101/pdb.prot4384. Mice were fed normal chow and water, ad libitum. All mouse work was performed under the guidelines of The University of Texas MD Anderson Cancer Center Institutional Animal Care and Use Committee. Liver tissue was collected at the 24 hours post PH or sham surgery; tissue resected during PH used as Time= 0.

2.2 Chromatin immunoprecipitation (ChIP)

ChIP was performed as previously described (67). Briefly, the ChIP liver lysate was prepared by 1% formaldehyde crosslinking of minced liver samples, followed by nuclei isolation. ChIP lysates were sonicated, using a Bioruptor (Diagenode), to generate chromatin fragments with DNA averaging around 500bp in length. Material from WT and p53-null liver chromatin lysates at specified time points using 5ug of either HNF4a antibody (ab41898, Abcam) or p53 antibody (OPO3, EMDMillepore) and were collected on Protein G magnetic beads (Invitrogen); nonspecific binding was

reduced by serial salt washes (300mM NaCl, 500mM NaCl, and 250mM LiCl). DNA was released from the immunoprecipitated material by treatment with RNase A, followed by Proteinase K digestion and reversal of formaldehyde crosslinks by >6 hours of heat shock at 65°C. DNA was further purified by phenol:chloroform extraction followed by ethanol precipitation.

2.3 RNA analysis

Total RNA isolation from liver tissue was realized with TriZOL (Invitrogen), following manufacturers recommended procedure. cDNA was prepared using Superscript II (Invitrogen) for Q-PCR and cDNA library was generated using ScriptSeq for RNA-Sequencing using the Illumina HiSeq2000 platform.

2.4 ChIP-Seq analysis

Bowtie mapping algorithm **(**version 0.12.8) (71) was utilized to map 36 bp raw reads in ChIP-Seq datasets to the NCBI build 37 (UCSC mm9) with following parameters: -n 1, -m 1, -S, --best, --strata, and --chunkmbs 320. The mapped reads were given as an input to peak calling algorithm MACS (1.4.0beta) to identify enriched regions (over Input) in the genome at a P value threshold of 10^{-8} (142). p53-enriched regions in sham liver was subtracted from the enriched regions in PH liver using

BEDTools (version 2.13.3) (96) to form the final set of p53-enriched regions specific to PH liver for further analysis.

2.5 Generation of ChIP-Seq signal density files for browser data visualization

After mapping ChIP-Seq tag sequences (reads) to the genome**,** aligned files were processed further for visualization. First, each aligned 36 bp ChIP-Seq tag sequence was extended to 200 bp, using the makeTagDirectory program in Homer (Hypergeometric Optimization of Motif Enrichment) software package (50). Second, ChIP-Seq signal density files in bedgraph format were generated using makeUCSCfile program. The signal density values were calculated as the total number of overlapping ChIP-fragments at each genomic position and normalized to the total number of 50 million tags. Then, normalized values were summarized as the average score at each 50 bp bin. Lastly, for visualization purposes, normalized signal density files in bedgraph format were converted to bigwig format, using UCSC bedGraphToBigwig program (http://genome.ucsc.edu) and uploaded to UCSC genome browser (http://genome.ucsc.edu) and IGV (Integrative Genomics Browser) (100, 124).

2.6 Heatmap generation for ChIP-Seq signal

The normalized ChIP-Seq signal density values were averaged at defined equally sized bins within ± 2.5 kb from p53-bound peak centers in Fig. 5. Then, p53-bound peaks were ranked according to their signal density values in a descending order and displayed in a heatmap using the heatmap program in Cistrome analysis pipeline (http://cistrome.org/ap/).

2.7 Distance plot calculations

Each p53-bound peak was assigned to the nearest annotated gene. Then p53-bound peaks were partitioned into a number of subgroups according to their relative distance to the nearest gene. The positions of p53-bound peaks were shuffled to obtain randomly generated sequences using shuffleBed by BEDTools (version 2.13.3) (96). The number of binding sites and randomly generated sequences in the defined distance interval i were denoted as, bs_i and r_i, respectively. The enrichment score in the distance interval i denoted as ES_i was defined in the below:

$$ES_{i=} \begin{cases} 1- r_i/bs_i & r_i \leq bs_i \\ 0 & r_i > bs_i \end{cases}$$

and plotted in Fig. 9.

2.8 Average gene profile calculations

For ChIP-Seq data set, the signal density values in PH liver and input as a control was calculated by seqMiner along the average gene profile. Each gene in RefSeq gene model was partitioned into 100 equally sized bins. Its 5' end and 3' end flanking regions were partitioned into 20 equally sized bins. The average ChIP-Seq signal density value in each bin were computed and plotted as an aggregate plot.

2.9 Conservation plot calculation

PhastCons conservation scores among 30 vertebrates were retrieved from the UCSC website (http://hgdownload.soe.ucsc.edu/goldenPath/mm9/phastCons30way/). Then, conservation scores were averaged at 100bp resolution within 3 kb of p53-bound peak centers and were plotted using the Sitepro program under CEAS(110).

2.10 Motif analysis

The enriched sequences within ± 300bp of the top 500 p53-bound peak centers were identified, utilizing transcription factor Affinity Prediction (TRAP) motif analysis tool with default P value threshold of 10^{-3} (123). In the analysis, mouse promoter sequences were chosen as background (control).

2.11 Gene ontology analysis

We identified the enriched Gene Ontology (GO) Biological Process terms and their associated P values for the gene lists obtained from ChIP-Seq and/or RNA-Seq analysis, making use of DAVID functional annotation tool (54).

2.12 RNA-Seq analysis.

TopHat (version 2.0.9) algorithm built on Bowtie mapping algorithm (version 0.12.8) (71) was applied to map 76 bp paired-end RNA-Seq reads to the NCBI build 37 (UCSC mm9) (64). After mapping the reads to the genome, the normalization procedure was performed in three steps. First, HTSeq (4) count tool was applied to compute the number of reads aligning to the exons of each gene. Then, to identify the genes with a significant alteration in their regenerative response (PH vs. sham liver), EdgeR (101) was employed to determine regenerative responsive genes at a false discovery rate (FDR) adjusted P value (q-value) of 1%. Second, to determine the genes that have a p53-mediated alteration in their regenerative response, expression values were calculated in terms of fragments per kilobase of exon per million fragments mapped (FPKM) values for each gene, using NGSUtils (12). The regenerative response ($\log_2$ fold change of normalized FPKM values in PH vs. sham liver) was computed in p53 WT and null liver,

separately. Lastly, permutation test was applied for each gene to determine p53-dependent regenerative responsive genes at a P-value cutoff 0.05.

2.13 Principal component analysis

The FPKM value was computed for each gene, using NGSUtils (12). These values were given as an input into principal component analysis. Principal component analysis was performed, utilizing prcomp function in R programming environment (http://www.r-project.org)

2.14 Binomial test

P value calculation for significance of overlapping regions in Venn diagrams was performed using Binomial test.

CHAPTER 3 RESULTS

3.1 p53 binds to a significant number of genomic sites in regenerating liver

Combining next generation technology with chromatin immunoprecipitation (ChIP-Seq) at specific time points of regeneration allows detection of genome-wide binding of p53 to *de novo* target genes in liver during cell cycle. We used liver regeneration after two-thirds PH as a model that enabled us to determine the change at the level of p53 response during liver regeneration. We determined p53-enriched regions (peaks) in regenerating liver by profiling of p53 interactions with chromatin (ChIP-Seq) at the G1-S transition of the cell cycle of normal proliferating cells. Our peak calling analysis with a stringent P value 10^{-8} revealed that a significant p53 signal is enriched at 14,071 and 1,705 genomic sites in PH and sham liver, respectively (Fig. 4).

A significant fraction (97%) of these sites were specifically enriched in PH liver (Fig. 4). These identified p53-bound peaks were specifically enriched with p53 signal in PH liver and not in any of other controls (Fig. 5). The centers of these peaks are highly conserved among 30 vertebrate species, compared to the flanking non-peak regions and to the equivalent number of randomly generated sequences (Fig. 6), suggesting that they are likely represent functional regulatory elements in the genome

(131). Identification of p53-bound peaks in regenerating liver at a genome-wide level offers an unprecedented opportunity to define a p53 DNA-binding motif at a global scale *in vivo.* We identified the sequences that are enriched within 300 bp of p53-bound peak centers and found one sequence that consists of two decameric half-sites and is significantly similar to the previously reported (84) p53-consensus motif ($P = 7.88*10^{-11}$) with a higher level of conservation at C and G bases in the CWWG core sequence of the right half-site (Fig. 7). Taken together, our ChIP-Seq data analysis suggested that p53 binds to a large number of genomic sites in normal proliferating cells *in vivo*, with a recognition site that has significant similarity to the p53 consensus motif obtained from the TRANSFAC database (84).

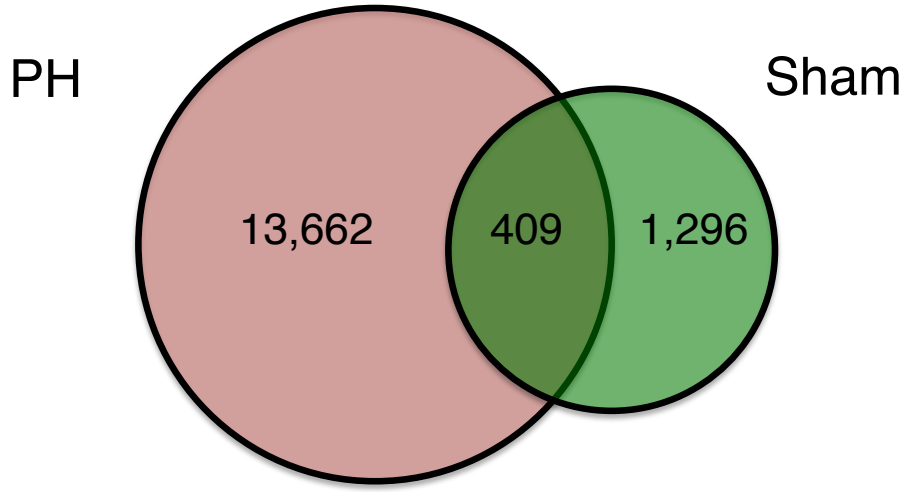

Figure 4. p53 gains a significant number of binding sites in regenerating liver

The numbers of p53-bound peaks in PH vs. sham liver are shown in Venn diagram.

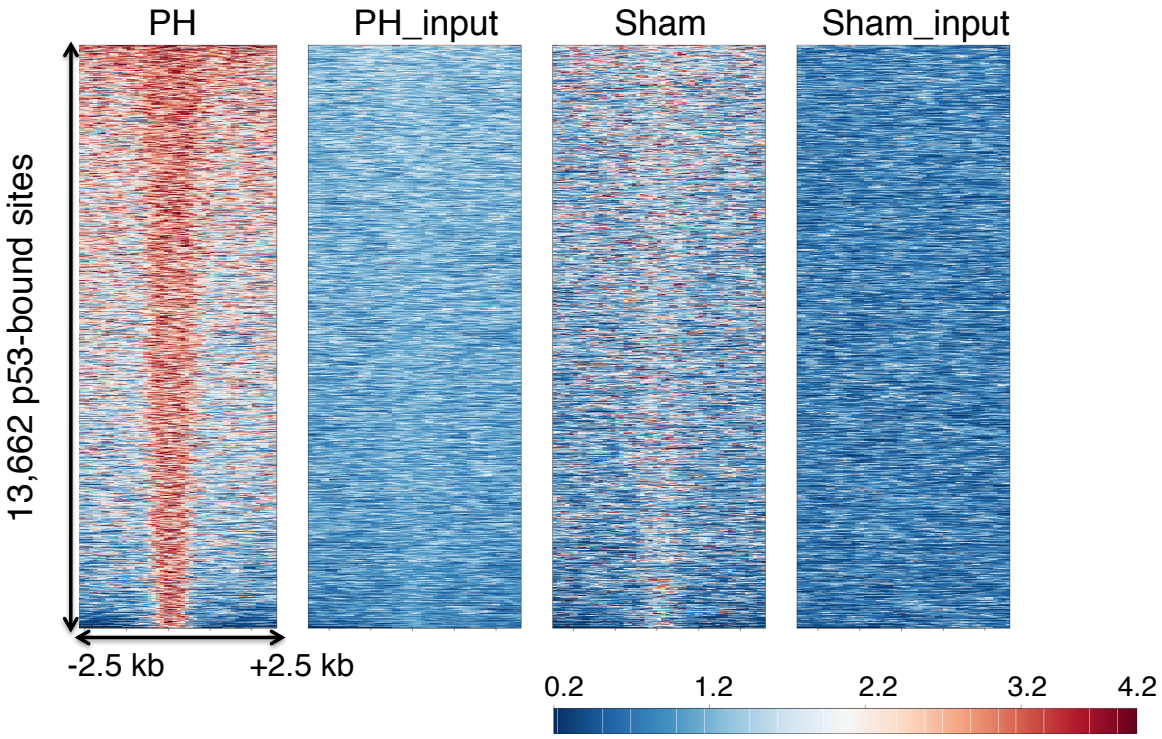

Figure 5. p53 exhibits binding at 13,662 sites in regenerating liver

p53 ChIP-Seq signal is specifically enriched at 13,662 sites in regenerating liver. The p53 ChIP-Seq signal densities of PH, sham and their inputs at 100 bp resolution within ± 2.5 kb of 13,662 p53-bound peaks are displayed in the heatmap.

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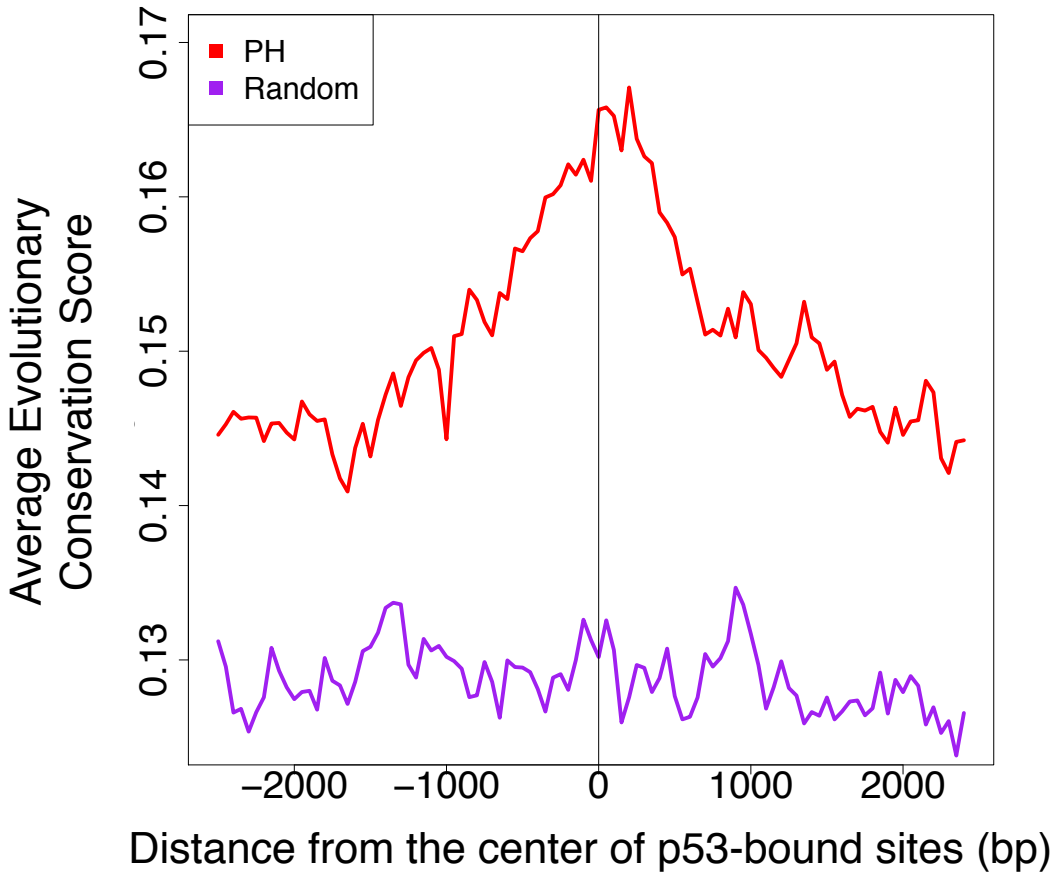


Figure 6. The centers of p53-bound peaks are well-conserved

The evolutionary conservation of p53-bound peak centers was compared to the flanking non-peak and the equivalent number of randomly generated sequences. The average evolutionary conservation score at 50 bp resolution was plotted within ±2.5 kb of the peak centers in the aggregate plot.

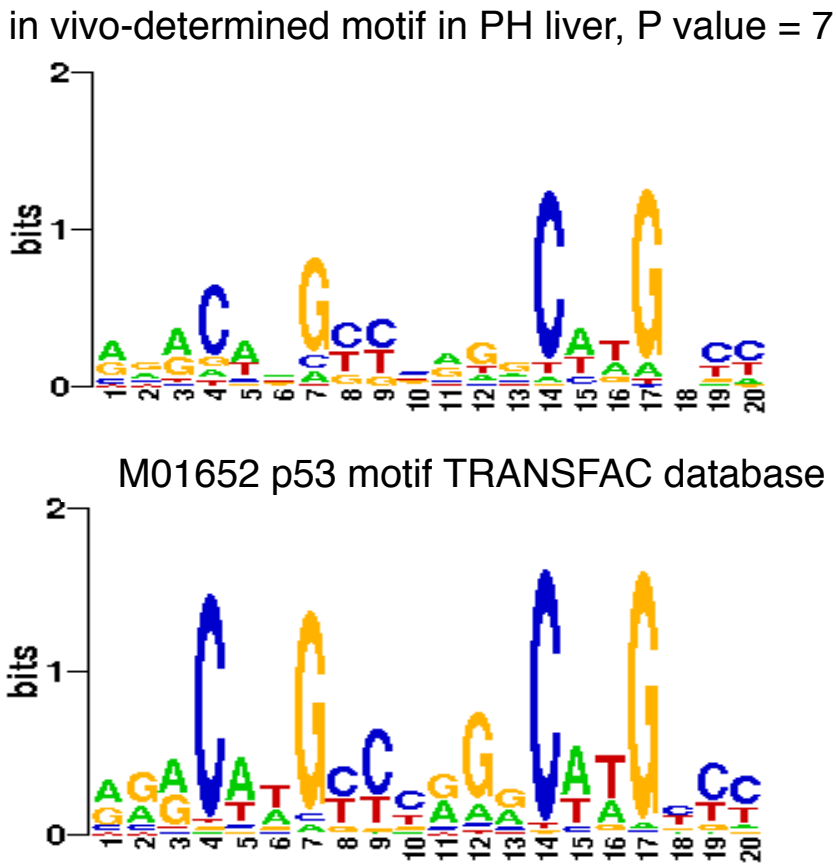


Figure 7. The centers of p53-bound peaks are significantly enriched with p53 consensus motif

Motif discovery analysis at p53-bound peak centers. Top: One of the mostly enriched sequences within 300 bp of the top 500 p53-bound peak centers with its associated P value. Bottom: p53 consensus motif sequence obtained from the TRANSFAC database (84).

3.1.2 p53 binds novel genes in regenerating liver

To define the p53-bound genes in regenerating liver, we first identified the genes that have p53 binding within a certain cutoff distance from each gene's transcription start site (TSS). First, each peak was assigned to the nearest annotated gene and the relative distance between each peak center and its assigned nearest gene transcription start site (TSS) was calculated. The overall distribution of p53-bound peaks revealed that a significant portion of them (38%) was localized within 10 kb of a gene TSS (Fig. 8). To determine whether their localization occurred more than expected by chance within a certain cutoff distance of a TSS, we normalized the peaks by the equivalent number of randomly generated sequences. At the end of the normalization procedure, we found that p53-bound peaks were localized most significantly within 1 kb of a TSS (Fig. 9). Similarly, the p53 binding density profile along an average gene structure revealed that p53 binding is most significantly enriched immediately downstream of the TSS of target genes (Fig. 10).

As examples of p53 binding and location relative to a TSS, I examined p53 binding at two most significantly enriched genomic sites at the TSS of *CDK2-Associated, Cullin Domain 1* (*Cacul1)* and in the promoter region of *olfactory receptor 1274, pseudogene (Olfr1274-ps)* (Fig. 11). Although p53 response elements can be found quite close to the TSS (within 300 bp), they are also found within exons and

introns (8). Therefore, we chose 10 kb as a cutoff distance and identified 5074 *de novo* p53 target genes. 413 target genes (8%) overlapped with a list of canonical p53 target genes retrieved from SABiosciences (http://www.genecards.org/cgibin/carddisp.pl?gene=TP53&sabio_targets=1911#sabio_targets) (P value= $2.54*10^{-9}$). Expectedly, this specific subgroup of canonical gene targets is enriched in well-established p53 functions, including regulation of apoptosis and cellular proliferation. Intriguingly, the remaining part of our *de novo* target genes, 4661 genes (92%) defined as non-canonical p53 target genes, is mainly involved in developmental processes (Fig. 12).

Numerous studies established p53 as a critical regulator of developmental processes, including the renewal of the embryonic stem cells (ESCs), adult neural stem cells, and hematopoietic stem cells (1, 76, 78, 85). In addition, p53 acts as an inducer of differentiation to protect mESCs from DNA damage (74, 76). To determine if p53 binds to a conserved subset of genes to regulate developmental processes in regenerating liver, we compared our non-canonical p53-bound genes in regenerating liver to the p53-bound genes in mESCs retrieved from a publicly available dataset (74). Our analysis revealed a significant overlap between p53-bound genes in regenerating liver and p53-bound genes in mESCs (Fig. 13) (61% overlap, P = $1.72*10^{-136}$), including E2F1 and KLF4 as the key players of the transcription factor network that

regulates stem cell renewal (15). In addition, the overlap encompassed a particular subgroup of genes, encoding crucial transcription factors that guide the specification of hepatic lineage, such as HNF4α, HNF3β (Foxa2), HNF6α, HNF6β, GATA6, CEBPα and CEBPβ (69). Taken together, these results led us to question whether p53-chromatin interactions at this specific subset of genes in regenerating liver would significantly alter their transcript levels.

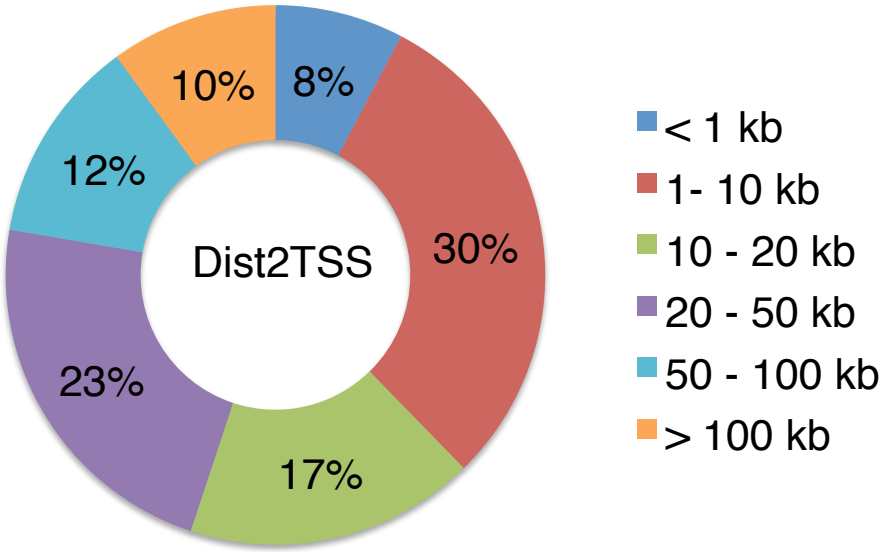


Figure 8. 38% of p53 binding sites are located within 10 kb of TSS

p53-bound peaks were partitioned into a number of subgroups by its relative distance to TSS of the nearest gene. The pie chart displays the fraction of p53-bound peaks in each distance interval.

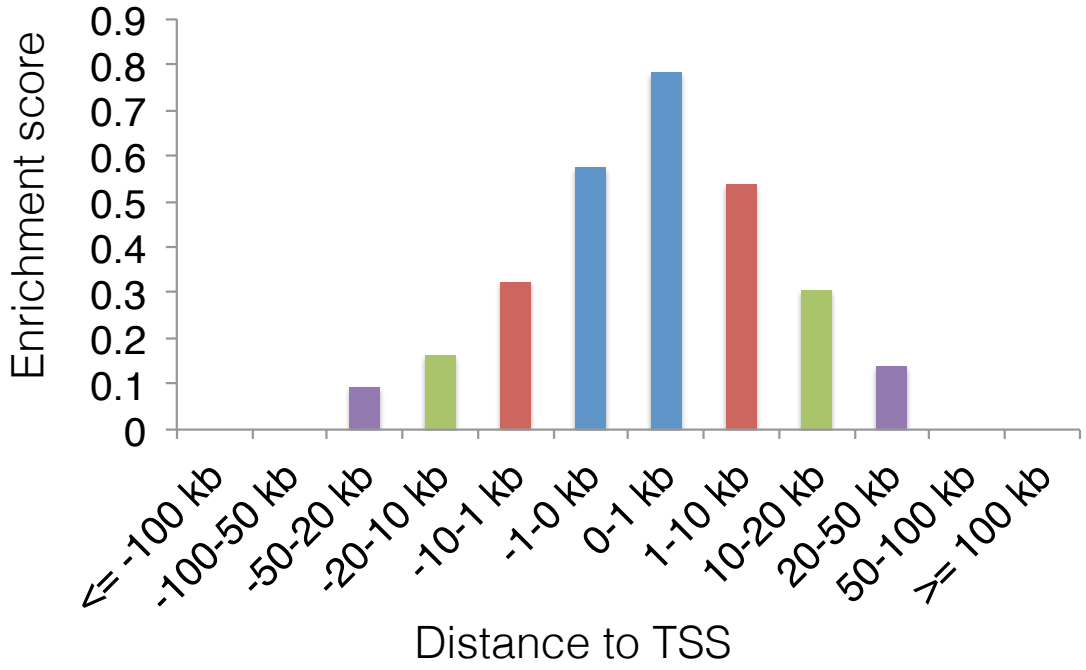


Figure 9. p53 binding is mostly enriched within 1 kb of TSS

Each p53-bound peak was assigned to the nearest annotated gene. Then p53-bound peaks were partitioned into a number of subgroups according to their relative distance to the nearest gene and normalized by the number of randomly generated sequences. An enrichment (normalization) score was assigned for each distance interval and plotted as a histogram.

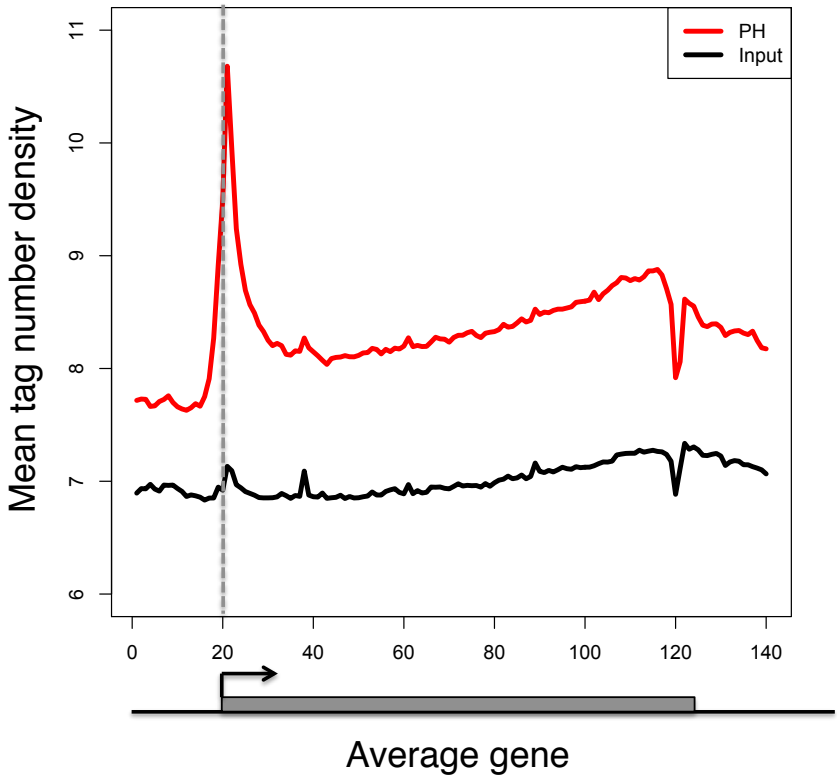


Figure 10. p53 binding signal peaks at TSS of genes

Average binding profile density of p53. The ChIP-Seq signal density at 100 bp resolution along average gene structure was plotted for PH liver (red) and as Input as control (black). X axis shows average gene structure, and y axis shows average tag number.

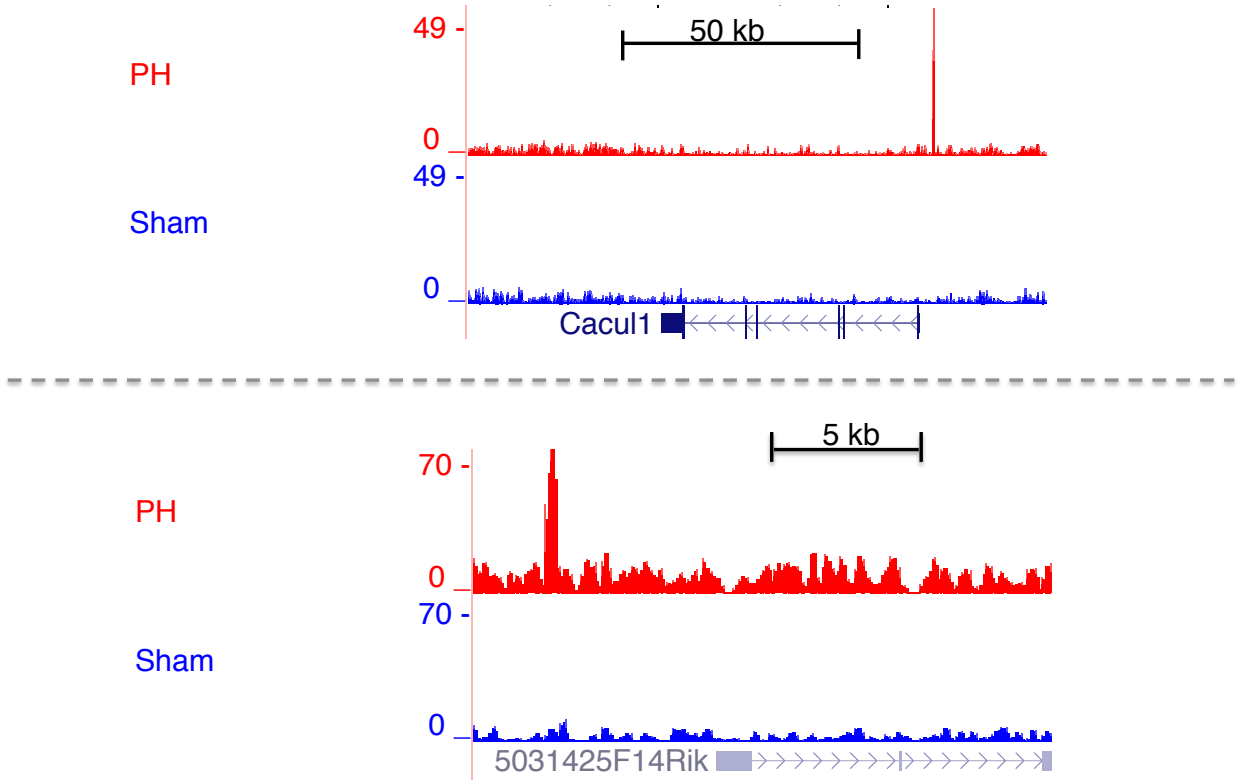
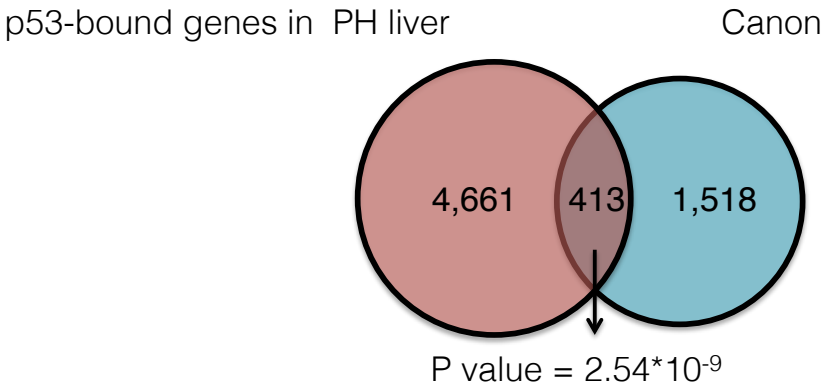


Figure 11. Individual examples of p53 binding at TSS of genes

Individual examples of PH-specific and common p53-bound genes. Top: Visualization of normalized p53 tag numbers in PH vs. sham liver at Cacul1 (PH-specific gene). Bottom: Visualization of normalized p53 tag numbers in PH vs. sham liver at Olfr1274-ps (common gene) in UCSC genome browser.



Non-canonical functions

Biological Functional Category	P-value
Cellular development	$4.40*10^{-16}$
Gene Expression	$1.10*10^{-14}$
Organismal development	$7.12*10^{-13}$
Embryonic development	$7.12*10^{-13}$
Molecular transport	$1.16*10^{-10}$

Canonical functions

Biological Functional Category	P-value
Regulation of apoptosis	$9.9*10^{-6}$
Cell adhesion	$3.7*10^{-4}$
Positive regulation of protein transport	$4.29*10^{-7}$
Embryonic morphogenesis	$1.2*10^{-3}$
Regulation of cell proliferation	$1.3*10^{-3}$

Figure 12. A significant number of non-canonical p53-bound genes

Venn diagram indicates the overlap between p53-bound genes in PH liver and canonical p53-bound genes. Tables below show enriched GO Biological Functions with associated P values for Left: non-canonical p53-bound genes and Right: canonical p53-bound genes in regenerating liver.

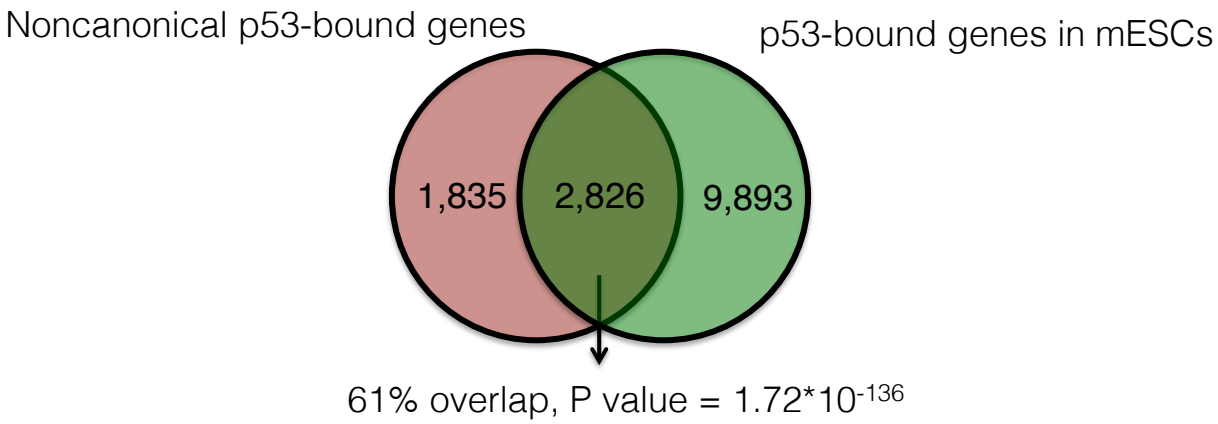


Figure 13. Non-canonical p53-bound genes has a significant overlap with p53-bound genes in mESCs

Venn diagram indicates the overlap between non-canonical p53-bound genes in PH liver and p53-bound genes in mESCs.

3.1.3 A novel role of p53 in regulating lipogenesis

Global profiling of gene expression using RNA Sequencing (RNA-Seq) in regenerating liver allowed us to identify genes with significant p53-mediated changes in their expression levels following PH. An unsupervised clustering of RNA-Seq samples by principal component analysis revealed a broad picture of transcriptional differences among samples due to regenerative response (PH vs. sham) and p53 response (WT vs. null) (Fig. 14). To identify p53-mediated alterations in regenerative response, we obtained 1305 genes with diminished (p53-activated) and 1573 genes with elevated levels of regenerative response (p53-repressed) in the absence of p53 (Fig. 15). p53-activated genes play a role in biological functions such as oxidation reduction, response to unfolded protein, response to nutrient levels, steroid metabolic process, and fatty acid metabolic process. Furthermore, a specific subset of genes involved in the regulation of immune response has a higher level of regenerative response in the absence of p53 (Fig. 16). A closer inspection of p53-activated genes enriched in oxidation reduction revealed that they are mostly included in the cytochrome P450 superfamily (CYP). CYP enzymes catalyze the detoxification reactions in the liver on a wide range of substances such as drugs, toxic chemicals, steroids, prostaglandins, and fatty acids (22). The metabolic activity of CYP enzymes

is a definitive marker of mature hepatic cell identity along with expression of alpha-feto protein (AFP) and Albumin (ALB) (114).

In addition, p53-activated genes are highly represented in steroid metabolism and fatty acid metabolic process (Fig. 17). p53-mediated alterations in steroid and fatty acid metabolic process at 24h after PH occurred concomitantly with a transient accumulation of lipids (transient steatosis) observed during the early phase of liver regeneration prior to the peak of hepatocyte proliferation in mice (111). Previous reports emphasized that disruption of this process is associated with impaired liver regeneration (29, 40, 102, 111, 129). Several reasons justify the necessity of a transient steatosis during liver regeneration, including utilization of lipids as an energy source through β-oxidation of fatty acids and as constituents of the nuclear cell membranes of newly proliferating hepatocytes (102).

Our study also reveals novel p53 direct target genes involved in lipogenesis. p53-activated direct target genes include a catalyzer for the synthesis of monounsaturated fatty acids *stearoyl-CoA desaturase 1* (*Scd1)*. Overexpression of Scd1 leads to hepatic steatosis (77). We further examined the biological output of p53-mediated activation of these genes involved in lipogenesis. The Oil Red O staining of neutral triglycerides and lipids in WT and null regenerating liver tissues indicated that

loss of p53 leads to a significant decrease in lipid accumulation at 24h after PH (Fig. 18).

Intersection of p53-responsive (p53-activated/-repressed) gene set with p53-bound genes (ChIP-Seq) provided a list of functional p53-bound genes defined as direct target genes. p53-activated direct target genes play roles in epithelial cell development and include a number of genes encoding transcription factors that are critical for the specification of hepatic lineage, such as HNF6α, HNF6β, CEBPα, and CEBPβ (69). On the other hand, p53-repressed target genes are highly represented in a totally different function, in regulation of immune response (Fig. 19).

One mechanism that may underlie the difference between a repressive vs. activating binding of p53 to its target genes is the DNA sequence of the p53 binding sites may differ. To address this possibility, I calculated the length of spacer sequences between two half-sites in p53 response element. According to a previously established model, the length of spacer in p53 response element is one of the determining factors for p53-mediated activation and repression (99). This model suggested that p53-mediated activation has a greater preference for no spacer sequence. However, our computational analysis indicated that the length of spacer sequences at activator and repressor sites are similarly distributed (Fig. 20). In

addition, we did not observe any significant difference between p53-bound motifs at the activator vs. the repressor sites (Fig. 21)

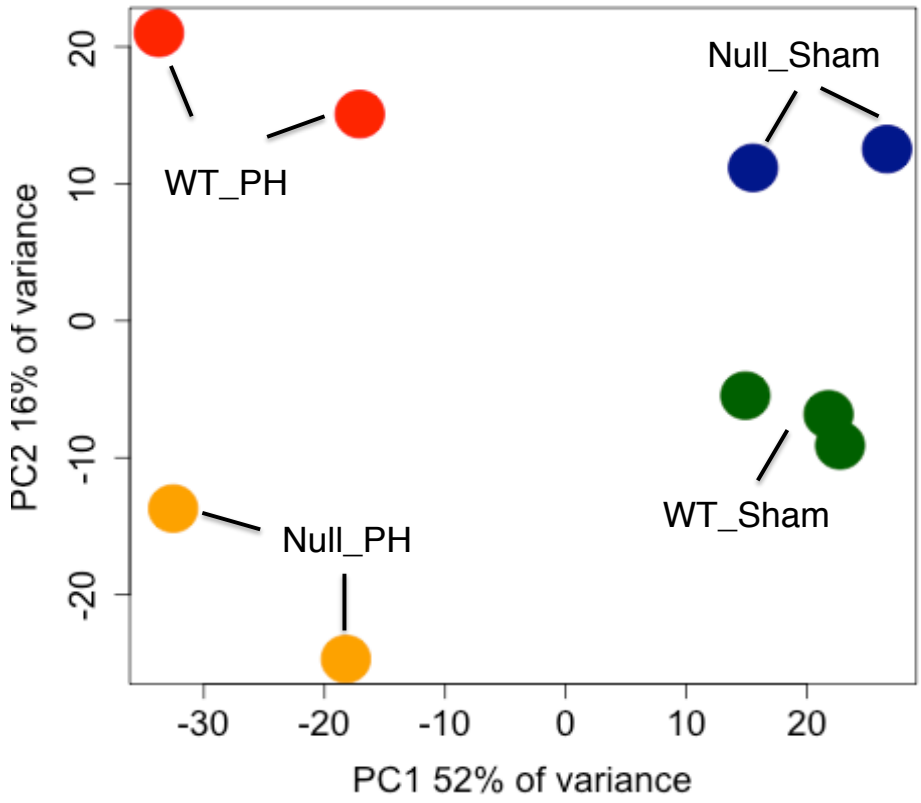


Figure 14. Principal component analysis of RNA-Seq samples

Unsupervised clustering of gene expression profiles in WT PH, WT Sham, p53-null PH, and p53-null Sham samples. Principal component analysis on expression levels of all genes in the genome, enabled us to obtain a broad picture of the transcriptional differences among WT PH, WT Sham, p53-null PH, and p53-null Sham samples (*n=2,2,2,3*).

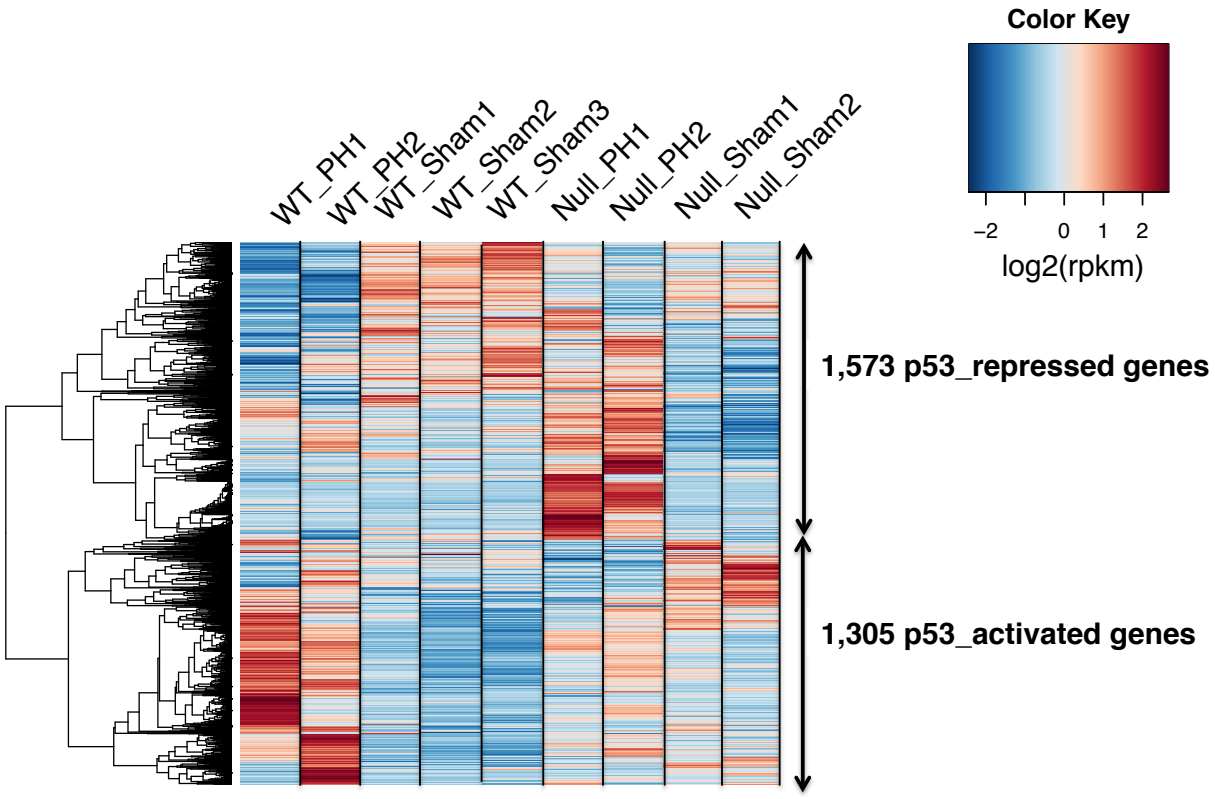


Figure 15. Hierarchical clustering of p53-responsive genes

Heatmap displays the clustering of p53-dependent regenerative responsive genes based on log2 expression values.

p53-activated genes

Identifier	GO term	P-Value
GO:0055114	Oxidation reduction	$2.77*10^{-7}$
GO:0006986	Response to unfolded protein	$2.67*10^{-4}$
GO:0031667	Response to nutrient levels	$4.14*10^{-3}$
GO:0008203	Cholesterol metabolic process	$2.55*10^{-3}$
GO:0006631	Fatty acid metabolic process	$3.10*10^{-3}$

p53-repressed genes

Identifier	GO term	P-Value
GO:0006955	Immune response	$9.32*10^{-10}$
GO:0050778	Positive regulation of immune response	$3.76*10^{-9}$
GO:0048584	Acute inflammatory response	$4.05*10^{-8}$
GO:0016064	Inflammatory response	$1.81*10^{-7}$
GO:0002253	Leukocyte mediated immunity	$2.23*10^{-7}$

Figure 16. p53-responsive genes are involved in liver-specific functions and regulation of immune response

Biological functional annotation of differentially expressed genes (p53-activated/-repressed). Tables demonstrate top 5 enriched GO Biological Process (BP) terms with associated identifiers and P values for Left: p53-activated and Right: p53-repressed genes.

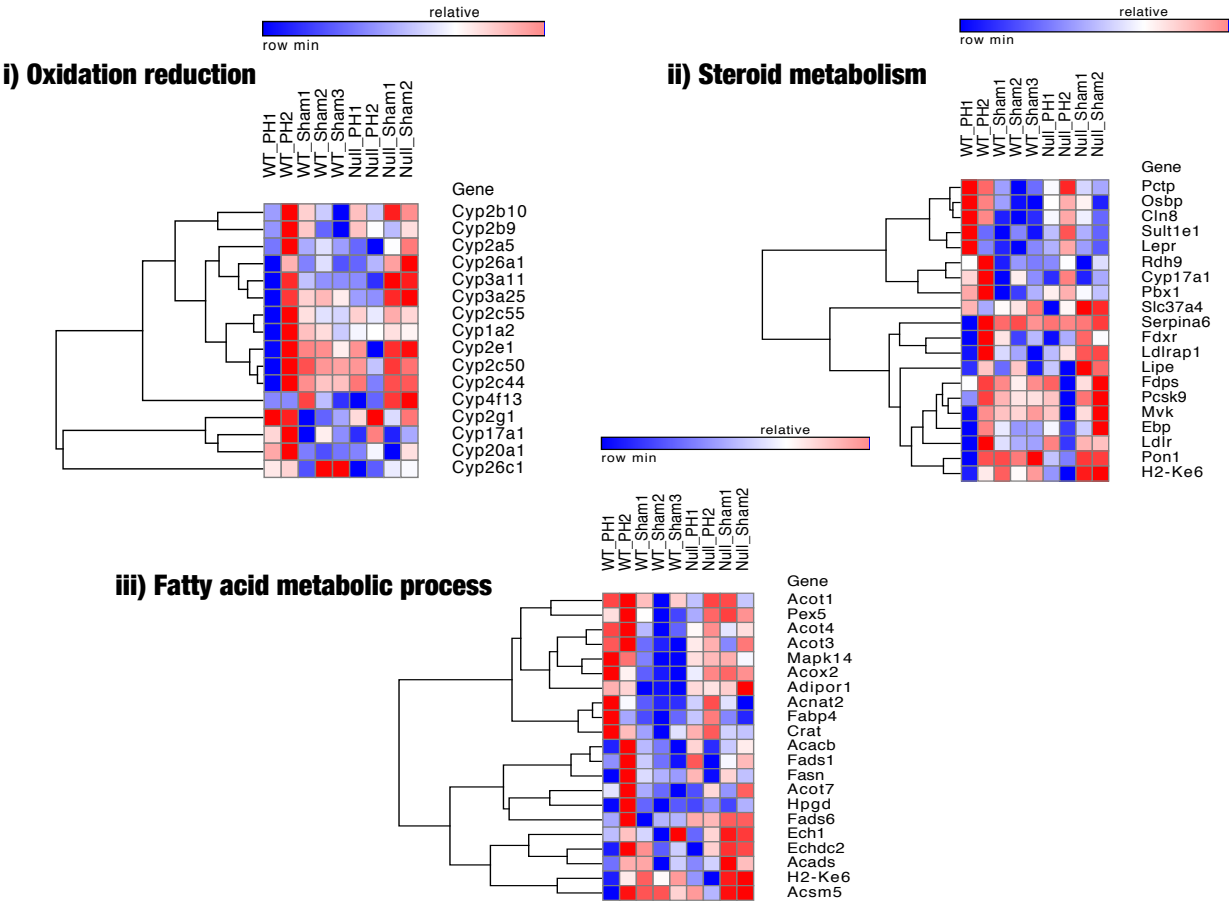


Figure 17. p53-activated genes are enriched in oxidation reduction, steroid metabolism, and fatty acid metabolic process

Heatmaps display the clustering of p53-activated genes involved in i) oxidation ii) steroid metabolism, and iii) fatty acid metabolic process, based on log2 expression values.

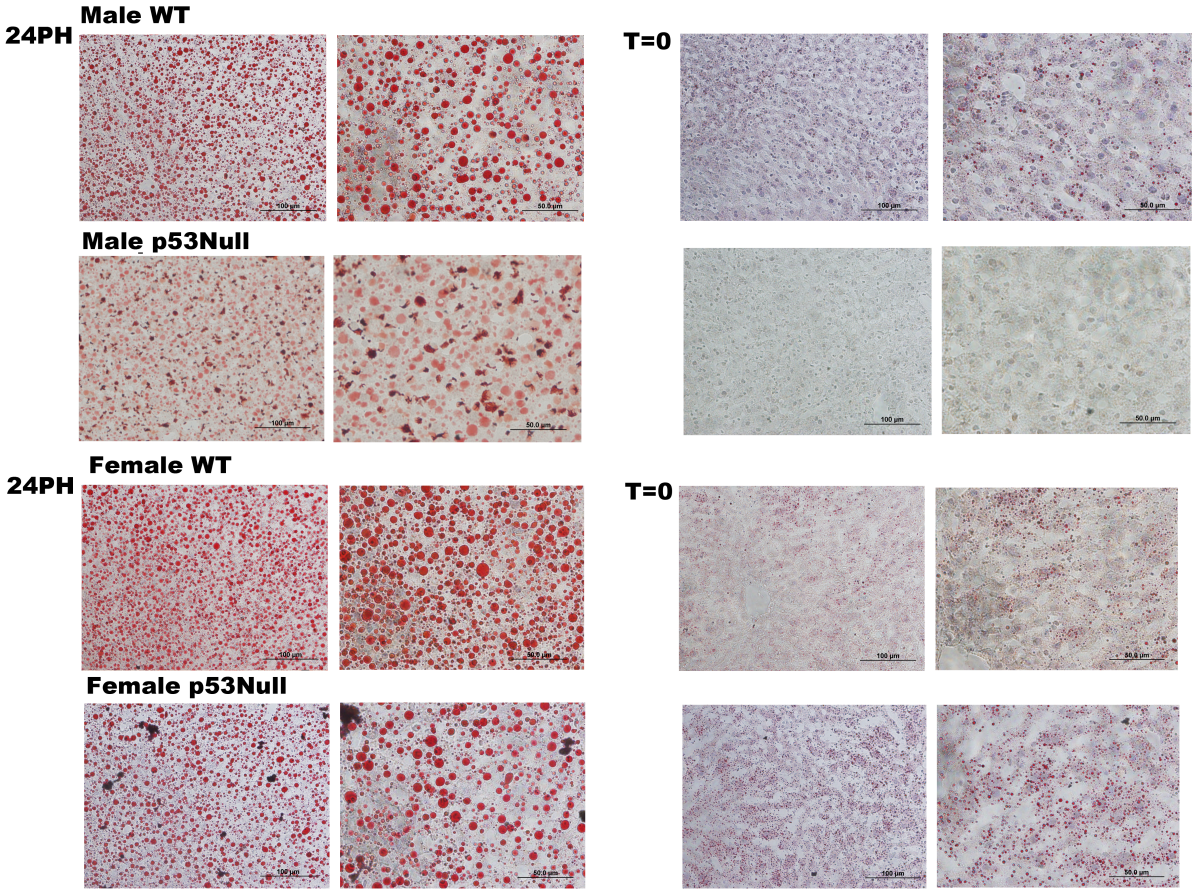


Figure 18. p53-dependent lipid accumulation at the G1-S transition of the cell cycle in regenerating liver

Top: Oil Red O staining of male p53 WT and null samples at T=0 and T=24 after PH. Bottom: Oil Red O staining of female p53 WT and null samples at T=0 and T=24 after PH (*n=3 in each group*).

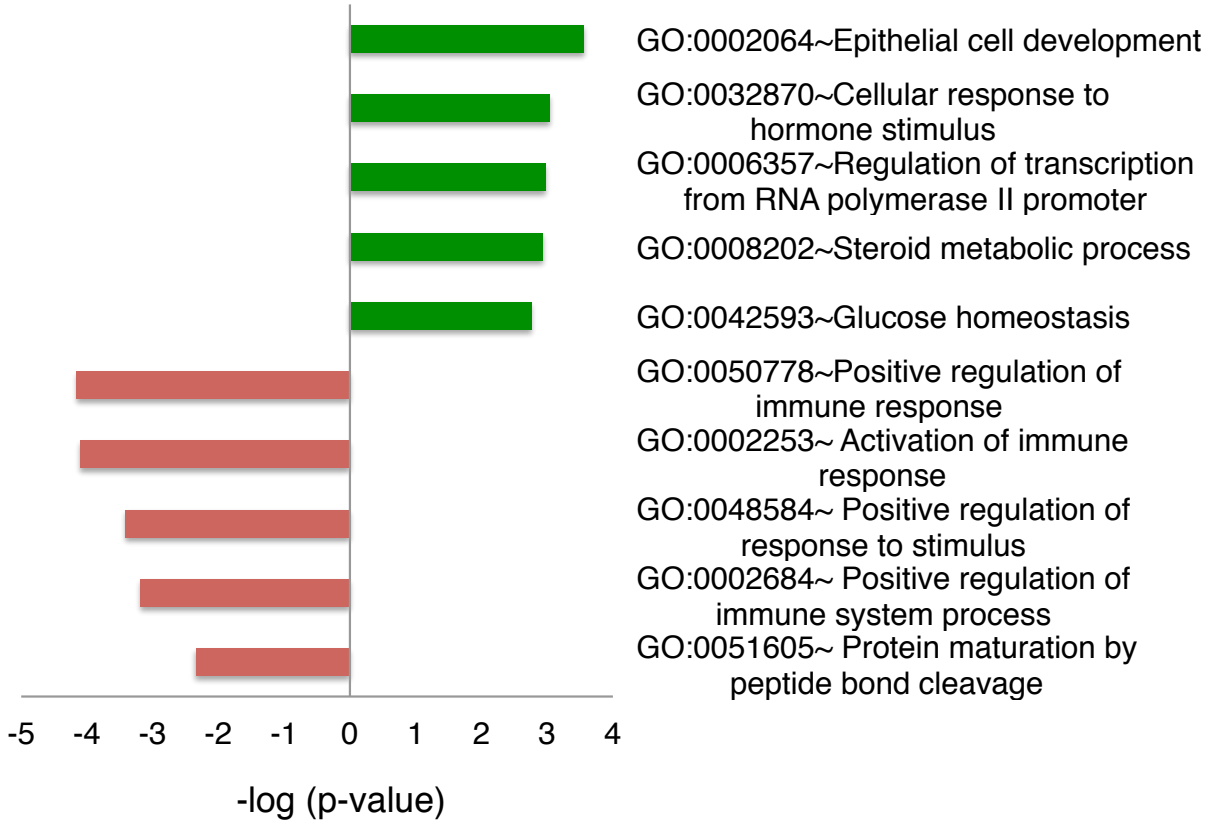


Figure 19. p53 direct target genes are involved in liver-specific functions and regulation of immune response

Functional annotation of p53-activated/-repressed direct target genes. Top 5 enriched GO Biological Process (BP) terms for p53-activated target genes (left) and p53-repressed target genes (right) (x axis in -log(p-value) scale).

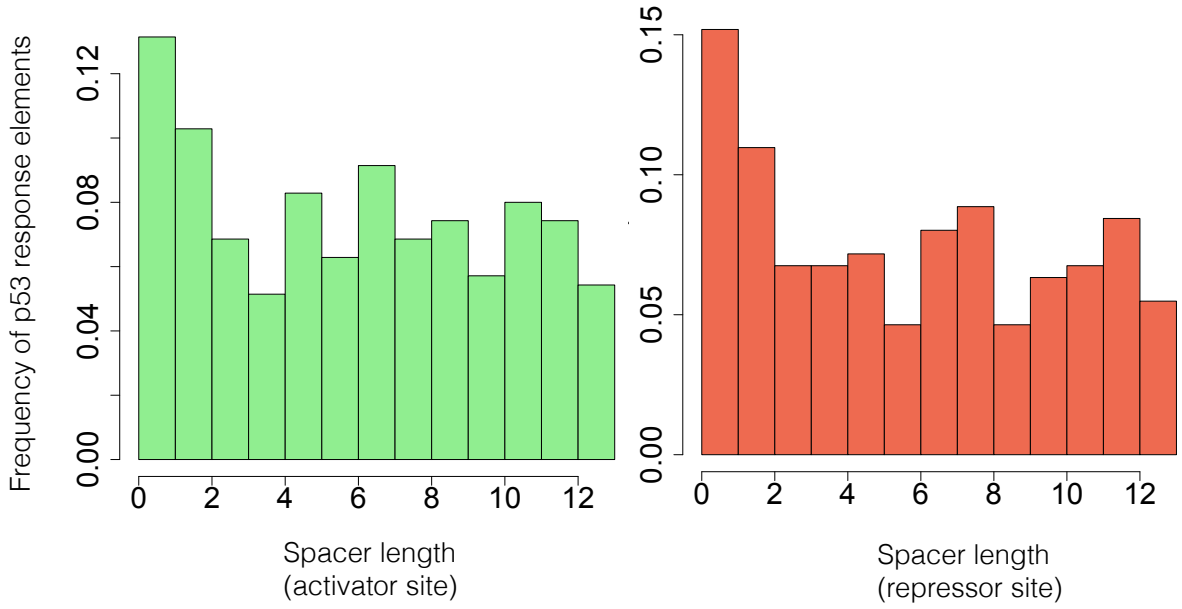


Figure 20. There is no major difference between spacer lengths of p53 response elements at activator and repressor sites

The histograms of all spacer lengths of p53 response elements (REs) within 300 bp of p53-bound peak centers at the activated (left) and the repressed (right) target genes.

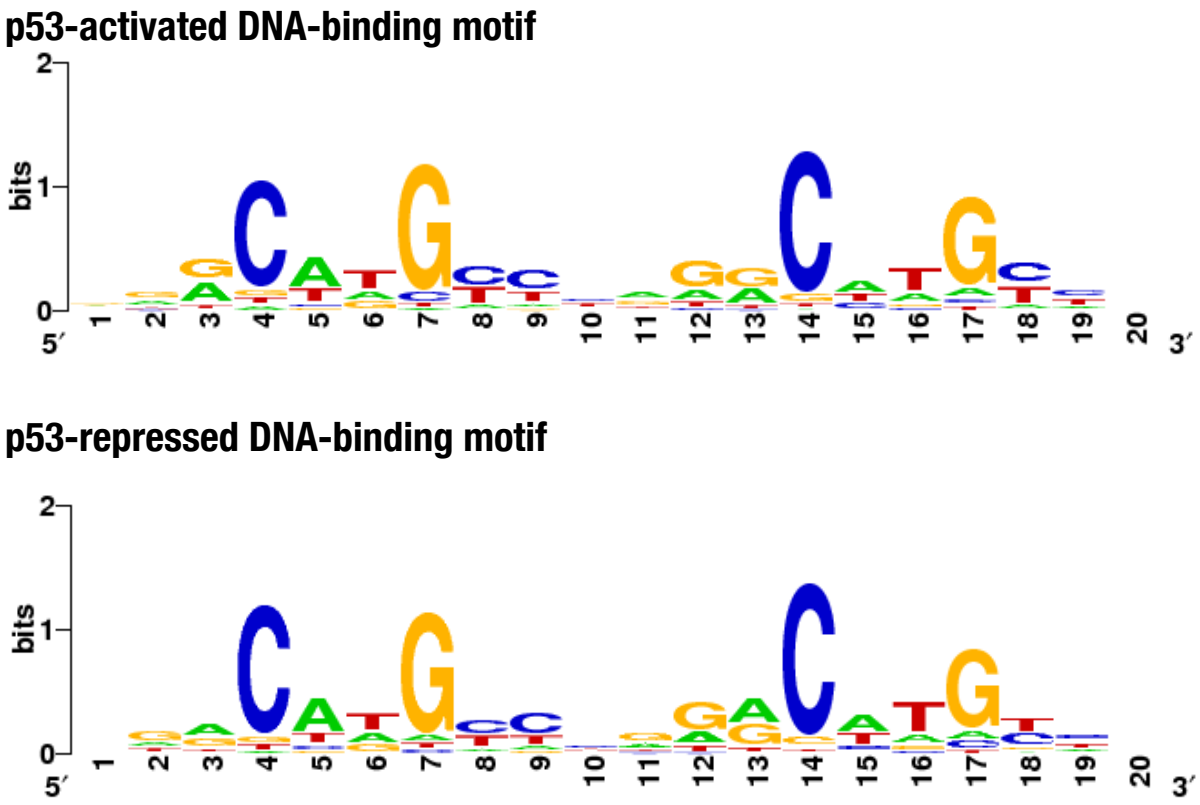


Figure 21. There is no major difference between p53 DNA-binding motifs at activator and repressor sites

Motif analysis to determine p53-activated and p53-repressed DNA-binding motif. The DNA binding motif identified within 300 bp p53-bound peak centers at the activated (top) and the repressed (bottom) target genes.

3.1.4 p53 and HNF4α shares a significant portion of their target genes

To further determine which transcription factors cooperate with p53 to regulate its target genes during liver regeneration, we performed *de novo* motif analysis of the DNA sequences at the center of p53-bound peaks. The most strongly enriched sequences within 300 bp of p53 peak summits were remarkably similar to the HNF4α DNA-binding motif with an adjusted P value of 10^{-85} (Fig. 22), suggesting HNF4α as a potential co-regulator. Indeed, taking advantage of publicly available datasets (106), we found that 40% of p53-bound genes overlapped with HNF4α-bound genes (Fig. 23, $P = 1.94*10^{-10}$). In addition, a majority (78%, $P = 1.04*10^{-43}$) of p53-activated direct target genes enriched in developmental processes and steroid metabolism, was also included in an HNF4α target gene list. On the other hand, 63% ($P = 1.57*10^{-9}$) of p53-repressed direct target genes, involved in regulation of immune response, overlapped with HNF4α target genes (Fig. 24). In aggregate, these correlation analyses suggest that HNF4α is a potential co-regulator of p53 during liver regeneration.

Motif_ID	Transcription Factor	Adjusted P-value
M01033	HNF4α	10^{-85}
M00468	LFA1	10^{-43}
M00646	KLF12	10^{-43}
M00943	TCF3	10^{-39}
M01596	MYOD	10^{-30}

Figure 22. p53-bound peak centers are enriched with HNF4α DNA-binding motif

Table showing the mostly significantly enriched DNA binding motifs identified within 300 bp of the top 500 p53 peak summits with associated P values.

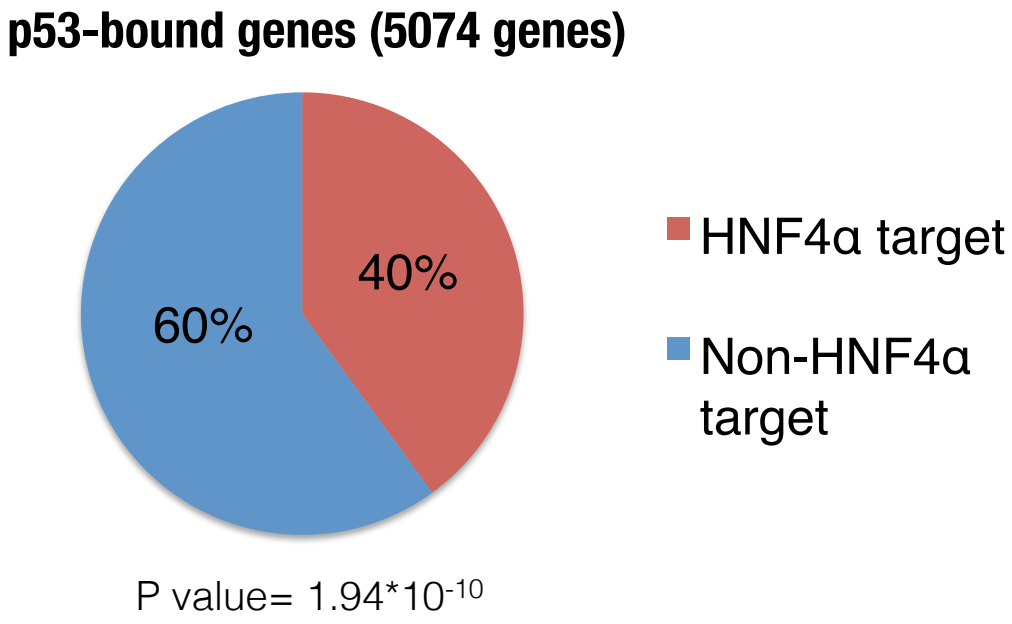
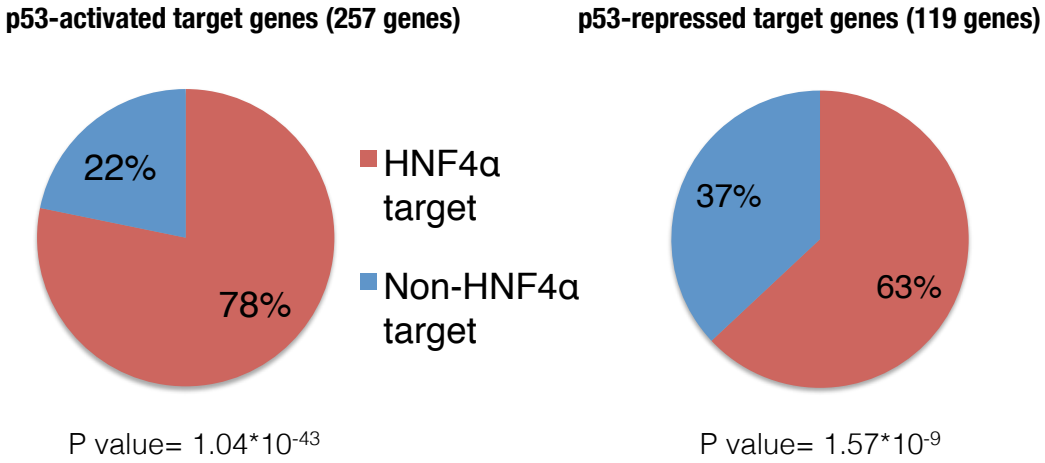


Figure 23. p53 and HNF4α shares a significant portion of their target genes

Identification of the overlap between genes bound both by p53 and HNF4α. The pie chart categorizes p53-bound genes into two subgroups (HNF4α target) and (Non-HNF4α target) with the associated P value for significance of the overlap.



Epithelial cell development	$1.15*10^{-4}$
Positive regulation of transcription	$1.31*10^{-3}$
Steroid metabolic process	$1.38*10^{-3}$

Positive regulation of immune response	$1.22*10^{-3}$
Activation of immune response	$3.24*10^{-3}$
Positive regulation of immune response	$3.83*10^{-3}$

Figure 24. A significant portion of p53 direct target genes coincide with HNF4α target genes

Identification of the overlap between p53 direct target genes and HNF4α-bound genes. Top: The pie chart categorizes p53-activated target genes (left) and p53-repressed target genes (bottom) into two subgroups (HNF4α target) and (Non-HNF4α target). Bottom: Enriched GO terms and associated P values for HNF4α-bound p53-activated target genes (left) and p53-repressed target genes (right).

3.1.5 p53 binds to tissue-specific enhancers in regenerating liver

A specific combinatorial chromatin signature, which consists of high levels of H3K4me1 and low levels of H3K4me3, defines putative transcriptional enhancers (48, 49). In addition, active enhancer sites show increased levels of H3K27ac (20). Enhancers as transcriptional regulatory regions are mostly tissue specific, usually marked by the binding of transcription factors specifically expressed in the differentiated tissue (48, 50). To define a particular subset of liver-specific enhancers bound by p53 in regenerating liver, we examined H3K4me1 and H3K27ac profiling in quiescent liver and compared these profiles at the center of the p53-bound peaks. We found that 1107 p53 peak summits were enriched with higher levels of H3K4me1 and H3K27ac marks relative to the flanking non-peak regions and to the equivalent number of randomly generated sequences (Fig. 25). Interestingly, a majority of these sites (74%) were also bound by HNF4α. I present one of the p53 and HNF4α co-bound enhancer regions at the upstream of *Pleckstrin Homology Domain Containing, Family A Member 1* (*Plekha1*) gene promoter as an example (Fig. 26). Furthermore, 33% of HNF4α/p53 co-bound enhancers, identified here, coincided with liver-specific enhancers, which were reported by a previous study (109). Altogether, our findings suggest that p53 and HNF4α co-bound enhancers serve as pivotal regulatory elements to control liver-specific gene expression.

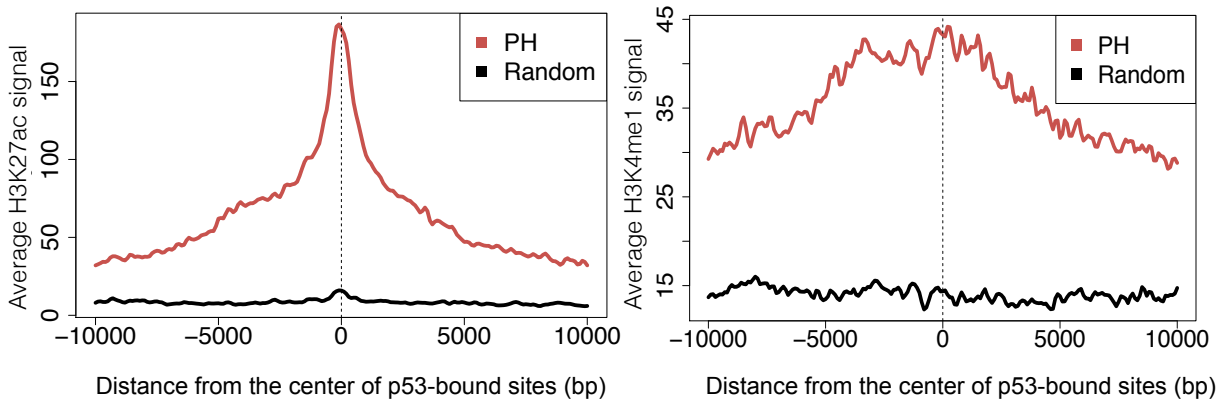


Figure 25. p53 binds to tissue-specific enhancers in regenerating liver

Active enhancer marks are enriched at the center of a specific subset of p53 peak summits. Left: H3K27ac binding signal is higher at p53 peak summits relative to random locations. The aggregate plot indicates the average H327ac signal density around the center of p53 peak summits. Right: H3K4me1 binding signal is higher at p53 peak summits relative to random locations. The aggregate plot indicates the average H3K4me1 signal density around the center of p53 peak summits.

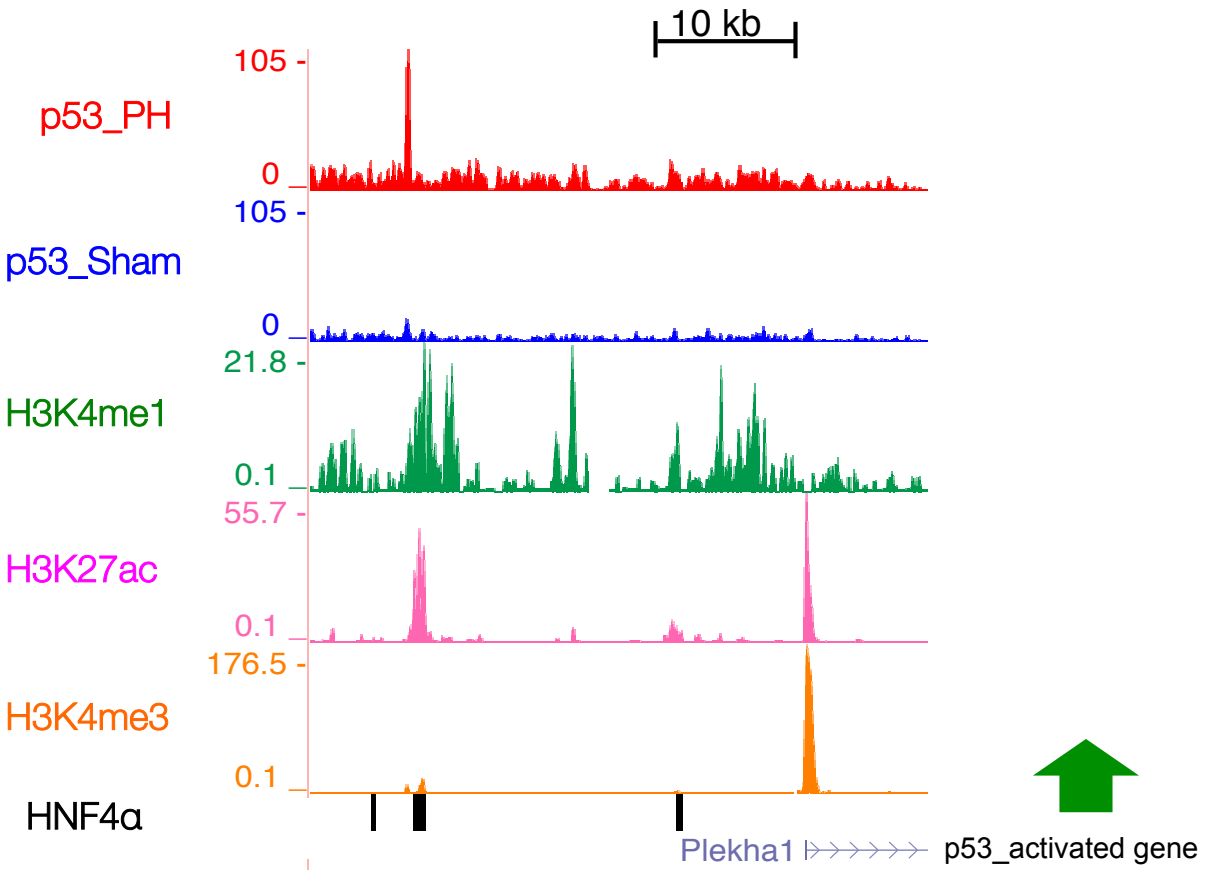


Figure 26. An individual example of p53 and HNF4α co-bound enhancer regions

Visualization of normalized p53 tag numbers in PH vs. sham liver as well as normalized tag numbers of H3K4me1, H3K27ac and H3K27me3 at the upstream of *Plekha1* gene promoter. Black bars indicate HNF4α binding sites.

3.1.6 p53 is required for HNF4α-mediated maintenance of hepatic cell identity during liver regeneration

We next investigated whether p53 is required for HNF4α recruitment to chromatin. We first selected a subset of p53-activated direct target genes that exhibit HNF4α co-binding either at their proximal promoter or distal enhancer sites. Next, we performed ChIP experiments for HNF4α binding at these genes. Our results indicated that p53 promotes HNF4α binding to this specific subset of genes. As examples, at the *Plasminogen* (*Plg)* and *Fibrinogen alpha chain* (*Fga)* genes, encoding major coagulation proteins plasminogen and fibrinogen in liver, respectively, we observed a p53-mediated increase in HNF4α binding to their proximal promoter regions during liver regeneration. In addition, at the *Fga* liver-specific enhancer site [114], p53 promotes an elevated level of HNF4α recruitment to chromatin (Fig. 27 and Fig. 28). When we examined the overall effect of p53 on HNF4α binding to chromatin, loss of p53 mediates a significant level of decrease in HNF4α binding during liver regeneration (Fig. 29).

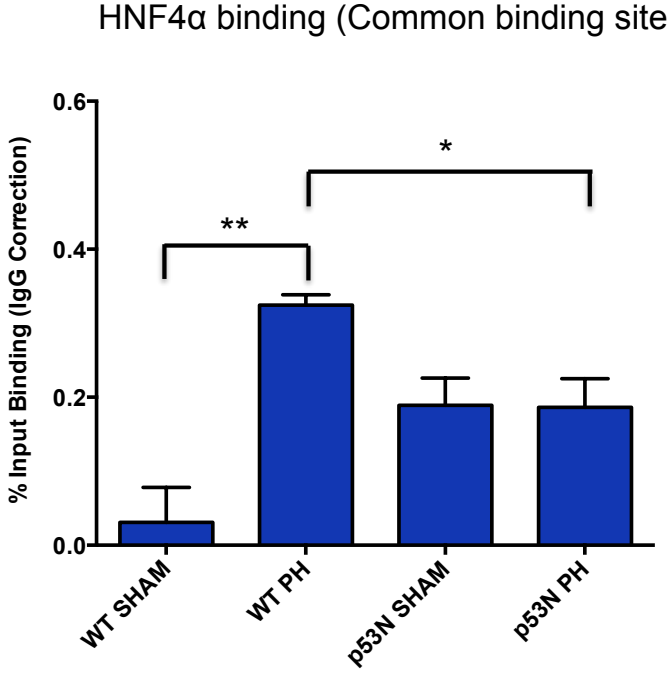


Figure 27. p53 promotes HNF4α binding to the *Plg* proximal promoter during liver regeneration

p53 mediates an increase in HNF4α binding at the proximal *Plg* promoter during liver regeneration (-140bp). (* $p<0.05$, ** $p<0.01$; Student's t-test).

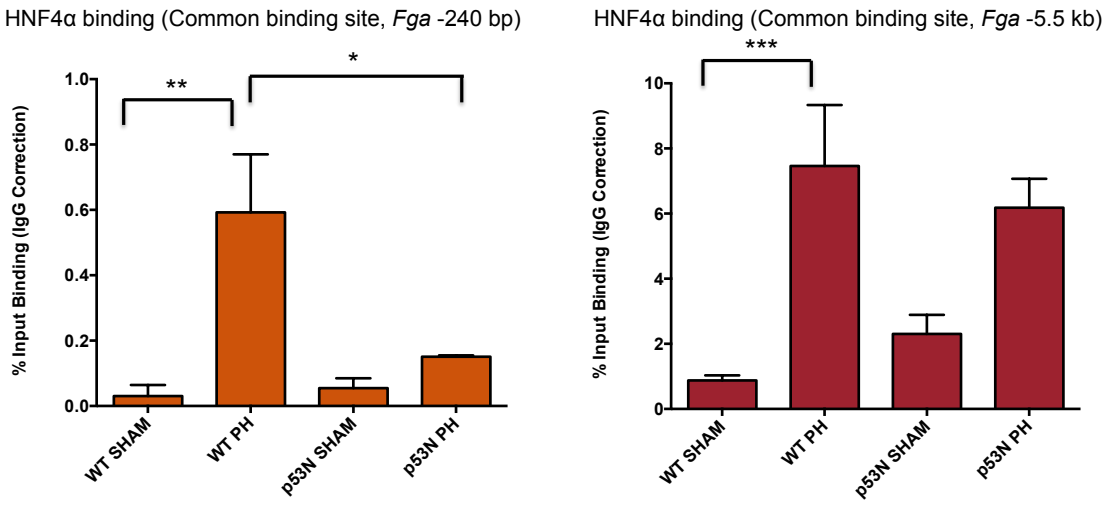


Figure 28. p53 promotes HNF4α binding both at the *Fga* proximal promoter and the distal *Fga* enhancer

p53 mediates an increase in HNF4α recruitment both to Left: the proximal promoter (-240bp) and Right: the distal enhancer (-5.5kb) of *Fga* during liver regeneration. P-values were calculated by direct comparison between WT and null liver (*p<0.05, ** p<0.01, *** p<0.001, Student's t-test).

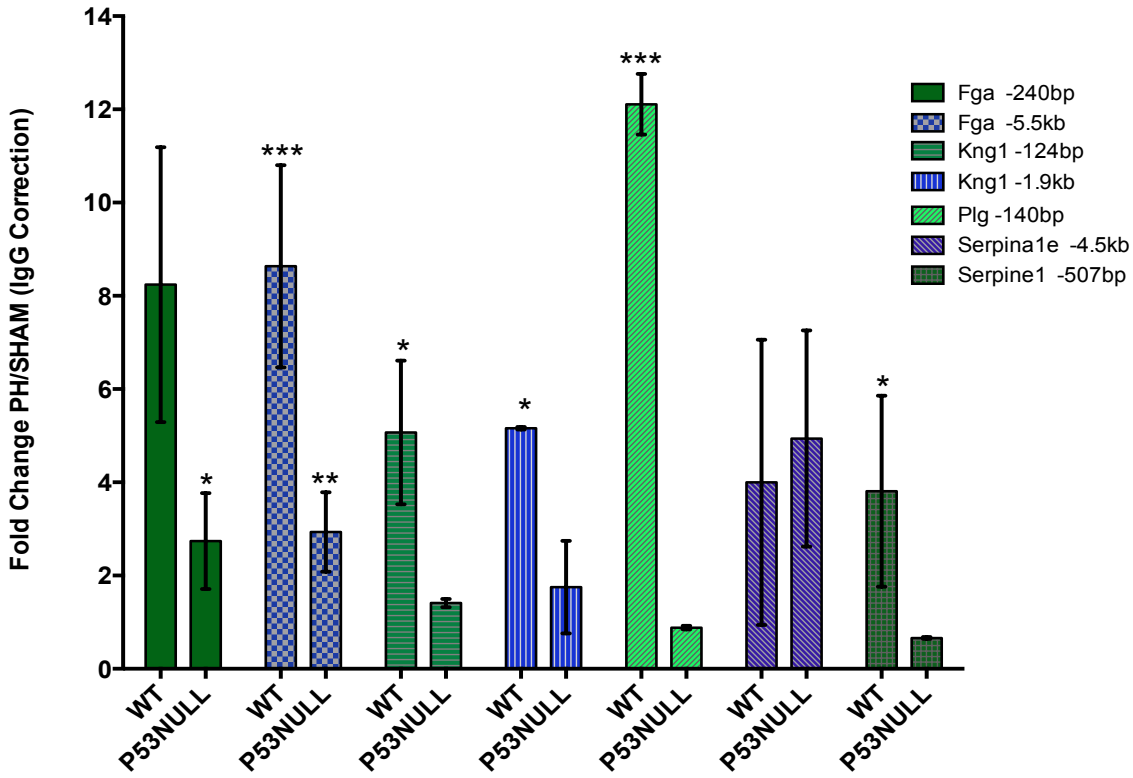


Figure 29. p53 regulation of HNF4α binding during liver regeneration

p53 promotes an increase in HNF4α recruitment to proximal promoters and/or distal enhancers of co-target genes during liver regeneration. ChIP experiments were performed in WT and null liver at 24h following PH and sham surgeries. P-values were calculated by internal comparison between PH and sham surgeries, determined by Student's t-test. ($p<0.05$ *, $p<0.01$ **$p<0.001$***). Y axis shows the fold change of HNF4α binding between PH and sham liver.

CHAPTER 4 DISCUSSION AND FUTURE DIRECTIONS

4.1 DISCUSSION

Liver regeneration in response to PH is one of the best models to study large populations of quiescent liver cells that re-enter the cell cycle in a synchronized wave. Unlike the model of liver regeneration induced by chemical injury, liver regeneration following PH does not involve an extensive level of necrosis and inflammation, which would cause hepatocyte damage. After PH, undamaged hepatocytes are able to achieve a significant portion of liver mass restoration (62) and this process is governed by an interconnected network of signaling pathways. Genetic manipulations in transgenic and knockout mouse models provide an invaluable resource to understand this intricate network of events regulating liver regeneration (53). Here, we use a p53-null mouse model to study loss of p53 function during liver regeneration (57)

It is interesting that not only stimulators but also repressors of the cell cycle such as p53 and p21 which function as safeguards of the cell cycle process, are activated during the PH-induced regenerative process (32, 66). This suggests that the cell cycle process during liver regeneration is tightly regulated. This highly controlled cell cycle process consists of two critical steps: the quiescent hepatocytes are first primed to re-enter the cell cycle (priming phase) and then pass through the restriction point in the

G1 phase of cell cycle to progress through the S phase to proliferate. Hence, the G1-S transition represents a crucial checkpoint after the quiescent hepatocytes re-enter the cell cycle and its regulation needs to be well characterized. This can be partly achieved by elucidating the transcriptional network that orchestrates regulation of this critical checkpoint in regenerating liver. In response to a range of genomic stress, the tumor suppressor p53 induces expression of genes that lead to cell cycle arrest or programmed cell death at the G1-S transition of cell cycle, a process well established in cultured cells (103, 127). Here, we determine the response of p53 *in vivo* during the G1-S transition of the cell cycle in normal proliferating cells isolated from regenerating liver. A phase-specific comparison of p53 binding to chromatin in regenerating vs. sham liver (control) cells provides a full perspective of p53-related changes in regenerating liver. Our ChIP-Seq analysis suggests that p53 specific target genes in regenerating liver are also genes highly represented during developmental processes, including a subset of transcription factors required for the maintenance of hepatic cell identity. Integration of these alterations in p53 binding to chromatin with gene expression profiling studies led us to uncover novel functions of p53 *de novo* target genes in normal proliferating cells *in vivo*.

It has previously been reported that p53 enhances lipid catabolism in response to glucose deprivation in human cell lines and mouse liver (6, 55). In cancer cells,

lipogenesis is usually favored to meet increasing biosynthetic demands. In this condition, p53 acts as a barrier to lipogenesis, repressing a vast array of genes involved in lipogenesis. Conversely, p53 also induces a particular subset of genes represented in fatty acid oxidation as a way of lipid catabolism (41). However, it remains unknown whether p53 plays any role in any of the conditions where fat accumulation is favored in normal proliferating cells, including the early phase of liver regeneration. Several studies suggested that transient fat accumulation is critical for normal liver regeneration in a rodent model (29, 40, 102, 111). In mice, transient hepatic fat accumulation was observed at a specific period during the time course of liver regeneration, 12-24 hours after PH prior to the peak of DNA synthesis. Reversal of hepatic fat accumulation by various means in regenerating liver, diminished the proliferative response in hepatocytes (111). Our data show that the multitude of genes involved in lipogenesis and lipid catabolism exhibit p53-mediated activation in normal proliferating cells at 24h after PH. Importantly, p53-null mice display markedly decreased hepatic fat accumulation compared to WT mice at the G1-S transition of the cell cycle, corresponding to 24h after PH. Taken together, the p53 response to PH could be associated with the regulation of metabolic homeostasis to meet the demands of newly proliferating cells in regenerating liver.

The remaining liver after PH is not only able to reestablish its mass but also performs its normal liver functions for the body, including maintenance of glucose homeostasis, synthesis of blood coagulation factors, bile acid secretion and detoxification of toxic compounds. This generates a massive metabolic load imposed on the remnant liver. It was hypothesized that this extensive metabolic load is one of the mechanisms that triggers the initiation of regenerative process in the remnant liver (39). A significant number of studies have shown that the regenerating liver exhibits specific metabolic alterations, including low blood glucose levels and transient fat metabolic accumulation (102). However, we still need a better understanding of transcriptional networks governing these metabolic alterations in the remnant liver and how they are linked to the proliferative response.

While the remaining liver maintains its hepatic cell identity during liver regeneration *in vivo*, this has not been observed *in vitro*. In contrast, hepatocytes in culture loose their hepatic cell identity accompanied by a decrease in expression of liver-enriched transcription factors (97). This suggests that the maintenance of metabolic homeostasis in the remaining liver requires the induction of liver-enriched transcription factors that help liver cells preserve their hepatic cell identity (120) (44). These liver-enriched transcription factors are transcriptionally activated in an orchestrated fashion to induce hepatocyte differentiation during organ development and

to maintain liver function in adult hepatocytes (107). HNF4α, as the master regulator of the hepatocyte differentiation process, directly controls the expression levels of other liver-enriched transcription factors required for normal liver function (69). Here, our data analysis reveals that p53 cooperates extensively with HNF4α to activate genes involved in liver-enriched functions such as steroid metabolic process. Among them, there are liver-enriched transcription factors such as CEBPβ, HNF6α and HNF6β. Further analysis uncovered a subset of genomic sites bound by both p53 and HNF4α, which exhibit distinguished features of active enhancers. Integration of our data with publicly available datasets revealed that 31% of these enhancer sites are liver-specific (109). In aggregate, these observations led us to hypothesize that p53 is required for HNF4α-mediated maintenance of liver-enriched transcriptional networks during the regenerative process. Indeed, our ChIP experiments further revealed that HNF4α binding significantly diminishes at a subset of liver-specific genes in the absence of p53. Altogether, we uncovered a novel function of p53 during the G1-S transition of the cell cycle in normal proliferating cells *in vivo.* p53 acts in concert with HNF4α to sustain hepatic cell identity and maintain metabolic homeostasis during liver regeneration. This modulation in p53 function at the G1-S checkpoint during liver regeneration may be partially explained by phosphorylation of p53 at Ser389 (80). Compelling evidence suggests that posttranslational modifications of p53 could alter its function in cells (10,

17). For instance, a previous study suggested that Ser46 phoshorylation of p53 leads to altered promoter selectivity and induces p53 to target proapoptotic genes but not cell cycle arrest genes (117).

Understanding the role of p53 in normal proliferating liver cells may allow us to better target it in diseases such as hepatocellular carcinoma (HCC) where p53 is one of the most commonly mutated genes. As one of the major causes of cancer-related deaths worldwide, hepatocellular carcinoma (HCC) is the most common type of human liver cancer (28, 140). Although liver resection is the most common treatment for HCC, it comes with major complications. For instance, 75% to 100% tumors recur within five years after liver resection (79, 108). The remarkable regenerative ability inherent in the liver may actually augment tumor recurrence rate after resection due to the shared molecular mechanisms between liver regeneration and liver cancer. Understanding these shared molecular mechanisms is crucial for development of therapeutic interventions that prevent liver carcinogenesis while allowing normal liver regeneration (16).

Identification of signaling pathways and gene regulatory networks in liver regeneration of a model organism is likely to suggest molecular mechanisms deregulated in HCC. A functional study of p53 response in liver showed that tissue-specific deletion of p53 induced liver tumors with bi-lineal differentiation (63). Using

publicly available global gene expression profiling dataset retrieved from the noted study, we identified genes with significantly altered expression levels in liver tumors induced by p53 deletion. Similar to p53-mediated changes in cellular processes in regenerating liver, p53 induces a vast array of genes involved in sterol and lipid biosynthetic process, whereas it represses a subset of genes enriched in leukocyte activation (Fig. 30). Based on these shared and conserved cellular processes, identification of p53 *de novo* target genes in liver during cell cycle of normal proliferating cells is very likely to identify a great number of genes important for liver growth and cancer. Taken together, this study elucidates the role of p53 in normal proliferating liver cells, providing further insights into its association with hepatocarcinogenesis.

4.1.2 FUTURE DIRECTIONS

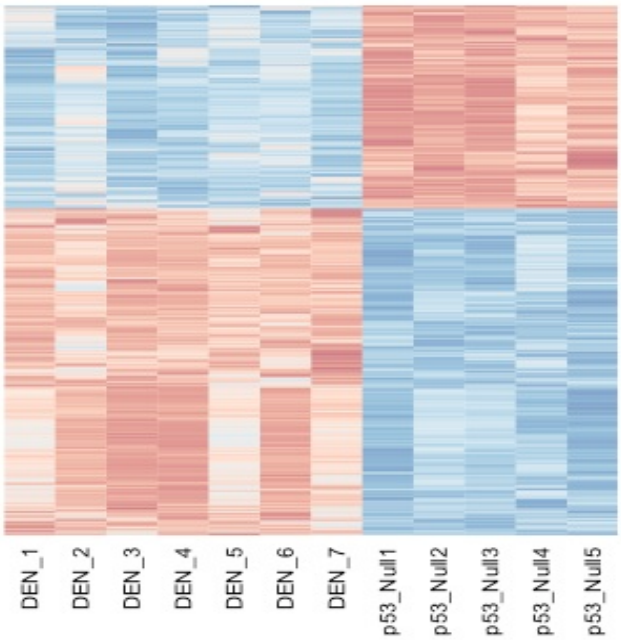
Our data analysis revealed that a specific subset of functional p53 target genes is included in Cyp gene family, a definitive marker of mature hepatic cell identity (114) . These genes have significantly decreased expression levels during liver regeneration mediated by a loss of p53 binding to chromatin in null liver. These results led us to hypothesize that p53 induction of a specific subset of hepatocyte differentiation markers in regenerating liver promotes the maintenance of hepatic cell identity. To

support our hypothesis, we will determine if there is any loss of hepatic cell identity in p53-null liver compared to WT liver by immunohistochemical staining of liver cells with hepatocyte differentiation markers. Next, we will quantify hepatocyte cell populations in p53-null liver compared to WT liver, using CyTOF mass cytometry. These experiments may allow us to address a quite significant question in the field that whether hepatocytes could preserve their cell identity in the absence of p53 while liver mass is reestablished during the PH-induced regenerative process. Our study has been the first one to elucidate the transcriptional role of p53 in a possible loss of hepatocyte differentiation during liver regeneration.

p53 shares a considerable amount of structural homology with its family members, p63 and p73 (60). p53 and p73 exhibit hepatic-specific functions in development of liver and tumor suppression (68). Mice with heterozygous deletion of p53 along with p73 are highly susceptible to spontaneous liver tumor development (37). Previous studies also demonstrated that p73 could maintain genomic integrity in certain conditions in the absence of p53 (118). Here, we show that p53 cooperates extensively with HNF4α to maintain hepatic cell identity during liver regeneration. Due to the significant role of p73 in liver development and function, further studies are required to investigate p73 function during liver regeneration. These studies should address if p73

could compensate for p53 function and could act in concert with HNF4α to sustain hepatic cell identity at certain time points of liver regeneration in the absence of p53.

Our analysis revealed that p53 and HNF4α co-bind to a substantial amount of tissue-specific enhancers to regulate liver-specific genes during liver regeneration. The recent discovery that enhancer-RNAs (e-RNAs), transcripts derived from enhancers (26, 65, 130), play a critical role in tissue-specific functions (70). Taking these studies into account, the identification of e-RNAs produced from p53 and HNF4α co-bound enhancers could provide further insights into the mechanism by p53 maintains hepatic cell identity during liver regeneration.



p53-activated genes

Sterol biosynthetic process	**$1.72*10^{-13}$**
Oxidation reduction	**$1.76*10^{-11}$**
Cholesterol biosynthetic process	**$3.50*10^{-11}$**
Lipid biosynthetic process	**$1.31*10^{-7}$**
Cofactor metabolic process	**$1.14*10^{-6}$**

p53-repressed genes

Cell cycle	**$3.98*10^{-9}$**
Cell adhesion	**$2.40*10^{-8}$**
Cytoskeletal organization	**$4.68*10^{-8}$**
Chemotaxis	**$5.50*10^{-8}$**
Leukocyte activation	**$6.68*10^{-7}$**

Figure 30. Tissue-specific p53 deletion induces liver carcinoma

Identification and functional annotation of p53-responsive genes in liver tumors induced by tissue-specific deletion of p53. Right: Heatmap displays expression levels of p53-responsive genes in null vs. N-nitrosodiethylamine (DEN)-treated liver tumors. Left: Enriched GO Biological Process (BP) terms and associated P values for p53-activated (top) and p53-repressed (bottom) genes in liver tumors.

Table 1: HNF4α ChIP primer sites

Fga -230bp 5'	CATCTCCCCAGCTTCCAA
Fga -230bp 3'	TTGTTTGTTTCCGATAAGTTGTTG
Fga -5.5kb 5'	CTCCTCATCAGTCTGGTTGTTG
Fga -5.5kb 3'	CCCAATGTTAGCTCCCTTCTTT
Kng1 -124bp 5'	CTCCTGGCTTCAAACTCT
Kng1 -124bp 3'	TCTCTGCTGGGTTCTATTG
Kng1 -1.9kb 5'	CCTGTTAGCCTGTTCTGG
Kng1 -1.9kb 3'	GCATCCACACCTTCAACA
Plg -140bp 5'	GTAAGAGGGAAGAGGGAGGA
Plg -140bp 3'	TGTGGTAGATGCTGGAAGTG
Serpina1e -4.5kb 5'	GGACACCCACTCAGTTAT
Serpina1e -4.5kb 3'	CCTCTTCTCGGAAATGGA
Serpine1 -507bp 5'	GCAGTAACCCAAGAGAAAG
Serpine1 -507bp 3'	ACAGCCATCACAGAGAAG

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