Direct Activation of Forkhead Box O3 by Tumor Suppressors p53 and p73 Is Disrupted During Liver Regeneration in Mice

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The p53 family of proteins regulates the expression of target genes that promote cell cycle arrest and apoptosis, which may be linked to cellular growth control as well as tumor suppression. Within the p53 family, p53 and the transactivating p73 isoform (TA-p73) have hepatic-specific functions in development and tumor suppression. Here, we determined TA-p73 interactions with chromatin in the adult mouse liver and found forkhead box O3 (*Foxo3*) to be one of 158 gene targets. Global profiling of hepatic gene expression in the regenerating liver versus the quiescent liver revealed specific, functional categories of genes regulated over the time of regeneration. *Foxo3* is the most responsive gene among transcription factors with altered expression during regenerative cellular proliferation. p53 and TA-p73 bind a *Foxo3* p53 response element (p53RE) and maintain active expression in the quiescent liver. During regeneration of the liver, the binding of p53 and TA-p73, the recruitment of acetyltransferase p300, and the active chromatin structure of *Foxo3* are disrupted along with a loss of *Foxo3* expression. In agreement with the loss of Foxo3 transcriptional activation, a decrease in histone activation marks (dimethylated histone H3 at lysine 4, acetylated histone H3 at lysine 14, and acetylated H4) at the *Foxo3* p53RE was detected after partial hepatectomy in mice. These parameters of *Foxo3* regulation are reestablished with the completion of liver growth and regeneration and support a temporary suspension of p53 and TA-p73 regulatory functions in normal cells during tissue regeneration. p53-dependent and TA-p73–dependent activation of *Foxo3* was also observed in mouse embryonic fibroblasts and in mouse hepatoma cells overexpressing p53, TA-p73α, and TA-p73β isoforms. *Conclusion:* p53 and p73 directly bind and activate the expression of the *Foxo3* gene in the adult mouse liver and murine cell lines. p53, TA-p73, and p300 binding and *Foxo3* expression decrease during liver regeneration, and this suggests a critical growth control mechanism mediated by these transcription factors *in vivo*. (HEPATOLOGY 2010;52:1023-1032)

Abbreviations: Afp, alpha-fetoprotein; Alb, albumin; BCL, B cell lymphoma; Cdkn1, cyclin-dependent kinase inhibitor 1; ChIP, chromatin immunoprecipitation; DAVID, Database for Annotation, Visualization, and Integrated Discovery; Foxo, forkhead box O; H3K14, histone H3 at lysine 14; H3K14Ac, acetylated histone H3 at lysine 14; H3K4me2, dimethylated histone H3 at lysine 4; H3K9, histone H3 at lysine 9; H3K9Ac, acetylated histone H3 at lysine 9; H4Ac, acetylated H4; HA, hemagglutinin; IPA, Ingenuity Pathway Analysis; Jak1, Janus kinase 1; MEF, mouse embryonic fibroblast; mRNA, messenger RNA; n.s., nonspecific site; p53RE, p53 response element; PANTHER, Protein Analysis through Evolutionary Relationships; PCR, polymerase chain reaction; Pea15, phosphoprotein enriched in astrocytes 15; PH, partial hepatectomy; TA-p73, transactivating p73 isoform; Trp73, tumor protein p73; TSS, transcription start site; Tuba1, tubulin alpha 1; WT, wild type. Brn3B, brain specific protein 3B, KAT3A/KAT3B, Lysine acetyltransferases 3A/3B

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Tumor suppressors p53 and p73 are members of a family of proteins with both unique and shared primary functions as transcription factors in mammalian cells.[1] $p53^{+/-}/p73^{+/-}$ mice develop hepatocellular carcinoma at 5 to 7 months of age, and this suggests a pivotal and cooperative role for p53 and p73 in the regulation of hepatic gene expression.[2] Approximately 90% of $p53^{+/-}/p73^{+/-}$ mice with hepatocellular carcinoma have a loss of heterozygosity in tumor protein p73 (*Trp73*), and this further emphasizes the importance of tissue-specific functions of p73 in the liver.[2]

Studies of cancer cell lines, mouse models, and patient samples have clearly established that a loss of p53 and p73 functions is causative in tumor development[2]; however, much less is known about the status and functions of p53 and p73 in normal, quiescent tissues in the absence of cellular stress. Our previous studies have shown that p53 protein levels are developmentally regulated in the mouse liver because p53 is undetectable in newborn mice but increases within 2 weeks and is maintained throughout adulthood.[3] Both p53 and p73 bind to the p53 response element (p53RE) of alpha-fetoprotein (*Afp*) in the liver; they target corepressor proteins and repressive histone modifications to chromatin at the p53RE and *Afp* transcription start site (TSS) and repress *Afp* within 2 to 3 weeks of age.[4,5]

Tumor suppressors p53 and transactivating p73 isoform (TA-p73) regulate the cell cycle, cell death, and senescence through transcriptional activation or repression of target genes.[6] These processes are highly regulated during regeneration of the liver when mature, quiescent hepatocytes reenter the cell cycle, proliferate, and grow in an effort to reestablish liver mass after surgical or chemical removal of liver tissue.[7] To uncover potential liver-specific targets of p53 family members, we combined chromatin immunoprecipitation (ChIP) of TA-p73 in the adult mouse liver and hybridization to microarrays (ChIP/chip) with determinations of global expression during liver regeneration in response to partial hepatectomy (PH). We found a highly restricted number of TA-p73 target genes that changed expression during liver regeneration, and we identified the Forkhead box transcription factor forkhead box O3 (*Foxo3*) as a new target gene of p53 and TA-p73 in the normal quiescent liver.

FoxO3 is a bona fide tumor suppressor that regulates the expression of genes inhibiting the cell cycle and activating apoptosis.[8] Ectopic expression of transcription factor E2F1 has been shown to activate *Foxo3*-driven reporter expression, but direct regulation of endogenous *Foxo3* transcription has not been demonstrated.[9] 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, the FoxO3 protein fails to bind and activate *FOXO3*.[10] Thus, the mechanisms of transcriptional regulation of *FOXO3* remain to be identified.

Our work has shown that p53 and TA-p73 bind to the p53RE of the endogenous *Foxo3* gene in the adult mouse liver and recruit acetyltransferase p300 to activate the chromatin structure and expression of *Foxo3*. In response to PH, the binding of p53, TA-p73, and p300 to the *Foxo3* p53RE is lost, and *Foxo3* expression is decreased during the proliferative stage of liver regeneration; restoration occurs with recovery of liver mass. Our findings establish a direct regulatory link between p53, TA-p73, and FoxO transcription factors, which are growth suppressors in normal tissues: an axis of homeostasis in hepatic cells that is temporarily disrupted during regeneration of the liver.

Materials and Methods

Liver Regeneration in Mice. PH (70% removal of the total liver) or sham control surgery was performed with isoflurane anesthesia.[11] Five to seven C57Bl6/Sv129 mice, 2 months of age, were used for each experimental condition according to the guidelines of the Institutional Animal Care and Use Committee of the M.D. Anderson Cancer Center. Mice were sacrificed 1, 2, 3, 4, or 7 days after surgery; remnant liver tissue was harvested, flash-frozen, and processed for RNA and ChIP analyses.

Cell Culture and Plasmids. Hepa1-6 cells were obtained from the American Type Culture Collection and cultured under standard, suggested conditions. The plasmid encoding wild-type (WT) hemagglutinin (HA)-tagged p53 was previously described.[12] Plasmids encoding HA–TA-p73α and HA–TA-p73β were gifts from G. Melino.[4] Cells were transfected with 4 μg of total DNA per 100-mm plate with Lipofectamine (Invitrogen). Val5 mouse embryonic fibroblasts (MEFs) stably expressing a temperature-sensitive p53 R135V mutant were kindly provided by M. Murphy.[13]

ChIP/Chip Analyses. ChIP was performed on liver tissue lysates from 2-month-old C57Bl6/Sv129 mice with TA-p73 antibody.[4] TA-p73 antibody–enriched DNA and input genomic DNA were differentially labeled and hybridized to an Agilent promoter array

representing one or two 60-mer oligomeric probes within −5.5 and +2.5 kb of 17,000 genes or predicted gene regulatory regions of the mouse genome. Ligation-mediated amplification was used before labeling and hybridization; amplified material was shown to have TA-p73 interaction sites by an analysis for *Afp* and cyclin-dependent kinase inhibitor 1a (*Cdkn1a*) binding.

Microarray Analysis. Liver tissue was collected 0, 0.5, 1, 2, 4, 24, 38, and 48 hours post-surgery. Total RNA (5 μg) from each animal was analyzed with an Affymetrix MGU74 gene array (at 0.5, 1, 2, and 4 hours) and an Affymetrix 430.2 gene array (at 24, 38, and 48 hours) with 12,488 and 45,000 probe sets, respectively, for mouse genes and expressed sequence tags. The variation between arrays using the same RNA sample in a quality control check revealed a difference of less than 2%. Data quality control was performed with Affymetrix Microarray Suite 5.0 and was normalized with Robust Multichip Analysis software. Genes with a negative or positive fold change of 1.5 times or more between time zero and the indicated post-PH time points, with a significance cutoff of $P < 0.005$ (MGU74) or $P < 0.0001$ (430.2), were further analyzed. These microarray data are available at the Gene Expression Omnibus database (accession number GSE20427).[14] ChIP/chip and microarray data sets were annotated and analyzed with Ingenuity Pathway Analysis (IPA),[15] Database for Annotation, Visualization, and Integrated Discovery (DAVID) bioinformatics resources,[16] and the Protein Analysis through Evolutionary Relationships (PANTHER) classification system.[17]

ChIP and RNA Analyses. Chromatin lysate was precleared and incubated overnight with the following specific antibodies for ChIP: histone H3 (Abcam), dimethylated histone H3 at lysine 4 (H3K4me2; Active Motif), acetylated histone H3 at lysine 9 (H3K9Ac; Active Motif), acetylated histone H3 at lysine 14 (H3K14Ac; Upstate/Millipore), acetylated H4 (H4Ac; Upstate/Millipore), p53 (Novocastra), TA-p73 (Santa Cruz), p300 (Santa Cruz), and normal sheep immunoglobulin G (Upstate/Millipore). To analyze antibody protein–bound DNA, primers for real-time quantitative polymerase chain reaction (PCR) and TaqMan (Applied Biosystems, ABI, Foster City, CA) were used (Supporting Table 5). The percentage of the input DNA that was bound was calculated with the cycle threshold difference methodology and was averaged over at least three experiments.

Primers and reverse-transcription PCR determinations of RNA expression were as previously described.[4] Briefly, total RNA from the homogenized mouse liver was extracted with the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Complementary DNA was obtained by the reverse transcription of 2 μg of RNA with the SuperScript system (Invitrogen). Real-time PCR primers for indicated genes are listed in Supporting Table 5.

Immunoblotting. Immunoblotting of whole cell and nuclear lysates was performed with the standard sodium dodecyl sulfate–polyacrylamide gel electrophoresis methodology. Nuclear extracts from liver tissue, collected 2 days after the sham or PH surgery, were prepared as described.[5] The primary antibodies were p53 (Santa Cruz), TA-p73 (Santa Cruz), HA tag (Cell Signaling), FoxO3 (Cell Signaling), and β-actin (Santa Cruz).

Statistical Analysis. Results are expressed as means and standard errors of the mean. Statistical analyses were performed with a t test; P values < 0.05 were considered significant.

Results

Comparative Analysis of Genes Bound by TA-p73 in the Quiescent Liver and Genes That Change Expression During Liver Regeneration. We performed ChIP of liver tissue from 2-month-old WT mice with antibody against TA-p73 and isolated TA-p73–bound chromatin fragments for ChIP/chip. We uncovered 158 genes among the arrayed promoters as potential targets of TA-p73 binding activity in the liver (Supporting Table 1). In a separate set of experiments, we determined genes that changed expression after PH. Functional annotations of genes among the 158 TA-p73 target genes (Fig. 1A) and those that changed expression in response to PH (Fig. 1B,C) were performed with IPA. The analysis yielded 12 categories of function, with cancer, cell death, and cell proliferation receiving the highest number of hits with the lowest P value, as determined by IPA (Fig. 1). As expected, TA-p73–bound genes in the quiescent liver also included development, inflammatory response, cell signaling, and cell cycle categories (Fig. 1A).[18,19] Gene ontology analysis using DAVID and PANTHER bioinformatics tools yielded similar categories and additional ones, such as sensory perception and transcription (Supporting Figs. 1A and 2A). None of these gene targets had been previously reported as p73-regulated, and eight are known targets of p53 [e.g., annexin A1,[20] Notch homolog 1 translocation-associated,[21] and brain-specific angiogenesis inhibitor 1[22]; Supporting Table 1]. Thus, many of the TA-p73 target genes identified by

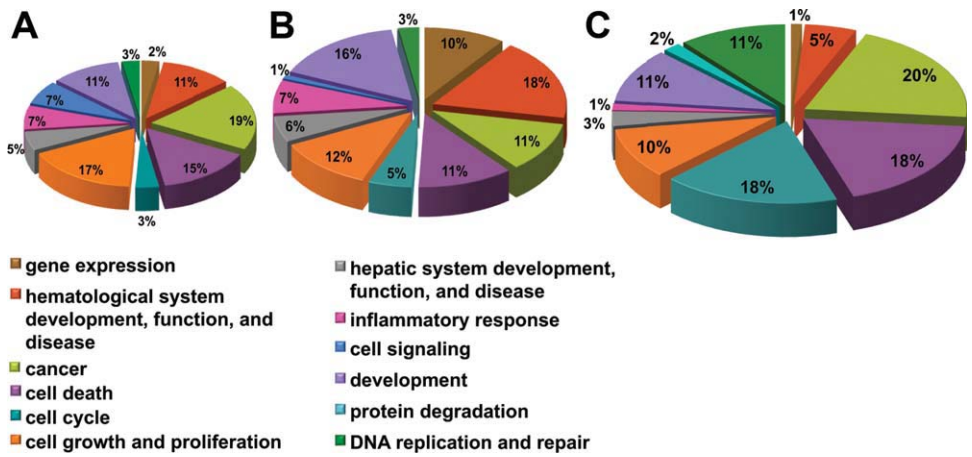


Fig. 1. Functional annotations of p73-bound genes and genes that change expression during liver regeneration. (A) Genes from TA-p73 ChIP/chip, (B) genes that change expression 0.5 to 4 hours post-PH, and (C) genes that change expression 24 to 48 hours post-PH were annotated and analyzed with IPA. Functional categories were selected on the basis of the highest number of genes and lowest *P* values. Fisher's exact test (right-tailed) was used to calculate the *P* value determining the probability that each biological function and/or disease assigned to that data set was due to chance alone. Pie charts were created according to the number of genes in each category.

ChIP/chip are not known to be p53/p73-regulated and potentially define a specific set of TA-p73–regulated genes in the quiescent liver.

Two-thirds PH promotes proliferation of liver cells and rapid growth of the remaining liver tissue and results in complete restoration of the organ mass in approximately 7 days after PH in mice. We collected liver tissue from mice 0, 0.5, 1, 2, 4, 24, 38, and 48 hours after PH for microarray analysis of gene expression. We found 434 genes that changed expression between 0.5 and 4 hours after surgery (Supporting Table 2) and 3807 genes that changed expression between 24 and 48 hours after PH versus time zero (Supporting Table 3).

In agreement with previous observations from more limited expression analyses,[23,24] our microarray analysis of livers 0.5 and 4 hours post-PH showed a significant increase in the expression of early response genes, such as CCAAT/enhancer binding protein beta, Jun oncogene, myelocytomatosis oncogene, tumor necrosis factor receptor superfamily member 1A, hypoxia inducible factor 1 alpha, activating transcription factor 3, and v-ets erythroblastosis virus E26 oncogene homolog 2 (Supporting Table 2). Several responsive genes, not previously reported to be regulated in the context of liver regeneration, included pluripotency regulator Kruppel-like factor 4, transcription factors MAX interacting protein 1 and SIN3 homolog A transcription regulator, and antiapoptotic B cell lymphoma 2 (Bcl2) family member *Bcl2l1* (Supporting Table 2). Functional annotations of genes that changed expression in response to PH at 0.5 to 4 hours included categories also identified for TA-p73–bound genes (Fig. 1B and Supporting Figs. 1B and 2B).

Similar to TA-p73–bound genes in the quiescent liver, genes that changed expression (either increasing or decreasing) during 24 to 48 hours of liver regeneration were associated with cancer, cell death, and cell proliferation (Fig. 1C). Cell signaling and inflammatory response, represented among TA-p73–bound genes, had a hit rate of less than 1% among genes that changed expression in the 24 to 48 hours after PH, and this suggests unique functions for TA-p73 in the liver that are not executed during the 24 to 48 hours of regeneration.

In comparison with earlier time points, genes that changed expression in the regenerating liver 24 to 48 hours post-PH had a significant increase in targets within the cell cycle and DNA replication categories (Fig. 1C and Supporting Figs. 1C and 2C). A direct comparison of gene IDs from a microarray analysis of genes with altered expression 24 to 48 hours post-PH to TA-p73–bound genes yielded 17 TA-p73–bound genes up-regulated or down-regulated in response to PH. This list included a group of transcription factors (cyclin D binding myb-like transcription factor 1; *Foxo3*; and nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 3) as well as cell cycle regulators and plasma membrane receptors (Supporting Table 4).

We hypothesized that the 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 TA-p73. TA-p73 can bind to the same consensus site, simultaneously with p53, at the p53RE of hepatic gene *Afp*.[4] We analyzed TA-p73–bound genes, uncovered by

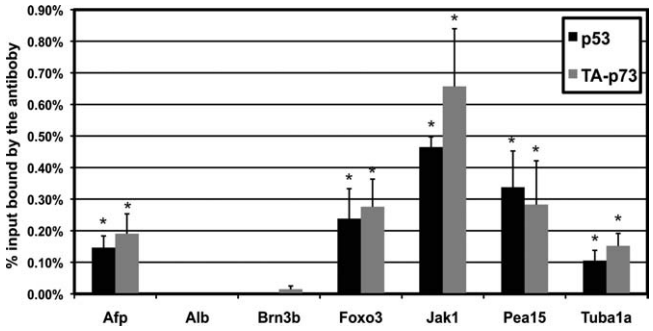


Fig. 2. p53 and TA-p73 binding to genes from TA-p73 ChIP/chip analysis. ChIP from adult mouse liver tissue was performed with antibodies against each p53 and TA-p73. The binding of p53/p73 to the previously identified p53RE upstream of *Afp* served as a positive control; primers to Alb and Brn3B genes were used as negative controls. Averages of at least three independent ChIPs for each p53 and TA-p73 are shown as percentages of the input bound by the antibody. Differences between p53/p73 binding to *Afp*, *Foxo3*, *Jak1*, *Pea15*, and *Tuba1a* genes and *Alb* and *Brn3B* were statistically significant and are marked with asterisks ($*P < 0.05$).

ChIP/chip, as potential p53-regulated gene targets in the normal, quiescent liver. Using a published algorithm to find p53 consensus sites,[25] we mapped potential, shared p53 and TA-p73 (p53/p73) binding sites upstream of four TA-p73–bound genes that changed expression during the 24 to 48 hours of liver regeneration: *Foxo3*, Janus kinase 1 (*Jak1*), phosphoprotein enriched in astrocytes 15 (*Pea15*), and tubulin alpha 1 (*Tuba1*; Supporting Table 4 and Supporting Fig. 3). Binding of p53 and TA-p73 was observed for all examined genes at identified p53REs, and this confirmed that putative targets uncovered by TA-p73 ChIP/chip may be bound by both p53 and TA-p73 in the quiescent liver *in vivo* (Fig. 2). *Afp* p53RE served as a positive control for p53/p73 binding in the quiescent liver, whereas upstream regions of albumin (*Alb*) and brain-specific protein 3B (*Brn3B*) genes served as negative controls for p53 and TA-p73 binding.[4,26] Taken together, these results suggest that p53 and TA-p73 activate or repress target genes in the quiescent liver and that regulatory activities of p53 and TA-p73 change during liver regeneration.

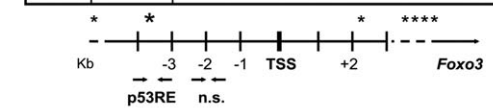
Foxo3 ***Gene Is a New p53/p73 Target.*** Among the 17 TA-p73 gene targets revealed by ChIP/chip, *Foxo3* had the most significant change in expression in response to PH and strong p73 binding (Supporting Table 4). We found a p53 consensus site −3.7 kb upstream of the TSS of *Foxo3* as well as several other potential p53 binding sites within the second and third introns (Fig. 3A). We detected binding of both p53 and TA-p73 to the p53RE −3.7 kb upstream of *Foxo3* (Fig. 3B). To confirm the specificity of p53/p73 binding to the *Foxo3* p53RE, we used primers for a region that contains no p53REs (located −2.0 kb upstream of the *Foxo3* TSS) and saw background levels of interaction (nonspecific region; Fig. 3A,B).

TA-p73 compensates for a loss of p53 by binding to the *Afp* p53RE in the absence of p53[4] and promotes a delayed but significant reduction of *Afp* expression in the liver by 4 months of age in $p53^{-/-}$ mice.[26] We performed ChIP from liver tissue collected from $p53^{-/-}$ mice at 2 months of age and found that TA-p73 binds the p53RE of *Foxo3* in the absence of p53 (Fig. 3C). Thus, both p53 and TA-p73 regulate transcription of *Foxo3* in the adult mouse liver at time zero.

p53 and TA-p73 Activate Expression of the **Foxo3** ***Gene.*** On the basis of known functions of FoxO3 as a tumor suppressor, we hypothesized that p53 and TA-

A

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



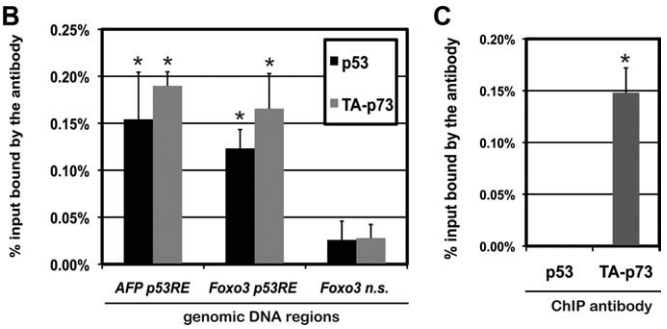


Fig. 3. Foxo3 is a new p53/p73 target gene. (A) p53 DNA binding motifs (p53REs labeled with asterisks) were found upstream and downstream of the *Foxo3* TSS with an algorithm.[41] Primers used for subsequent ChIP experiments for the p53RE site (marked in the table in bold) and the nonspecific site (n.s.) are shown with pairs of arrows. (B) p53 and TA-p73 were found to bind to the p53RE located in the −3.7-kb 5′ *Foxo3* region. ChIP from adult mouse liver tissue was performed with antibodies against each p53 and TA-p73. The 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* (n.s.) were used as negative controls. Averages of at least three independent ChIPs for each p53 and TA-p73 are shown as percentages of the input bound by the antibody. The difference between p53/p73 binding to *Afp* and *Foxo3* p53REs and the n.s. site was statistically significant and is marked with asterisks ($*P < 0.05$). (C) TA-p73 was found to bind Foxo3 p53RE in the absence of p53. ChIP from $p53^{-/-}$ adult liver tissue was performed with antibodies against p53 and TA-p73. The average of three independent ChIPs is shown; TA-p73 binding to *Foxo3* p53REs was statistically significant and is marked with an asterisk ($*P < 0.05$).

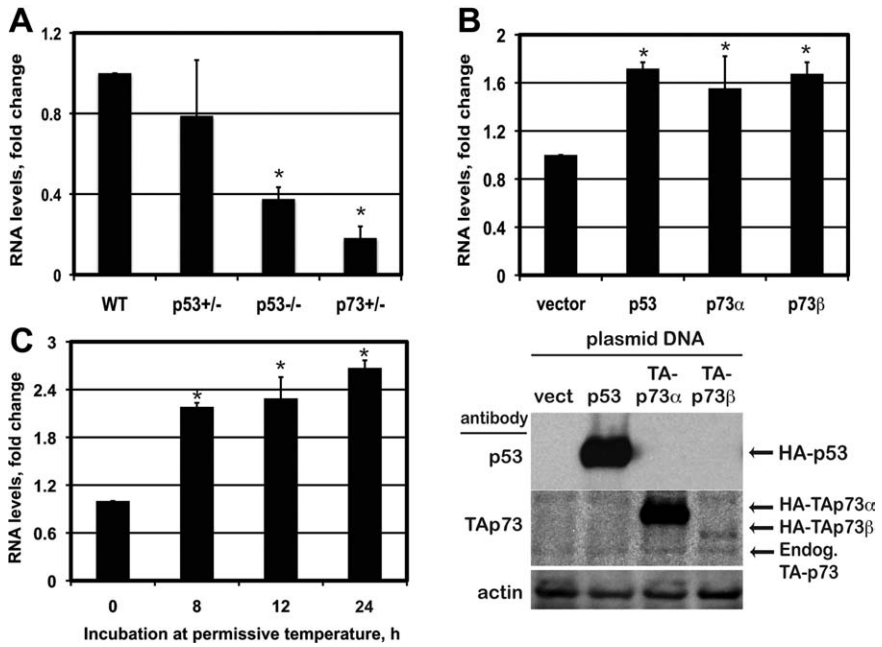


Fig. 4. p53 and p73 activate the expression of the *Foxo3* gene. (A) Foxo3 mRNA levels in $p53^{+/-}$, $p53^{-/-}$, and $p73^{+/-}$ mice (five mice for each genotype) were compared to the Foxo3 expression in WT mice by real-time PCR. The difference in Foxo3 mRNA levels between WT and $p53^{-/-}$ mice and between WT and $p73^{+/-}$ mice was statistically significant and is marked with asterisks ($*P < 0.05$). (B) Hepa1-6 mouse hepatoma cells were transiently transfected with HA-p53, HA–TAp73α, and TA-p73β; mRNA levels were measured by real-time PCR. The average of three independent transfection experiments is shown; the difference between Foxo3 expression in vector-transfected cells and cells overexpressing p53 and TA-p73 was statistically significant ($*P < 0.05$). The immunoblotting in the lower panel shows HA-p53, HA–TAp73α, TA-p73β, and endogenous TA-p73 in transfected Hepa1-6 cells. (C) *Foxo3* expression was measured in immortalized MEFs overexpressing a temperature-sensitive p53 R135V mutant. RNA was isolated from cells incubated at the permissive temperature (32°C) for 0, 8, 12, and 24 hours. The average of three independent experiments is shown; the difference between each time point and time zero was statistically significant and is marked with an asterisk ($*P < 0.05$).

p73 act as positive regulators of *Foxo3* at the level of transcription. We determined levels of *Foxo3* messenger RNA (mRNA) isolated from liver tissue collected from $p53^{+/-}$, $p53^{-/-}$, and $p73^{+/-}$ mice in comparison with WT littermates, and we observed a significant decrease in *Foxo3* expression in $p53^{-/-}$ and $p73^{+/-}$ mice (Fig. 4A).

Transcription of *Trp73* from multiple promoters, together with alternative mRNA splicing, results in at least 28 isoforms of p73.[27] We performed transient transfection of a mouse hepatoma–derived cell line (Hepa1-6)[28] with plasmids that expressed transactivating TA-p73 isoforms, HA–TA-p73α and HA–TA-p73β or HA-p53. A significant increase in endogenous *Foxo3* mRNA levels was observed in cells expressing p53 or TA-p73 (Fig. 4B). Immunoblotting with antibodies against p53 and all TA-p73 isoforms (Fig. 4B, lower panel) showed that HA–TA-p73β was expressed at a lower level than HA–TA-p73α, but the induction of endogenous *Foxo3* expression was comparable (Fig. 4A). This is consistent with increased transcriptional activity previously reported for TA-p73β, which lacks a previously identified, repressive *S*-adenosyl methionine domain, versus other TA-p73 isoforms.[29-31]

To establish cause and effect in the direct transcriptional regulation of *Foxo3* by p53, we used immortalized MEFs expressing $p53^{val135}$, a temperature-sensitive p53 conformational mutant (Val5MEFs; Fig. 4C).[12] In this model system, Val5MEFs that are incubated at a restrictive temperature (37°C) have only cytoplasmic-localized p53, $p53^{val135}$, which is unable to regulate target gene expression. 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* (Fig. 4C). Together, these results demonstrate that endogenous *Foxo3* is activated by p53 and TA-p73 in the mouse liver and by nuclear translocation of p53 or ectopic expression of p53 or TA-p73.

Binding of p53 and p73 to the* Foxo3 *Gene Is Decreased During Liver Regeneration. Our analysis of global gene expression levels (Supporting Tables 2 and 3) suggested that *Foxo3* expression decreased in the 24 to 48 hours following PH. We determined whether the loss of *Foxo3* expression in the regenerating liver occurred as a result of decreased p53/p73 binding to chromatin at the p53RE of *Foxo3*. We performed ChIP analysis of liver tissue (collected 1, 2, 4,

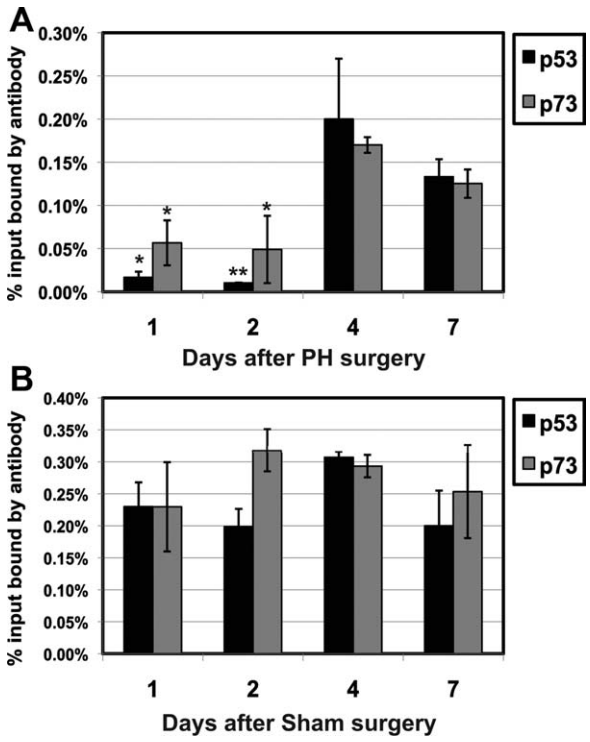


Fig. 5. p53/p73 binding to the *Foxo3* 5′ region changes during liver regeneration. (A) ChIP from mouse liver tissue collected 1, 2, 4, and 7 days after PH surgeries was performed with antibody against p53 and TA-p73, and the results are shown as percentages of the input bound by the antibody. (B) ChIP from mouse liver tissue collected 1, 2, 4, and 7 days after sham surgeries was performed in the same way. Statistically significant differences between p53/p73 binding in the PH liver and the sham-operated liver at corresponding time points are marked with asterisks (*$P < 0.05$, **$P < 0.01$).

and 7 days after PH and sham surgeries) with antibodies recognizing p53 and TA-p73. The chromatin interaction of p53 at the *Foxo3* p53RE was dramatically reduced on days 1 and 2 after PH, and this was accompanied by an equally significant reduction in TA-p73 binding (Fig. 5A). Binding of both p53 and TA-p73 was partially restored on days 4 and 7 of liver regeneration (Fig. 5A), but it was not equivalent to the level of binding observed in sham-operated mice (Fig. 5B); this suggests that regulatory mechanisms in addition to those mediated by p53 and TA-p73 may activate *Foxo3*.

Expression of* Foxo3 *Is Lost During the Proliferative Stage of Liver Regeneration and Is Restored During Termination of Liver Growth. Microarray analysis of early time points (0.5-4 hours) showed no significant change in *Foxo3* expression (Supporting Table 2) in comparison with time zero; a significant decrease in *Foxo3* expression was observed in livers collected 24, 38, and 48 hours after PH (Supporting Table 3). This result suggests that a loss of *Foxo3* expression occurs specifically during the cell cycle G_1-S-G_2 transition. We performed sets of PH and sham surgeries on 2-month-old WT mice and collected their livers at 1, 2, 3, 4, and 7 days. We observed a significant decrease in *Foxo3* mRNA levels between 1 and 3 days after PH in comparison with time zero, with the lowest *Foxo3* expression on day 2 (Fig. 6A). FoxO3 protein levels were also reduced in hepatic nuclei on day 2 after PH (Supporting Fig. 4), and this suggests that transcriptional functions of FoxO3 are inhibited during the proliferative stage of regeneration. Importantly, several known target genes of FoxO3 changed expression during days 1 and 2 of liver regeneration (*Bcl6*, cyclin D1, cyclin G2, sirtuin 1, and superoxide dismutase 2; Supporting Table 3).[32] *Foxo3* expression gradually returned to the time zero level on day 4 after PH (Fig. 6A); final adjustments of the regenerating liver tissue restored a normal liver/body weight index by 7 days in the mice (data not shown). No significant difference in *Foxo3* expression was observed after sham surgeries in comparison with time zero (Fig. 6B).

Foxo3 ***Expression Is Regulated by p53/p73-Mediated Recruitment of Acetyltransferase p300.*** Gene expression is associated with an active chromatin structure [e.g., dimethylation of histone H3 at lysine 4

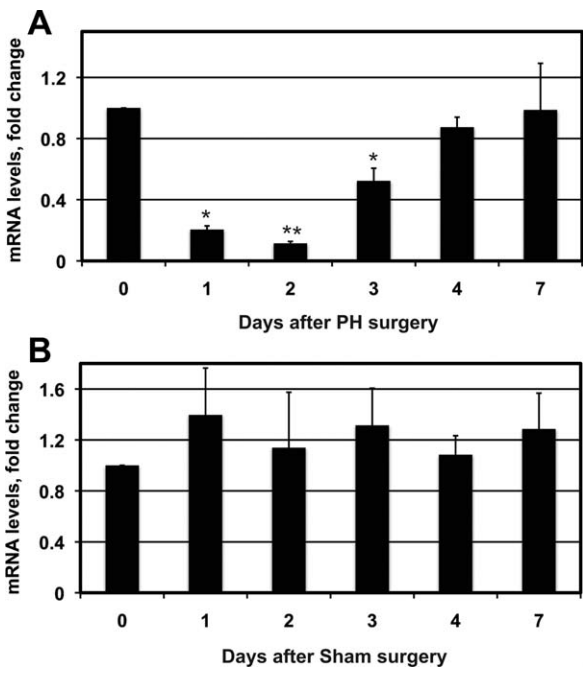


Fig. 6. Foxo3 expression decreases 1 to 3 days after PH and increases when regeneration is complete (4-7 days). Foxo3 mRNA was isolated from mouse liver tissue at the indicated time points after surgery. Relative expression levels were measured by real-time PCR with primers to *Foxo3*. (A) Foxo3 expression in response to 65% PH. Time points after PH with statistically significant differences in Foxo3 expression versus the sham surgery are marked with asterisks (*$P < 0.05$, **$P < 0.01$). (B) Foxo3 expression in response to the sham surgery. There was no statistically significant difference in Foxo3 mRNA levels between time zero and all time points after sham surgeries.

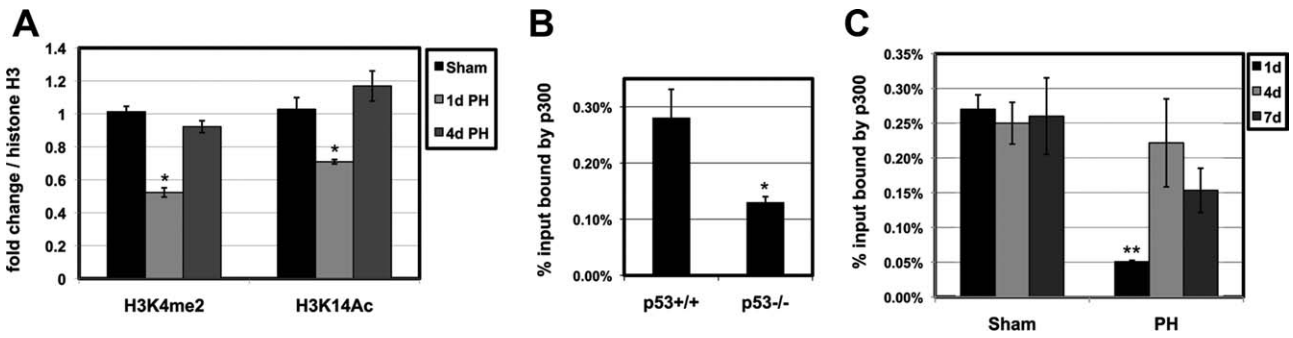


Fig. 7. Acetyltransferase p300 binds *Foxo3* p53RE. (A) Histone modifications in the *Foxo3* p53RE region during liver regeneration. ChIP from mouse liver tissue collected 1 and 4 days after sham and PH surgeries was performed with antibody against histone H3 and modified histone H3 [dimethylated on lysine 4 (H3K4me2) and acetylated on lysine 14 (H3K14Ac)]. The average of three independent ChIPs for each time point is shown as a fold change in H3K4me2 and H3K14Ac over the total H3 bound to *Foxo3* p53RE in PH versus the sham surgery. The statistically significant differences between PH and sham surgery are marked with asterisks (*$P < 0.05$). (B) p53/p73 recruited p300 to the p53RE of the 5′ *Foxo3* region. ChIP was performed with antibodies against p300. The average of three independent ChIPs for each genotype is shown as the percentage of the input bound by the antibody. The difference between p300 binding to *Foxo3* p53REs in the WT mice and the $p53^{-/-}$ mouse liver was statistically significant and is marked with an asterisk (*$P < 0.05$). (C) p300 binding to *Foxo3* p53RE was lost during the proliferative stage of liver regeneration. ChIP experiments with liver tissue collected 1, 4, and 7 days after PH and sham surgeries were performed with antibodies against p300. The average of three independent ChIPs for each time point of PH and sham surgeries is shown; the decrease in p300 binding to *Foxo3* p53REs on day 1 post-PH was statistically significant and is marked with asterisks (**$P < 0.01$).

(H3K4me2) and acetylation of histone H3 at lysine 9 (H3K9), histone H3 at lysine 14 (H3K14), and several lysines of histone H4]. Using ChIP analysis of liver tissue collected 1 day after PH and sham surgeries, we tested whether histone modifications, associated with active chromatin, decreased with a loss of p53 and TA-p73 binding in the *Foxo3* p53RE region. We observed decreased levels of H3K4me2 and H3K14Ac (Fig. 7A) and a decrease in global H4 acetylation (Supporting Fig. 5) without a significant change in H3K9 acetylation (data not shown) at the *Foxo3* p53RE in the regenerating liver on day 1 versus day 4 and the sham-operated mice. Although several methyltransferases and acetyltransferases modify H3K4, H3K14, and H4 residues, p53 and p73 are known to recruit CBP/p300 (lysine acetyltransferase 3A/3B [KAT3A/KAT3B]) to activate the transcription of target genes.[33,34] Using an antibody against p300, we observed a significant decrease in p300 binding to the *Foxo3* p53RE region of chromatin in the quiescent liver of $p53^{-/-}$ mice (Fig. 7B). Binding of p300 to the *Foxo3* p53RE decreased even further in the regenerating liver of WT mice on 1 day post-PH (Fig. 7C), and this suggests that both p53 and p73 contribute to the recruitment of p300 to activate expression of *Foxo3* in the quiescent liver. Recruitment of p300 was restored on day 4 after PH along with p53 and p73.

Discussion

A genome-wide evaluation of p53-bound and p73-bound sequences by ChIP/chip analysis, using cultured cells, has shown that 72% of p53-bound sites are also bound by p73.[35] In the current study, we uncovered 158 genes bound by TA-p73 in normal quiescent liver cells. Ten genes were known p53 targets, with the remainder not previously connected to p53/p73-mediated transcriptional regulation. In a recent review, Riley et al.[36] identified stringent criteria for bona fide p53 target genes and generated a list of 129 genes containing at least one p53RE per gene. In our ChIP/chip experiments, we identified a similar number of TA-p73 target genes and confirmed binding of both p53 and TA-p73 to the p53REs of four target genes: *Foxo3*, *Jak1*, *Pea15*, and *Tuba1a*. Annotation of the 158 hepatic target genes yielded physiological categories of established p53/p73 functions (e.g., cancer, cell death, and cell proliferation). Other TA-p73–bound genes, associated with inflammatory response and development, are supported by the phenotype of $p73^{-/-}$ mice, which display severe developmental and inflammatory defects and die soon after birth.[18]

Our work identifies the *Foxo3* gene as a new target of p53 and TA-p73 in the quiescent mouse liver. Loss of FoxO3-mediated regulation of transcription is directly linked to increased proliferation and tumorigenesis in human cancers[8,37]; however, mechanisms that control *Foxo3* itself remain poorly defined. We suggest that p53, p73, and FoxO3 function along the same axis in a tumor suppressor network because many target genes of FoxO3 are also known p53/p73 targets [e.g., *Cdkn1a* (p21), *Cdkn1b* (p27), and growth arrest and DNA-damage-inducible alpha]. In turn, functions of FoxO3 as a transcription factor augment

p53 proapoptotic activity.[38] Previous studies have suggested that p53 and FOXO3 proteins interact as a protein complex.[39] Whether feed-forward regulation of antiproliferative genes by a p53-FoxO3 complex in the quiescent liver is disrupted during regeneration because of decreased levels of FoxO3 protein and a loss of p53–target gene interactions requires further study. Overall, our results establish a direct, linear pathway between p53 family members and FoxO tumor suppressors in normal tissues.

Despite many studies of p53/p73 target genes, mechanisms of endogenous p53 and TA-p73–mediated transcriptional regulation are understood for a very limited number of target genes *in vivo*. In quiescent liver cells, we found p53-dependent recruitment of histone acetyltransferase p300 at the *Foxo3* p53RE to be a mechanism of histone modification and gene activation *in vivo*, and this was in agreement with the direct interaction of p53/p73 with p300 observed in cultured cells.[33,34] Binding of p300 at the *Foxo3* p53RE is decreased but not lost in the livers of $p53^{-/-}$ mice, and this suggests that endogenous p73, independently of p53, relies on similar coregulators of transcription and partially compensates for a loss of p53. Recruitment of p300 and activation of *Foxo3* expression significantly decrease during day 1 of liver regeneration when both p53 and p73 show a loss of binding to the *Foxo3* p53RE; this provides additional evidence for p53/p73-mediated recruitment of p300 as a critical mechanism to activate expression of the *Foxo3* gene in the quiescent liver. Thus, we conclude that both p53 and TA-p73 lose the ability to bind and regulate expression of specific hepatic genes during the G_0-G_1-S transition.

Importantly, p53/p73 binding to the *Foxo3* p53RE is restored when liver regeneration is complete, and this leads to activation of *Foxo3* expression as hepatocytes reenter G_0. Similarly, p53 binding to *Afp* is restored 7 days after PH,[5] but transcriptional activity of p53 is not required for termination of liver regeneration.[40] TA-p73 compensates for a lack of p53 in the repression of *Afp* during liver development,[4] and similar mechanisms may be in play during the regeneration of hepatocytes in $p53^{-/-}$ mice. This hypothesis cannot be tested in $p73^{-/-}$ mice because these mice succumb to developmental and inflammatory defects soon after birth.[18] As a bona fide tumor suppressor, FoxO3 is aligned with p53 and p73 in regulating transcription in normal tissues. Their functions in the surveillance of normal cells are temporarily disrupted during liver regeneration; mechanisms that restore their regulatory functions are of considerable interest for future studies.

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