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Direct activation of *Foxo3* by tumor suppressors p53 and p73 is disrupted during liver regeneration in mice

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Abstract

The p53-family of proteins regulates 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 TA-p73 have hepatic-specific functions in development and tumor suppression. Here, we determined TA-p73-interactions with chromatin in adult mouse liver and found Forkhead factor *Foxo3* as one of 158 gene targets. Global profiling of hepatic gene expression in regenerating versus quiescent liver revealed specific, functional categories of genes regulated over 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 and maintain active expression in quiescent liver. During regeneration of liver, binding of p53 and TA-p73, recruitment of acetyltransferase p300 and an active chromatin structure of *Foxo3* are disrupted, alongside loss of *Foxo3* expression. Consistent with the loss of *Foxo3* transcriptional activation, a decrease of histone activation marks (H3K4me2, H3K14Ac and H4Ac) at *Foxo3* p53RE was detected following partial hepatectomy in mice. These parameters of *Foxo3* regulation are reestablished at completion of liver growth and regeneration, supporting a temporary suspension of p53 and TA-p73 regulatory functions in normal cells during tissue regeneration. p53 and TA-p73-dependent activation of *Foxo3* is also observed in mouse embryonic fibroblasts and in mouse hepatoma cells overexpressing p53, TA-p73 α and TA-p73 β isoforms.

Conclusion—p53 and p73 directly bind and activate expression of *Foxo3* gene in adult mouse liver and murine cell lines. p53, TA-p73, and p300 binding and *Foxo3* expression decrease during liver regeneration, suggesting a critical growth control mechanism mediated by these transcription factors *in vivo*.

Keywords

transcription; partial hepatectomy; acetyltransferase; quiescence

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). The p53^{+/-}; p73^{+/-} mice develop hepatocellular carcinoma (HCC) at 5-7 months of age, suggesting a pivotal and cooperative role for p53 and p73 in regulation of hepatic gene expression (2). Approximately 90% of p53^{+/-};p73^{+/-} mice with HCC have loss of heterozygosity in *Trp73*, further emphasizing the importance of tissue-specific functions of p73 in liver (2).

Studies of cancer cell lines, mouse models, and patient samples clearly establish that 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 show that p53 protein levels are developmentally regulated in mouse liver, as p53 is undetectable in newborn mice but increases within two weeks and maintained throughout adulthood (3). Both p53 and p73 bind to the p53 response element (p53RE) of *Afp* in liver, targeting co-repressor proteins and repressive histone modifications to chromatin at the p53RE and *Afp* transcription start site (TSS), and repressing *Afp* within 2-3 weeks of age (4, 5).

Tumor suppressors p53 and TA-p73 regulate cell death, cell cycle, and senescence through transcriptional activation or repression of target genes (6). These processes are highly regulated during regeneration of liver when mature, quiescent hepatocytes re-enter cell cycle, proliferate and grow, in efforts 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 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 identified the Forkhead box transcription factor *Foxo3* as a new target gene of p53 and TA-p73 in normal quiescent liver.

FoxO3 is a *bona fide* tumor suppressor that regulates expression of genes that inhibit cell cycle and activate apoptosis (8). Ectopic expression of transcription factor E2F1 was 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, FoxO3 protein fails to bind and activate *FOXO3* (10). Thus, the mechanisms of transcriptional regulation of *FOXO3* remain to be identified.

Our work shows that p53 and TA-p73 bind to the p53RE of the endogenous *Foxo3* gene in adult mouse liver and recruit acetyltransferase p300 to activate chromatin structure and expression of *Foxo3*. In response to PH, 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, and 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 liver.

Materials and Methods

Liver regeneration in mice

PH (70% removal of total liver) or Sham control surgery was performed using isoflurane anesthesia (Cold Spring Harb. Protoc.; 2006; doi:10.1101/pdb.prot4384). 5-7 C57Bl6/Sv129

mice, 2 months of age, were used for each experimental condition according to the MD Anderson Cancer Center Institutional Animal Care and Use Committee guidelines. Mice were sacrificed 1, 2, 3, 4, or 7 days following surgeries; remnant liver tissue was harvested, flash-frozen, and processed for RNA and ChIP analyses.

Cell Culture and Plasmids

Hepa1-6 cells were obtained from ATCC and cultured under standard, suggested conditions. The plasmid encoding wild type HA-tagged p53 was previously described (11). Plasmids encoding HA-TA-p73 α and HA-TA-p73 β were gifts of G. Melino (4). Cells were transfected with 4 μ g of total DNA/100-mm plate using Lipofectamine (Invitrogen). Val5 mouse embryonic fibroblasts (MEFs) stably expressing temperature-sensitive p53 R135V mutant were kindly provided by M. Murphy (12).

ChIP/chip analyses

Chromatin immunoprecipitation (ChIP) was performed on liver tissue lysate 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 Agilent promoter array representing 1-2 60-mer oligomeric probes within -5.5kb and +2.5kb of 17,000 genes or predicted gene regulatory regions of the mouse genome. Ligation-mediated amplification was used prior to labeling and hybridization; amplified material was shown to have TA-p73-interaction sites by analysis for *Afp* and *Cdkn1a* binding.

Microarray analysis

Liver tissue was collected at T=0, 0.5, 1, 2, 4, 24, 38, and 48 hours post-surgery. 5 μ g of total RNA, from each individual animal, were analyzed by Affymetrix MGU74 (T = 0.5, 1, 2, 4 hours) and Affymetrix 430.2 (T = 24, 38, 48 hours) gene arrays, 12,488 and 45,000 probe sets respectively, for mouse genes and expressed sequence tags. Variation between arrays using the same RNA sample in a quality control check revealed less than 2% difference. Data quality control was done with Affymetrix Microarray Suite (MAS) 5.0 and normalized by Robust Multichip Analysis (RMA) software. Genes with a negative or positive fold change of 1.5 times or more between T=0 and indicated post-PH time points, with a significance cut-off of p-value < .005 (MGU74) or < 0.0001 (430.2), were further analyzed. These microarray data are available on the GEO database (accession number GSE20427) at <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE20427>. ChIP/chip and microarray data sets were annotated and analyzed by Ingenuity Pathway Analysis (IPA, Ingenuity® Systems, www.ingenuity.com), DAVID Bioinformatics Resources (13) and Panther Classification System (14).

ChIP and RNA analyses

Chromatin lysate was precleared and incubated overnight with the following specific antibodies for ChIP: histone H3 (Abcam), H3K4me2 (Active Motif), H3K9Ac (Active Motif), H3K14Ac (Upstate/Millipore), H4Ac (Upstate/Millipore), p53 (Novocastra), TA-p73 (Santa Cruz), p300 (Santa Cruz), and normal sheep IgG (Upstate/Millipore). To analyze antibody-protein-bound DNA, primers for real-time quantitative PCR (qPCR), and TaqMan (Applied Biosystems, ABI, Foster City, CA) were used (Supplementary Table 5). The percentage of input DNA bound was calculated by dCt methodology and averaged over at least three experiments.

Primers and reverse transcription-PCR determinations of RNA expression were as previously described (4). Briefly, total RNA from homogenized mouse liver was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was

obtained by reverse transcription of 2 ug of RNA using the SuperScript system (Invitrogen). Real-time PCR primers for indicated genes are in Supplementary Table 5.

Immunoblotting

Immunoblotting of whole-cell and nuclear lysates was performed using standard SDS-PAGE methodology. Nuclear extracts from liver tissue, collected at 2 days following Sham and PH surgery, were prepared as described (5). The primary antibodies used are: 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 standard error of the mean (SEM). Statistical analyses were performed by t-test; p values <0.05 were considered significant.

Results

Comparative analysis of genes bound by TA-p73 in 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 liver (Supplementary Table 1). In a separate set of experiments, we determined genes that change expression after PH. Functional annotations of genes among the 158 TA-p73 target genes (Figure 1A) and those that changed expression in response to PH (Figure 1B and 1C) were performed using 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 (Figure 1). As expected, TA-p73-bound genes in the quiescent liver also included development, inflammatory response, cell signaling, and cell cycle categories (Figure 1A) (15, 16). Gene ontology analysis using DAVID and Panther bioinformatics tools yielded similar categories, including additional ones, such as sensory perception and transcription (Supplementary Figure 1A and 2A). Of these gene targets, none were previously reported as p73-regulated and 8 are known targets of p53 (e.g., *Annexin A1* (17), *Notch1* (18), and *Bai1* (19), Supplementary Table 1). Thus, many of the TA-p73 target genes identified by ChIP/chip are not known as p53/p73-regulated and potentially define a specific set of TA-p73-regulated genes in quiescent liver.

Two-thirds PH promotes proliferation of liver cells and rapid growth of the remaining liver tissue, resulting in complete restoration of organ mass in approximately 7 days following PH in mice. We collected liver tissue from mice at T=0, 0.5, 1, 2, 4, 24, 38, and 48 hours following PH for microarray analysis of gene expression. We found 434 genes that changed expression between 0.5 – 4 h after surgery (Supplementary Table 2), and 3807 genes that changed expression between 24 - 48 h following PH, compared to T=0 (Supplementary Table 3).

Consistent with previous observations utilizing more limited expression analyses (20, 21), our microarray analysis of 0.5-4 h post-PH livers showed a significant increase in expression of ‘early response’ genes, e.g., *C/EBP β* , *Jun*, *Myc*, *Tnfrsf1a*, *Hif1a*, *Atf3*, *Ets2* (Supplementary Table 2). Several responsive genes, not previously reported as regulated in the context of liver regeneration, included pluripotency regulator *Klf4*, transcription factors *Mxi1* and *Sin3a*, as well as anti-apoptotic Bcl2 family member *Bcl2l1* (Supplementary Table 2). Functional annotations of genes that changed expression in response to PH at 0.5-4 h

included categories also identified for TA-p73-bound genes (Figure 1B, Supplementary Figure 1B and 2B).

Similar to TA-p73-bound genes in quiescent liver, genes that changed expression (either increasing or decreasing) during 24-48 hours of liver regeneration were associated with cancer, cell death, and cell proliferation (Figure 1C). Cell signaling and inflammatory response, represented among TA-p73-bound genes, had less than 1% hits among genes that changed expression at 24-48 hours following PH, suggesting unique functions for TA-p73 in liver that are not executed during 24-48 hours of regeneration.

Compared to earlier time points, genes that changed expression in regenerating liver at 24-48 hours post-PH had a significant increase in targets within cell cycle and DNA replication categories (Figure 1C, Supplementary Figure 1C and 2C). Direct comparison of gene IDs, from microarray analysis of genes with altered expression 24-48 hours post-PH to TA-p73-bound genes, yielded seventeen TA-p73-bound genes up- or down-regulated in response to PH. This list included a group of transcription factors, *Dmtf1*, *Foxo3*, and *Nfatc3*, as well as cell cycle regulators and plasma membrane receptors (Supplementary 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 ChIP/chip, as potential p53-regulated gene-targets in normal, quiescent liver. Using a published algorithm to find p53 consensus sites (22), we mapped potential, shared p53 and TA-p73 (p53/p73) binding sites upstream of four TA-p73-bound genes that changed expression during 24-48 h of LR: *Foxo3*, *Jak1*, *Pea15*, and *Tuba1* (Supplementary Table 4 and Supplementary Figure 3). Binding of p53 and TA-p73 was observed for all examined genes at identified p53REs, thus confirming that putative targets uncovered by TA-p73 ChIP/chip may be bound by both p53 and TA-p73 in quiescent liver *in vivo* (Figure 2). *Afp* p53RE served as a positive control for p53/p73 binding in quiescent liver, whereas upstream regions of *Alb* and *Brn3B* genes served as negative controls for p53 and TA-p73 binding (4, 23). Taken together, these results suggest that p53 and TA-p73 activate or repress target genes in quiescent liver, and that regulatory activities of p53 and TA-p73 change during liver regeneration.

Forkhead Transcription Factor O3 (*Foxo3*) gene is a new p53/p73 target

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

TA-p73 compensates for 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 liver by 4-months of age in p53^{-/-} mice (23). 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 (Figure 3C). Thus, both p53 and TA-p73 regulate transcription of *Foxo3* in adult mouse liver at T=0.

p53 and TA-p73 activate expression of Foxo3 gene

Based on known functions of FoxO3 as a tumor suppressor, we hypothesized that p53 and TA-p73 act as positive regulators of *Foxo3* at the level of transcription. We determined levels of *Foxo3* mRNA, isolated from liver tissue collected from p53^{+/-}, p53^{-/-}, and p73^{+/-} mice, in comparison to *wt* littermates, and observed a significant decrease of *Foxo3* expression in p53^{-/-} and p73^{+/-} mice (Figure 4A).

Transcription of *Trp73* from multiple promoters, together with alternative mRNA splicing, results in at least 28 isoforms of p73 (24). We performed transient transfection of a mouse hepatoma-derived cell line Hepa1-6 (25), with plasmids that express “transactivating” TA-p73 isoforms, HA-TA-p73 α and HA-TA-p73 β or HA-p53. A significant increase in endogenous *Foxo3* mRNA levels is observed in cells expressing p53 or TA-p73 (Figure 4B). Immunoblotting with antibodies against p53 and all TA-p73 isoforms (Figure 4B, lower panel) shows that HA-TA-p73 β is expressed at a lower level than HA-TA-p73 α , but induction of endogenous *Foxo3* expression is comparable (Figure 4A). This is consistent with increased transcriptional activity previously reported for TA-p73 β , which lacks a previously identified, repressive SAM domain, versus other TA-p73 isoforms (26-28).

To establish cause-and-effect in direct transcriptional regulation of *Foxo3* by p53, we used immortalized MEFs that express a temperature-sensitive p53 conformational mutant: p53^{val135} (Val5MEFs, Figure 4C) (12). In this model system, Val5MEFs incubated at a restrictive temperature (37°C) have only cytoplasmic-localized p53, p53^{val135}, which is unable to regulate target gene expression. At the permissive temperature of 32°C, p53^{val135} assumes a *wt* conformation and moves to the nucleus to activate or repress its target genes, including endogenous *Foxo3* (Figure 4C). Together, these results demonstrate that endogenous *Foxo3* is activated by p53 and TA-p73 in mouse liver, as well as by nuclear translocation of p53 or ectopic expression of p53 or TA-p73.

Binding of p53 and p73 to Foxo3 gene is decreased during liver regeneration

Our analysis of global gene expression levels (Supplementary Table 2 and 3) suggested that *Foxo3* expression is decreased at 24-48 hours following PH. We determined if loss of *Foxo3* expression in regenerating liver, occurs as a result of decreased p53/p73 binding to chromatin at the p53RE of *Foxo3*. We performed ChIP analysis of liver tissue, collected at 1, 2, 4 and 7 days after PH and Sham surgeries, with antibodies that recognize p53 and TA-p73. Chromatin interaction of p53 at the *Foxo3* p53RE was dramatically reduced at day 1 and 2 after PH, accompanied by an equally significant reduction in TA-p73 binding (Figure 5A). Binding of both p53 and TA-p73 is partially restored at days 4 and 7 of liver regeneration (Figure 5A), but is not equivalent to the level of binding observed in Sham (Figure 5B), suggesting 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 h) showed no significant change in *Foxo3* expression (Supplementary Table 2), compared to T=0; a significant decrease in *Foxo3* expression was observed in liver collected at 24, 38, and 48 hours following PH (Supplementary Table 3). This result suggests that loss of *Foxo3* expression occurs specifically during cell cycle G₁-S-G₂ transition. We performed sets of PH and Sham surgeries on 2-month-old *wt* mice, collecting liver at 1, 2, 3, 4, and 7 days. We observed a significant decrease in *Foxo3* mRNA levels between 1-3 days after PH, compared to T=0, with lowest *Foxo3* expression on day 2 (Figure 6A). FoxO3 protein levels were also reduced in hepatic nuclei on day 2 following PH (Supplementary Figure 4), suggesting that

transcriptional functions of FoxO3 are inhibited during the proliferative stage of regeneration. Importantly, several known target genes of FoxO3 changed expression during day 1 and 2 of liver regeneration (*Bcl6*, *Ccnd1*, *Ccng2*, *Sirt1*, and *Sod2*, Supplementary Table 3) (29). *Foxo3* expression gradually returned to T=0 level on day 4 following PH (Figure 6A), when final adjustments of regenerating liver tissue restores a normal liver/body weight index by 7 days in mice (data not shown). No significant difference in *Foxo3* expression is observed following Sham surgeries, compared to T=0 (Figure 6B).

Foxo3 expression is regulated by p53/p73-mediated recruitment of acetyltransferase p300

Gene expression is associated with active chromatin structure, *e.g.* dimethylation of histone H3 at lysine 4 (H3K4me2) and acetylation of H3K9, 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, decrease with loss of p53 and TA-p73 binding at the *Foxo3* p53RE region. We observed decreased H3K4me2 and H3K14Ac (Figure 7A) and a decrease of global H4 acetylation (Supplementary Figure 5) without a significant change in H3K9 acetylation (data not shown), at the *Foxo3* p53RE, in regenerating liver on day 1, compared to day 4 and Sham. While several methyltransferases and acetyltransferases modify H3K4, H3K14, and H4 residues, p53 and p73 are known to recruit CBP/p300 (KAT3A/KAT3B) to activate transcription of target genes (30, 31). Using an antibody against p300, we observed a significant decrease in p300 binding to the *Foxo3* p53RE region of chromatin, in quiescent liver of p53^{-/-} mice (Figure 7B). Binding of p300 to the *Foxo3* p53RE decreased even further in regenerating liver of *wt* mice at 1 day post-PH (Figure 7C), suggesting that both p53 and p73 contribute to the recruitment of p300 to activate expression of *Foxo3* in quiescent liver. Recruitment of p300 was restored at day 4, following PH, alongside p53 and p73.

Discussion

Genome-wide evaluation of p53- and p73-bound sequences by ChIP/chip analysis, using cultured cells, showed that 72% of p53-bound sites are also bound by p73 (32). 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.* identified stringent criteria for *bona fide* p53 target genes, and generated a list of 129 genes containing at least one p53RE per gene (33). 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 (15).

Our work identifies the *Foxo3* gene as a new target of p53 and TA-p73 in quiescent mouse liver. Loss of FoxO3-mediated regulation of transcription is directly linked to increased proliferation and tumorigenesis in human cancers (8, 34); 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, since many target genes of FoxO3 are also known p53/p73 targets, *e.g.*, *Cdkn1a* (p21), *Cdkn1b* (p27), and *Gadd45a*. In turn, functions of FoxO3, as a transcription factor, augment p53 pro-apoptotic activity (35). Previous studies suggest that p53 and FOXO3 proteins interact as a protein complex (36). Whether feed-forward regulation of anti-proliferative genes by a p53-FoxO3 complex in quiescent liver is disrupted during regeneration, due to decreased levels of FoxO3 protein and 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, as a mechanism of histone modification and gene activation *in vivo* and in agreement with direct interaction of p53/p73 with p300 observed in cultured cells (30, 31). Binding of p300 at the *Foxo3* p53RE is decreased but not lost in liver of p53^{-/-} mice, suggesting that endogenous p73, independently of p53, relies on similar co-regulators of transcription and partially compensates for 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 loss of binding to the *Foxo3* p53RE, providing additional evidence for p53/p73-mediated recruitment of p300 as a critical mechanism to activate expression of the *Foxo3* gene in quiescent liver. Thus, we conclude that both p53 and TA-p73 lose the ability to bind and regulate expression of specific hepatic genes during G₀-G₁-S transition.

Importantly, p53/p73 binding to the *Foxo3* p53RE is restored when liver regeneration is complete, leading to activation of *Foxo3* expression as hepatocytes re-enter G₀. Similarly, p53 binding to *Afp* is restored at 7 days following PH (5), but transcriptional activity of p53 is not required for termination of liver regeneration (37). TA-p73 compensates for lack of p53, in repression of *Afp* during liver development (4), and similar mechanisms may be in play during regeneration of hepatocytes in p53^{-/-} mice. This hypothesis cannot be tested in p73^{-/-} mice, as these mice succumb to developmental and inflammatory defects soon after birth (15). As a *bona fide* tumor suppressor, FoxO3 is aligned with p53 and p73 in regulating transcription in normal tissues. Their functions in surveillance of normal cells are temporarily disrupted during liver regeneration; mechanisms that restore their regulatory functions are of considerable interest for future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HCC	Hepatocellular carcinoma
p53RE	p53 response element
ChIP	chromatin immunoprecipitation
PH	partial hepatectomy
FoxO3	Forkhead box O3
IPA	Ingenuity Pathway Analysis
TA-p73	transactivating p73 isoform
HA	hemagglutinin
HSC	hematopoietic stem cells

MEF mouse embryonic fibroblasts
TSS transcription start site

Figure 1.

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

Figure 2.

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

Figure 3.

Foxo3 is a new p53/p73 target gene. (A) p53-DNA binding motifs (p53 response elements, p53REs, labeled with asterisks) are found upstream and downstream of the *Foxo3* TSS using an algorithm <http://linkage.rockefeller.edu/p53/>. Primers used for consequent ChIP experiments for the p53RE site (marked in the table in bold) and to the non-specific site (n.s.) are shown with pairs of arrows below. (B) p53 and TA-p73 bind to the p53RE located at -3.7 kb 5' *Foxo3* region. ChIP from adult mouse liver tissue was performed using antibodies against each p53 and TA-p73. Binding of p53/p73 to the previously identified p53RE upstream of *Afp* served as a positive control. Primers to the -2 kb region of *Foxo3* (non-specific site, n.s.) was used as a negative control. Average of at least three independent ChIPs for each p53 and TA-p73 is shown as percentage of input bound by the antibody. The difference between p53/p73 binding to *Afp* and *Foxo3* p53REs as compared to n.s. site is statistically significant and marked with asterisks (* $p < 0.05$). (C) TA-p73 binds Foxo3 p53RE in the absence of p53. ChIP using p53^{-/-} adult liver tissue was done with antibodies against p53 and TA-p73. Average of three independent ChIPs is shown; TA-p73 binding to *Foxo3* p53REs is statistically significant and marked with an asterisk (* $p < 0.05$).

Figure 4. p53 and p73 activate expression of *Foxo3* gene. (A) *Foxo3* mRNA levels in p53^{+/-}, p53^{-/-}, and p73^{+/-} mice (5 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^{-/-} and between *wt* and p73^{+/-} is statistically significant and marked with asterisks (**p* < 0.05). (B) Hepa1-6 mouse hepatoma cells were transiently transfected with HA-p53, HA-TAp73 α and TA-p73 β ; mRNA levels were measured by real-time PCR. Average of three independent transfection experiments is shown; the difference between *Foxo3* expression in vector-transfected cells and cells overexpressing p53 and TA-p73 is statistically significant (**p* < 0.05). Immunoblotting on the lower panel shows HA-p53, HA-TAp73 α , TA-p73 β and endogenous TA-p73 in transfected Hepa1-6 cells. (C) *Foxo3* expression was measured in immortalized MEFs overexpressing temperature-sensitive p53 R135V mutant. RNA was isolated from cells incubated at permissive temperature (32°C) for 0, 8, 12, and 24 h. Average of three independent experiments is shown; the difference between each time point and T=0 is statistically significant and marked with an asterisk (**p* < 0.05).

Figure 5. p53/p73 binding to *Foxo3* 5' region changes during liver regeneration. (A) ChIP from mouse liver tissue collected at 1, 2, 4, and 7 days following PH surgeries were performed using antibody against p53 and TA-p73 and shown as percentage of input bound by the antibody. (B) ChIP from mouse liver tissue collected at 1, 2, 4, and 7 days following Sham surgeries were performed as in (A). Statistically significant differences between p53/p73 binding in PH liver compared to corresponding time points in Sham are marked with asterisks (* $p < 0.05$; ** $p < 0.01$).

Figure 6.

Foxo3 expression is decreased at 1-3 days following PH and increased when regeneration is complete (4-7 days). Foxo3 mRNA was isolated from mouse liver tissue at indicated time points following the surgery. Relative expression levels were measured by real-time PCR using primers to *Foxo3*. (A) Foxo3 expression in response to 65% PH. Time points following PH with statistically significant difference in Foxo3 expression as compared to Sham are marked with asterisks (* $p < 0.05$; ** $p < 0.01$). (B) Foxo3 expression in response to Sham surgery. There is no statistically significant difference in Foxo3 mRNA levels between T=0 and all time points following Sham surgeries.

Figure 7.

Acetyltransferase p300 binds *Foxo3* p53RE. (A) Histone modifications at *Foxo3* p53RE region during liver regeneration. ChIP from mouse liver tissue collected at 1 and 4 days following Sham and PH surgeries were performed using antibody against histone H3 and modified histone H3 (dimethylated on lysine 4 (H3K4me2) and acetylated on lysine 14 (H3K14Ac). Average of three independent ChIPs for each time point is shown as fold change of H3K4me2 and H3K14Ac over total H3 bound to *Foxo3* p53RE in PH compared to Sham. The statistically significant difference between PH and Sham is marked with asterisks (* $p < 0.05$). (B) p53/p73 recruit p300 to the p53RE of 5' *Foxo3* region. ChIP was performed using antibodies against p300. Average of three independent ChIPs for each genotype is shown as percentage of input bound by the antibody. The difference between p300 binding to *Foxo3* p53REs in *wt* as compared to p53^{-/-} mouse liver is statistically significant and marked with an asterisk (* $p < 0.05$). (C) p300 binding to *Foxo3* p53RE is lost during proliferative stage of liver regeneration. ChIP experiments using liver tissue collected at 1, 4, and 7 days post-PH and Sham surgeries were done with antibodies against p300. Average of three independent ChIPs for each time point of PH and Sham surgeries is shown; decrease in p300 binding to *Foxo3* p53REs on day 1 post-PH is statistically significant and marked with asterisks (** $p < 0.01$).