p53-mediated redox control promotes liver regeneration and maintains liver function in response to CCl₄

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The p53 transcription factor coordinates wide-ranging responses to stress that contribute to its function as a tumour suppressor. The responses to p53 induction are complex and range from mediating the elimination of stressed or damaged cells to promoting survival and repair. These activities of p53 can modulate tumour development but may also play a role in pathological responses to stress such as tissue damage and repair. Using a p53 reporter mouse, we have previously detected strong induction of p53 activity in the liver of mice treated with the hepatotoxin carbon tetrachloride (CCl₄). Here, we show that p53 functions to support repair and recovery from CCl₄-mediated liver damage, control reactive oxygen species (ROS) and limit the development of hepatocellular carcinoma (HCC), in part through the activation of a detoxification cytochrome P450, CYP2A5 (CYP2A6 in humans). Our work demonstrates an important role for p53-mediated redox control in facilitating the hepatic regenerative response after damage and identifies CYP2A5/CYP2A6 as a mediator of this pathway with potential prognostic utility in human HCC.

Cell Death & Differentiation (2022) 29:514–526; https://doi.org/10.1038/s41418-021-00871-3

INTRODUCTION

The TP53 (p53) transcription factor coordinates diverse aspects of the cellular stress response and is capable of engaging both pro-survival and pro-death pathways [1]. Although p53 was initially identified through its association with cancer, it also has broader roles in organismal health. p53 is required for efficient implantation of embryos into the uterus [2], promotes stamina during exercise [3, 4], limits fibrosis after chronic liver injury [5], and protects against Listeria monocytogenes infection [6]. Conversely, unrestrained activation of p53 in embryos is rapidly lethal [7–9], p53 promotes B-cell apoptosis in type 2 diabetes mellitus [10], and in ischaemia, inhibition of p53 is protective [11–13]. These disparate outcomes suggest a nuanced balance between divergent aspects of p53 activity.

The liver provides an excellent setting in which to examine the intersection of p53 signalling in cancer and normal biology. Disruption of TP53 is observed in 30–40% of human hepatocellular carcinomas (HCCs) [14], suggesting tumour-suppressive functions for p53 in the liver. Indeed, the loss of hepatic p53 alone is sufficient to promote liver cancer in mice, albeit at long latency [15]. Even so, wild-type p53 is retained in more than half of human HCCs, with a previously identified TP53 gene expression signature characterising this group [14]. These observations suggest that aspects of p53 function may also support—or at least not directly antagonise—hepatic tumorigenesis.

Although the liver is largely quiescent in adults, it can undergo rapid regeneration after damage or resection [16]. As in HCC, the p53 pathway has been reported to support and antagonise liver regeneration. It has been shown that p53 limits liver damage after acetaminophen overdose and protects mitotic fidelity after partial hepatectomy, indicating protective roles for p53 in the hepatic injury response [17–19]. Similarly, p53-deficient mice exhibit enhanced sensitivity to high-dose irradiation during CCl₄-induced regeneration [20]. In addition, the loss of CDKN1A/p21, a p53 target, has been reported to impair liver regeneration in certain liver damage models [21, 22]. However, robust activation of p21 has also been shown to promote senescence and to limit the regenerative response, and p21 loss can allow for survival after severe liver damage—suggesting that this arm of the p53 response may also impede liver regeneration [21, 23]. More directly, unrestrained activation of p53 is lethal in hepatocytes, p53-mediated apoptosis contributes to disease progression in a model of non-alcoholic steatohepatitis, and p53 activity promotes fibrosis in a model of chronic regeneration in rats [24–27]. Thus, the role of p53 in the hepatic response to toxic damage is not clear and may be dependent on the nature and severity of the initiating damage.

Here, we utilise CCl₄-mediated liver regeneration as a model system to investigate the function of p53 in liver biology. Our work demonstrates a role for p53-mediated redox control in facilitating the hepatic regenerative response after damage. We identified CYP2A5/CYP2A6 as a mediator of this pathway with potential prognostic utility in human HCC.
RESULTS
Liver-specific loss of p53 exacerabates liver damage and increases ROS during CCl4-mediated liver regeneration

Using a p53 reporter mouse, we have previously detected strong induction of the p53 pathway, albeit without clear accumulation of p53 itself, in hepatocytes of mice treated with the hepatotoxic carbon tetrachloride (CCl4). To explore the potential roles for p53 function during this process, we created mice harbouring liver-specific deletion of Trp53 (p53) (Albumin-Cre; p53<sup>fl/fl</sup> mice), confirmed that recombination of the p53 floxed allele was highly efficient in the liver (Fig. S1A/B) and proceeded to characterise the acute response to CCl4-mediated liver toxicity using this model (Figs. 1A/B and S1C–H). Importantly, mice of either p53 genotype developed normally and were histologically indistinguishable prior to treatment (Fig. S1 C/D). Within the first 24 h following treatment with CCl4, Albumin-Cre; p53<sup>fl/fl</sup> mice (p53 WT) mice exhibited evident liver damage, including vacuolisation, neutrophil infiltration, and destruction of Glutamine Synthetase (GS)-positive peri-central vein hepatocytes (Figs. 1A/B and S1C–H). Damage progressed outward from the central vein over the first 48–72 hours after treatment before resolving within the remainder of the 168-h time-course (Figs. 1A/B and S1 C/D). In Albumin-Cre; p53<sup> WT/WT</sup> mice, although GS-positive hepatocyte destruction and damage-associated neutrophil infiltration were similar to p53 WT mice (Fig. S1E–H), liver damage progressed outward from the peri-central vein region more rapidly, coalescing into larger regions of injury at 24 h after CCl4 treatment (Figs. 1A/B and S1 C/D). Even so, the liver damage in Albumin-Cre; p53<sup> WT/WT</sup> mice also resolved within 168 h.

To confirm our histological assessment of liver damage, we examined the presence of alanine and aspartate transaminase (ALT/AST) activity in blood plasma, two markers of liver damage. In agreement with the liver histology, we noted extended elevation of plasma ALT and AST activity in Albumin-Cre; p53<sup> WT/WT</sup> mice (Fig. 1C/D), suggesting elevated liver damage in these mice. Even so, as observed in liver histology, plasma ALT and AST levels ultimately normalised by 168 h after treatment in mice of both genotypes, suggesting potentially significant but transient effects of p53 during CCl4-mediated liver regeneration. These findings are consistent with previous reports of p53 acting to limit liver damage after acetaminophen overdose [18, 19], where p53 exerted short-lived protective effects.

One of the features of damage-mediated liver regeneration is the dramatic but transient microvesicular steatosis that occurs prior to initiation of the proliferative phase of the regenerative response [28–30]. The resulting accumulation of lipids can be visualised as red puncta in oil-red-O-stained liver sections. In contrast to our liver damage assessments, we observed a similar peak accumulation of oil-red-O staining at 24 h after CCl4 treatment in the livers of Albumin-Cre; p53<sup> WT/WT</sup> and Albumin-Cre; p53<sup> fl/fl</sup> mice (Fig. 1F/F). However, while lipid levels rapidly normalised by 48 h after treatment in Albumin-Cre; p53<sup> WT/WT</sup> mice, elevated oil-red-O staining persisted in Albumin-Cre; p53<sup> fl/fl</sup> mice for an additional 2 days (Fig. 1F/F).

The initial reductive dehalogenation of CCl4 generates short-lived but highly reactive intermediates that potently oxidise lipids and cause DNA damage [31, 32]. Peroxidized lipids can impede mitochondrial function, including fatty acid oxidation, and impair lipid export—potentially contributing to lipid accumulation during CCl4 detoxification [33–35]. Considering the established role for p53 supporting the redox response [36], we investigated whether the disrupted lipid clearance in Albumin-Cre; p53<sup> WT/WT</sup> mice was a consequence of decreased ROS detoxification. Indeed, levels of malondialdehyde (MDA), a marker of lipid peroxidation, were elevated to a greater extent, and for an additional two days, in Albumin-Cre; p53<sup> fl/fl</sup> mice compared with Albumin-Cre; p53<sup> WT/WT</sup> mice (Fig. 1G/H).

To investigate the contribution of ROS stress to deficiencies in liver regeneration in Albumin-Cre; p53<sup> WT/WT</sup> mice, we compared CCl4-mediated regeneration between Albumin-Cre; p53<sup> WT/WT</sup> mice given normal drinking water to those provided with N-Acetylcycteine (NAC)-supplemented drinking water. NAC treatment is an established antidote to liver toxicity that results from acetaminophen overdose in humans and functions by maintaining liver glutathione levels during the detoxification process [37]. As expected, NAC treatment significantly attenuated lipid peroxidation in Albumin-Cre; p53<sup> WT/WT</sup> mice (Fig. 1I/J). NAC treatment also promoted significantly more rapid clearance of lipid droplets (Fig. 1K/L), suggesting that redox management is an important feature of the p53-mediated response to hepatic CCl4 toxicity.

Liver p53 engages Cyp2a5/Cyp2a6 to support redox control during CCl4-mediated liver regeneration

Given the differences in redox control between Albumin-Cre; p53<sup> WT/WT</sup> and Albumin-Cre; p53<sup> fl/fl</sup> mice at 24 h after CCl4 treatment, we focused on the early response to toxicity. At 8 h after CCl4 treatment, we observed similar oil-red-O staining to baseline and comparable lipid peroxidation between livers taken from Albumin-Cre; p53<sup> WT/WT</sup> and Albumin-Cre; p53<sup> fl/fl</sup> mice (Fig. 2A/B), suggesting that bifurcation of the regenerative response had not yet occurred. Bulk RNA-seq analysis at this time point identified 13 significant differentially regulated genes between Albumin-Cre; p53<sup> WT/WT</sup> and Albumin-Cre; p53<sup> fl/fl</sup> livers 8 h after CCl4 treatment (Fig. 2A/A). Clustering analysis stratified these genes into three groups, one of which contained p53 itself and four established p53 targets: Ccng1/Cyclin G1, Eda2r, Zmat3/Wig-1, and Abcb1a/Mdr1 [38–41] (Fig. 2C). Our attention was drawn to the remaining member of this cluster, Cyp2a5, encoding a cytochrome P450 enzyme that is induced by NFE2L2 (NRF2) to aid in the murine redox response during ethanol detoxification [42, 43]. CYP2A6, the human orthologue of Cyp2a5 [44], has been shown to be a transcriptional target of p53 [45], suggesting potential p53-directed functions for Cyp2a5 in the mouse as well.

Consistent with our RNA-seq data, we confirmed that expression of Cyp2a5 was induced in Albumin-Cre; p53<sup> WT/WT</sup> mice within the first 8–24 h after CCl4 treatment, with later induction observed in Albumin-Cre; p53<sup> WT/WT</sup> mice alongside increased levels of the NRF2 target gene Nqo1 (Fig. 2B/C). Through IHC staining, we confirmed higher levels of CYP2A5 protein in livers of Albumin-Cre; p53<sup> WT/WT</sup> compared to Albumin-Cre; p53<sup> fl/fl</sup> mice within 24 h after CCl4 treatment, as well (Fig. 2D/E). CYP2A5 levels remained significantly elevated in Albumin-Cre; p53<sup> WT/WT</sup> livers at 48 and 72 h after treatment, with a delayed increase evident in Albumin-Cre; p53<sup> fl/fl</sup> livers at 48–72 h after CCl4 treatment (Fig. 2D/E).

Interestingly, although we observed potent early induction of the p53 target gene Cdkn1a/p21 in Albumin-Cre; p53<sup> WT/WT</sup> mice, this was matched by similar induction in Albumin-Cre; p53<sup> fl/fl</sup> mice, a finding that we confirmed by IHC staining for p21 at 8 h after CCl4 treatment (Fig. 2D/E). These findings suggest that early expression of p21 after CCl4 treatment is p53-independent (Fig. 2D/E), in contrast to its p53-dependent induction later in the regenerative process (Fig. 2D). These results also explain why Cdkn1a was not differentially expressed in our RNA-seq analysis. Expression of CYP2A5, in contrast, was elevated in Albumin-Cre; p53<sup> WT/WT</sup> mice but significantly lower in Albumin-Cre; p53<sup> fl/fl</sup> livers at this time point, consistent with our RNA-seq data (Fig. S2F). Bbc3/Puma, a pro-apoptotic p53 target gene that has been shown to play a role in modulating liver metabolism in human HCC [46], was not differentially expressed between Albumin-Cre; p53<sup> WT/WT</sup> and Albumin-Cre; p53<sup> fl/fl</sup> mice during CCl4-mediated regeneration (Fig. S2G), reinforcing the idea that not all aspects of p53 activity are engaged during liver regeneration.

In the Mdm2<sup>Δex5/6</sup> mouse model, excision of Mdm2 exons 5 and 6 (Mdm2<sup>Δex5/6</sup>), comprising the p53-binding domain of MDM2,
leads to rapid stabilisation of p53, robust expression of p53 target genes, and ultimately in p53-dependent lethality within 4–5 days [24, 47]. RNA-seq analysis of mice sampled at two days after treatment with liver-specific AAV8-TBG-Cre [48] to induce expression of Mdm2Ex5/6Δ, a time point before widespread liver attrition, confirmed significant induction of Cyp2a5. Indeed, we identified all of the genes in our CCl4 RNA-seq analysis, alongside classical p53 targets such as p21 and Puma in this alternative model (Fig. S2H). As in the CCl4 liver regeneration model, we validated the induction of p21 and CYP2A5, as well as stabilisation of p53, via IHC (Fig. S2I–K). Combined, these findings confirm that activation of p53 engages CYP2A5 in the liver.
To explore the role of CYP2A5/CYP2A6 in damaged hepatocytes more fully in vitro, we turned to HepG2 and SK-Hep-1 cells, human HCC cell lines that maintain wild type p53 [46] (Fig. 2F/G). Treatment of these cells with Nutlin, a direct activator of p53 [49], induced expression of CDN1A/p21, as expected, as well as CYP2A6. Treatment of both HepG2 and SK-Hep-1 cells with CDN1 also induced p21 and CYP2A6 expression (Fig. 2F/G). This response was abrogated following siRNA-mediated depletion of TP53, confirming the role of p53 in the upregulation of p21 and CYP2A6 expression in response to CDN1 in vitro (Fig. 2F/G).

Functionally, both HepG2 and SK-Hep-1 cells treated with CDN1 exhibited increased ROS levels, and this was exacerbated in CYP2A5/CYP2A6 depleted cells (Fig. 2L/M). In HepG2 and SK-Hep-1 cells with cumene hydroperoxide (CH), a stable organic oxidising agent [50], similarly engaged CYP2A6 and CDN1A/p21 (Fig. S2 L/M), and CYP2A6-depletion also increased cellular ROS levels after CH treatment (Fig. 2J/K)—suggesting that downstream ROS, rather than CH, directly promotes activation of CYP2A6 to aid in ROS detoxification. Interestingly, although induction of CYP2A6 was p53-dependent in response to CDN1 treatment, CYP2A6 increased independently of p53 after CH treatment (Fig. S2L/M). Since hydroperoxides have been shown to activate NRF2 in HepG2 cells [51], this finding is consistent with an established role for NRF2-induced Cyp2a55 supporting the redox response during ethanol detoxification in mice [42, 43]. Based on these findings, we concluded that the p53-dependent activation of CYP2A5 in response to CDN1 treatment in vivo contributed to the enhanced detoxification of lipid ROS in support of rapid regeneration in Albumin-Cre; p53WT/WT mice.

**Hepatocyte p53 protects liver function and limits tumourigenesis following CCl4-mediated chronic regeneration**

A close relationship has been described between chronic regeneration and cancer—with tumourigenesis sometimes conceptualised as ‘a wound that does not heal’ [52]. While we detected clear defects in redox control and liver function during one round of CCl4 treatment and regeneration in Albumin-Cre; p53L/L mice compared to Albumin-Cre; p53WT/WT mice, these differences were transient and resolved within one week (Fig. 1). In contrast to acute damage, repeated regeneration resulting from regular CCl4 treatment causes lasting fibrotic liver damage, leading to cirrhosis and HCC [53, 54]. This progression is exacerbated by systemic DNA damage, chronic inflammation, and ROS stress [55, 56]. With these findings in mind, we investigated the effects of lack of liver p53 on fibrosis and HCC development in the well-established CCl4 chronic liver regeneration model [5] (Fig. S3A).

One week after the conclusion of the 10-week chronic regeneration regime, we observed striking generalised hepatocyte hypertrophy [57] throughout the livers of Albumin-Cre; p53WT/WT mice that was absent in similarly treated Albumin-Cre; p53L/L mice (Fig. 3B/D). Functionally, we also found that hepatocyte-specific p53 loss did not lead to differences in activated HSC content, as evaluated by IHC staining for alpha-smooth muscle actin (aSMA), or to increased fibrosis as assessed by picrosirius red staining (PSR) (Fig. 3B–D). In fact, we observed a modest decrease in fibrosis in Albumin-Cre; p53L/L mice (Fig. 3B/D), consistent with a previous report showing that hepatocyte p53 can enhance fibrosis during CCl4-mediated chronic regeneration in rats [27]. Although murine hepatocyte p53 does not appear to limit fibrosis after chronic regeneration either, we nevertheless detected higher levels of unresolved DNA damage, measured by IHC staining for phospho-histone H2AX (gH2AX), increased lipid peroxidation (measured by MDA) and—as expected—decreased levels of CYP2A5 in livers from Albumin-Cre; p53L/L mice (Fig. 3E/F). Functionally, we also found that plasma levels of ALT and AST enzyme activity were both elevated in Albumin-Cre; p53L/L mice after chronic regeneration, consistent with compromised liver function in these mice (Fig. 3G).

In wild-type mice, it can take up to 2 years for HCC to arise from chronic CCl4 treatment [58]. Consistent with this, few Albumin-Cre; p53WT/WT mice (3/14) reached clinical endpoint within 550 days after initial CCl4 treatment in our experimental cohorts (Fig. 4A). However, Albumin-Cre; p53L/L mice exhibited accelerated and highly penetrant development of liver tumours in this timeframe.
Fig. 2 Liver p53 engages Cyp2a5/CYP2A6 to support redox control during CCl4-mediated liver regeneration. Staining (A) and quantification (B) for malondialdehyde (MDA) and oil-red-O (ORO) in Albumin-Cre; p53\WT/WT (WT) and Albumin-Cre; p53\FL/FL (FL) mice at 8 h after CCl4 treatment. Scale bars 10\(\mu\)m. Representative of \(N=3\) WT and \(N=4\) FL mice. Data presented as mean ± SEM and analysed using two-tailed \(t\)-tests and the Sidak–Bonferroni method to account for multiplicity of tests. ns not significant. D) Clustering analysis of significant differentially expressed genes (adjusted \(p < 0.05\)) from RNA-seq analysis between Albumin-Cre; p53\WT/WT (WT) and Albumin-Cre; p53\FL/FL (FL) mice at 8 h after CCl4 treatment. Samples from \(N=3\) WT and \(N=4\) FL mice included in analysis. Positive Z-score values correspond to genes enriched in livers of mice of the genotype indicated in the sample name. Cyp2a5-associated cluster as indicated. For further information, see materials and methods. IHC staining (D) and quantification (E) of CYP2A5 in Albumin-Cre; p53\WT/WT (WT) and Albumin-Cre; p53\FL/FL (FL) mice at indicated times (hours) after CCl4 treatment. Scale bars 20\(\mu\)m. Quantification from \(N=4\) untreated (0 h) mice/group, \(N=5\) mice/group at 24 h, \(N=7\) mice/group at 48 and 72 h, and \(N=3\) mice/group at 168 h. Data presented as mean ± SEM and analysed using two-way ANOVA with Holm–Sidak’s multiple comparisons test and multiplicity-adjusted \(p\) values. *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\), ****\(p < 0.0001\). Measurement of cellular ROS levels relative to baseline using the CellROX fluorescent probe in HepG2 (H) or SK-Hep-1 (I) cells treated with non-targeting control (NT) or CYP2A6 (2A6) siRNA for 96 hours and additionally treated with either DMSO control (UT) or with CCl4 (4 mM) or with Nutlin (10 \(\mu\)M) for 24 h prior to analysis. Data presented as median ± SEM relative to untreated NT cells and analysed using two-way ANOVA with Holm–Sidak’s multiple comparisons test and multiplicity-adjusted \(p\) values. *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\), ****\(p < 0.0001\). Measurement of cellular ROS levels relative to baseline using the CellROX fluorescent probe in HepG2 (J) or SK-Hep-1 (K) cells treated with non-targeting control (NT) or CYP2A6 (2A6) siRNA for 96 h and additionally treated with either DMSO control (UT) or with cumene hydroperoxide (10 \(\mu\)M) (CH) for 24 h prior to analysis. Data presented as median ± SEM relative to untreated NT cells and analysed using two-way ANOVA with Holm–Sidak’s multiple comparisons test and multiplicity-adjusted \(p\) values. *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\), ****\(p < 0.0001\).
Liver tissue 7 days after conclusion of chronic CCl₄ dosing regime

A. Quantification of relative hepatocyte size in Albumin-Cre; p53<sup>WT/WT</sup> (WT) and Albumin-Cre; p53<sup>FL/FL</sup> (FL) mice at 7 days after completion of 10-week CCl₄ chronic regeneration regime. N = 9 mice/genotype. Data presented as mean ± SEM and analysed using an unpaired two-tailed t-test with Welch’s correction. ***p < 0.01. B. H&E and IHC staining for alpha-smooth muscle actin (αSMA), and picrosirius red (PSR) staining in Albumin-Cre; p53<sup>WT/WT</sup> (WT) and Albumin-Cre; p53<sup>FL/FL</sup> (FL) mice at 7 days after completion of 10-week CCl₄ chronic regeneration regime. Scale bars 10 μm. Images representative of N = 10 WT and N = 8 FL mice for H&E, N = 4 WT and N = 5 FL mice for αSMA, and N = 7 mice/group for PSR. C. Quantification of IHC staining for αSMA in Albumin-Cre; p53<sup>WT/WT</sup> (WT) and Albumin-Cre; p53<sup>FL/FL</sup> (FL) mice from (B) at either 7 days after completion of 10-week CCl₄ chronic regeneration regime (chronic) or in untreated age-matched mice (control), N = 5 control mice/genotype, N = 4 WT and N = 5 FL chronic mice. Data presented as mean ± SEM and analysed using two-way ANOVA with Holm–Šidák’s multiple comparisons test and multiplicity-adjusted p values. **p < 0.01. D. Quantification of staining for picrosirius red (PSR) in Albumin-Cre; p53<sup>WT/WT</sup> (WT) and Albumin-Cre; p53<sup>FL/FL</sup> (FL) mice as in (C), N = 4 control mice/genotype and N = 7 chronic CCl₄ mice/genotype. Data presented as mean ± SEM and analysed using two-way ANOVA with Holm–Šidák’s multiple comparisons test and multiplicity-adjusted p values. **p < 0.01, ***p < 0.0001. Images (E) and quantification (F) of IHC staining MDA, CYP2A5, and gH2AX in Albumin-Cre; p53<sup>WT/WT</sup> (WT) and Albumin-Cre; p53<sup>FL/FL</sup> (FL) mice at 7 days after completion of 10-week CCl₄ chronic regeneration regime as in (C). Scale bars 20 μm. N = 4 mice/group for MDA, N = 7 mice/group for CYP2A5, and N = 9 mice/group for gH2AX. Data presented as mean ± SEM and analysed using two-tailed t-tests and the Sidak–Bonferroni method to account for multiplicity of tests. **p < 0.01, ****p < 0.0001. G. Plasma ALT and AST activity (mU/mL) in Albumin-Cre; p53<sup>WT/WT</sup> (WT) and Albumin-Cre; p53<sup>FL/FL</sup> (FL) mice at 7 days after completion of 10-week CCl₄ chronic regeneration regime. N = 7 mice/group. Each data point represents the mean from technical duplicates per mouse. Data presented as mean ± SEM and analysed using two-tailed t-tests and the Sidak–Bonferroni method to account for multiplicity of tests. **p < 0.01.

The p53-mediated expression of BBC3/PUMA has been shown to promote a pro-cancer metabolic switch in human HCC, correlating with poor prognosis in patients [46]. However, we did not observe differential expression of murine Bbc3 in endpoint HCC tumours arising in Albumin-Cre; p53<sup>WT/WT</sup> mice after chronic CCl₄ treatment compared with those in Albumin-Cre; p53<sup>FL/FL</sup> mice (Fig. S3D). Expression of Cdkn1a/p21, in contrast, was elevated in the tumours of Albumin-Cre; p53<sup>WT/WT</sup> mice (Fig. S3D). These findings suggest that in murine HCC arising from chronic CCl₄ treatment the p53-PUMA mediated metabolic switch [46] is not a defining feature of p53 WT HCC.

CYP2A6 expression is prognostically favourable and clusters with a subset of p53-induced genes that correlate with increased survival in human HCC

Given the strength of the association between increased survival, retention of p53, and expression of CYP2A5 in the murine CCl₄ chronic regeneration model, we examined whether high expression of CYP2A6 correlated with increased survival in human HCC patients. Utilising the HCC dataset available through the cancer-genome atlas (TCGA-LIH dataset [59]), we confirmed that high expression of CYP2A6 was associated with significantly greater median survival in HCC (Fig. 4F). In addition, by stratifying the
TCGA dataset between patients with disrupted TP53 (mutation or loss) and those with WT TP53, we further determined that high expression of CYP2A6 and retention of WT TP53 coincided with increased survival compared with all other combinations of tumours harbouring low expression of CYP2A6 and/or loss of WT TP53 (Fig. 4G).

Previous work utilising TCGA data has focused on the genomic determinants of human HCC [14]. In these analyses, the authors identified a ‘p53-induced gene target expression signature’ as part of an aim to improve the clustering of HCC based on molecular and biological attributes [14]. Using the TCGA-LIHC dataset, we examined the relationship between these 20 identified p53-induced genes and
CYP2A6 expression and found that CYP2A6 expression clustered with a subset of the p53-induced genes including GADD45B, ALDH4A1, GADD45A, ESR1, PANK1, and ACAD11 (Fig. 4H). Interestingly, although high expression of the full p53-induced gene target expression signature correlated with significantly reduced median survival in the TCGA-LIHC dataset (Fig. 4I), we found that high expression of the CYP2A6-associated gene cluster instead correlated with improved median survival (Fig. 4J). Taken together, these observations are consistent with a role for p53 and CYP2A6 in limiting liver cancer.

**DISCUSSION**

Tissue regeneration recapitulates many features of tumourigenesis, including potent activation of proliferative signalling pathways, changes to cellular metabolism, and rapid cell growth [60]. With this overlap in mind, we have examined the function of the canonical tumour suppressor protein p53 during regeneration. Using the liver as a model system, we have interrogated non-tumour roles for p53 activity during both the acute and chronic phases of CCl4-mediated chronic liver regeneration, we found that the paradigms identified in acute regeneration persist. The presence of hepatic p53 does not limit, but rather leads to a slight increase in fibrosis in our model. Nevertheless, as in acute regeneration, p53 continues to engage CYP2A5, restrict lipid peroxidation, and maintain liver architecture and function. These protective actions are blunted in livers that lack p53, leading to pervasive hepatocyte hypertrophy, chronically increased ROS, unresolved DNA damage, and ultimately to mortality from liver cancer. Thus, in our system, increased fibrosis is not required for increased tumourigenesis. Further work is warranted to more carefully examine the relationship between fibrosis, p53 signalling, and liver tumourigenesis.

Our findings generalise to human HCC, where high expression of CYP2A6 correlates with increased median survival, as well as increased survival in the subset of patients that retain WT TP53 and maintain high expression of CYP2A6. These results suggest that increased CYP2A6 expression is an important component of p53’s tumour-suppressive function. Even so, our results also suggest that elevated CYP2A6 alone is not sufficient to substitute for p53 activity in limiting liver tumorigenesis, consistent with the diverse repertoire of p53 tumour-suppressive activities in the cell. We have further distinguished a group of six genes previously reported as part of a p53-induced gene signature in human HCC [14] whose expression clusters with CYP2A6 and together account for improved median survival—in contrast with the poor prognosis associated with high expression of the entire gene set.
Focusing on this point, we were surprised that high expression of the whole p53 gene signature significantly reduced median patient survival. However, it has been shown that p53 can help to protect cancer cells from nutrient starvation [63, 64], reduce cell death from ferroptosis [65, 66], and enhance redox control to limit ROS [67, 68]. In addition, common tumour-derived p53 mutants have been found to retain aspects of WT p53 function that promote adaptation to metabolic stress [69, 70]. With these findings in mind, and considering that ‘pro-tumourigenic’ p53 is an established paradigm in skin carcinogenesis [71–73], it is conceivable that aspects of p53 function can also enhance tumourigenesis in the liver. Future work investigating this possibility is warranted.

In humans, expression of CYP2A6 and of various CYP2A6 polymorphisms have been linked to higher rates pancreatic and colorectal cancer but to mostly reduced rates of lung and oesophageal cancer [74–78]. These findings suggest tissue, and potentially carcinogen-specific, functions for CYP2A6 in limiting or promoting tumourigenesis. In the liver, our findings suggest that expression of CYP2A6 is beneficial. One method to infer CYP2A6 activity non-invasively is through the analysis of CYP2A6-derived urinary metabolites of caffeine [74, 75]. Increased consumption of coffee reduces the risk of developing HCC [79]. As such, it would be interesting to examine whether caffeine consumption promotes CYP2A6 expression. If so, this pathway could account for some of the protective features of coffee consumption against HCC. Future work examining this relationship, as well as whether CYP2A6 activity in HCC patients has prognostic or stratification value, is warranted.

Taken together, our results underscore the importance of p53 for maintaining liver function following damage. Interestingly, in contrast to previous models showing that the tumour suppressor function of p53 is a reflection of its ability to drive the elimination of damaged cells [80], our work shows that the repair and survival activities of p53 can also suppress the development of HCC.

**MATERIALS AND METHODS**

**Mice**

Procedures involving mice were performed under Home Office licence numbers 70/8645, P63645023, and 70/8691. Experiments were conducted in accordance with the Animals (Scientific Procedures) Act 1986 and the EU Directive 2010 and sanctioned by Local Ethical Review Process (University of Glasgow). Mice were housed on a 12-h light/12-h dark cycle and provided with normal chow diet and water ad libitum. Mice were genotyped by Transnetyx (Cordova, TN). p53FL/FL; Albumin-Cre; p53WT/WT (Speer et al. [70]), Mdm2tm2.1Glo (Mdm2tm2.1Glo) mice were described previously [47, 81, 82]. For acute CCl4-mediated liver regeneration, mice were treated as previously described [53, 83]. In brief, young male Albumin-Cre; p53WT/WT and Albumin-Cre; p53FL/FL mice (approx. 70 days old) were given CCl4 (1 mL/kg from stock solution) or water, respectively, at 10% neutral buffered formalin (Solmedia), rinsed in tap water, and then briefly rinsed in 60% isopropanol (Fisher Chemicals). The analysis of IHC staining for p53 and p21 in Mdm2tm2.1Glo adult mice was performed as previously described [69, 85] with the reagents and staining platform used for each antibody as noted in the accompanying reactant and antibody information tables (Supplementary Tables 1 and 2).

**Analysis of liver damage**

The analysis of IHC staining in CCl4 experiments, and for CYP2A5 IHC staining in Mdm2tm2.1Glo mice, was performed as previously described [69, 85]. For the analysis of IHC staining for p53 and p21 in Mdm2tm2.1Glo mice, a Leica Aperio AT2 slide scanner (Leica Microsystems, UK) was used to scan stained sections at 20x magnification. Histological scoring was performed using HALO image analysis software (V3.1.1076.363, Indica Labs).

**Quantiﬁcation of liver damage**

A minimum of five random non-overlapping 4x magnification ﬁelds were taken from each H&E stained slide using an Olympus BX51 microscope with Zen Blue software (LS). From the tissue damage was manually traced and the total damaged area per slide was calculated using ImageJ software.

**Cell culture**

HepG2 (HB-8065) and SK-Hep-1 (HTB-52) cells were obtained from ATCC but were not authenticated. Mycoplasma testing was performed when cells were thawed and semi-regularly thereafter using the MycoAlert Mycoplasma Detection Kit (Lonza LT07-318). Independent experiments were performed on cells treated with siRNA and compounds from separate matched as much as possible, and all treated at the same time. Downstream analyses were performed on a random order of samples blinded to the genotype and treatment regime until the summation of results.

For Mdm2tm2.1Glo RNA-seq experiments, mice homozygous for the Mdm2tm2.1Glo allele were bred on a mixed background. 8–12 week old male mice were injected with either AAVV8.TBG.Pi.Cre.BG (Addgene, 107787-hal.8) or AAVV8.TBG.Pi.Null.BGH (Addgene, 105536-AAV8) at a dose of 2 x 1011 genetic copies/mouse, as described previously [23]. Male mice of the same age and genotype, but without AAV injection, served as baseline controls (Untreated/uninduced controls). All mice were euthanized at 48 h post-AAV injection via CO2 inhalation.

**Recombination PCR**

For recombination PCR, liver DNA from Albumin-Cre; p53WT/WT (mt2.1Glo) mice (WT) and liver and kidney DNA from Albumin-Cre; p53FL/FL (p53WT/mt2.1Glo) adult mice were isolated as previously described [85]. DNA was ampliﬁed using KOD Hot Start Master Mix (Merck Millipore cat# 71842) according to standard protocols. PCR primers were previously described [86].
passages of each cell line. Stock flasks were maintained in DMEM glucose, glutamine, and phenol red-free medium (Gibco, A1443001) supplemented with 4 mM glucose (Sigma cat# 49163), 1 mM pyruvate (Gibco cat# 11360088), 1 mM L-Glutamine (Gibco cat# 25030032), penicillin/streptomycin (Gibco cat# 15070063), Gentamycin (Gibco cat# 15750037), and 10% FBS (Gibco cat# 10091148). Cells were cultured at 37 °C in a humidified atmosphere of 5% CO2.

Cells were treated with 10 μM Nutilin-3a (Nutilin) (Sigma cat# SML0580) dissolved in DMSO, 4 mM CCl4 (Sigma cat# 289116) dissolved in DMSO, 10 μM cumene hydroperoxide (Thermo Fisher Scientific cat# C10445) dissolved in DMSO, or DMSO as vehicle control. For in vitro experiments, CCl4 was prepared by first combining an 80/20 (v/v) mixture of CCL4 and DMSO with media to make a 100× stock. The stock was then sonicated for 5 min to mix the CCL4 mixture and the resulting solution was added to cells.

Transfection with siRNA

Studies utilising siRNA knockdown were performed as previously described [69], with siGENOME SMARTpool siRNA constructs (Horn) used for the non-targeting siRNA control pool (D-001206-13-05) and to target P53 (M-003329-03-0005) and CYP2A6 (M-008781-02-0005). Constructs were used to transfect cell lines at 20 nM concentration using the Lullaby siRNA transfection reagent and the manufacturer’s recommended reverse transfection procedure (Oz Biosciences).

Flow cytometry

HepG2 and SK-Hep-1 cells were analysed for cellular ROS levels as previously described [69]. Data were analysed using FlowJo X 10.0.7r2 (FlowJo, LLC) and median fluorescence intensity values were obtained and compared across samples.

RNA-seq

Liver samples were isolated and preserved in Allprotect tissue reagent (Qiagen cat# 74104) (CCl4 samples) or snap frozen on dry ice and stored at −80 °C until RNA extraction (Mdm2Ex5/6 samples). To isolate RNA, tissue was homogenised using a Preccyls tissue homogeniser (Bertin Instruments) and RNA was extracted using the RNeasy Plus Universal mini kit (Qiagen cat# 76405) (CCl4 samples) or the RNeasy mini kit (Qiagen cat# 73404) (CCl4 samples) or the RNeasy mini kit (Qiagen cat# 74104) (Mdm2Ex5/6 samples), all according to the manufacturers’ recommendations. The quality of the purified RNA was tested on an Agilent 2200 Tapestation using RNA screen tape (Agilent). Libraries for cluster generation and DNA sequencing were prepared as previously described using an Illumina TruSeq Stranded mRNA HT kit (CC4 samples) or an Illumina TruSeq Stranded mRNA HT Kit (Mdm2Ex5/6 samples) [87]. The quality and quantity of the DNA libraries was assessed on an Agilent 2200 Tapestation (D1000 screenape) and Qubit (Thermo Fisher Scientific), respectively. The libraries were run on the Illumina Next Seq 500 using the High Output 75 cycles 2 × 36 cycles, paired-end reads, single index for CCl4 samples and 2 × 36 cycles, paired-end reads, dual index for Mdm2Ex5/6 samples.

Analyses of RNA-seq expression data

For CCl4 RNA-seq, Fastq files were generated from the sequencer output using Illumina’s bcl2fastq (version 2.15.0.4) and quality checks on the raw data were done using FastQC (version 0.10.1) [86] and FastQ Screen (version 0.4.2) [89]. Alignment of the RNA-Seq paired-end reads was to the GRCh38.75 [90] version of the mouse genome and annotation using Tophat (version 2.0.13) with Bowtie (version 2.2.6.0) [91]. Expression levels were determined and statistically analysed by a workflow combining HTSeq (version 2.2.4.0) [92], the R environment (version 3.4.2) [93], and packages from the Bioconductor data analysis suite [94]. Differential gene expression analysis was based on the negative binomial distribution using the DESeq2 package [95]. “Heatmap.” function of gplots package [96] was used for hierarchical clustering of significant hits.

For Mdm2Ex5/6 RNA-seq, quality checks and trimming on the raw RNA-Seq data files were done using FastQC (version 0.11.7) [88], FastP [97] and FastQ Screen (version 0.12.0) [89]. RNA-Seq paired-end reads were aligned to the GRCh38.92 [90] version of the mouse genome and annotated using Hisat2 (version 2.1.0) [98]. Expression levels were determined and statistically analysed by a combination of HTSeq version 0.9.1 [92] and the R environment version 3.4 [93], utilising packages from the Bioconductor data analysis suite [94] and differential gene expression analysis based on the negative binomial distribution using the DESeq2 package version 1.18.1 [95].

TCGA analysis

Survival, mutation and expression data were obtained via cBioPortal [99, 100]. The results here are in whole or part based upon data generated by the TCGA Research Network (http:// cancergenome.nih.gov/), using the TCGA-LIHC dataset [59].

The optimal cut-off point for high or low expression of CYP2A6 in survival analyses was determined using the “surv_cutpoint” function of survminer package in R (0.4.8) [93, 101]. Overall survival data from patients for each expression group was plotted and analysed using inbuilt tools as indicated in Prism 7 (Graph Pad). Correlations between CYP2A6 expression and the ‘PS3-induced gene target expression signature’ [14] were assessed using the “cor” function from base R [93]. Then, the resulting heatmap was plotted using the function “corplot” from the corplot package (Version 0.84) to plot heatmaps [102].

Quantitative RT-PCR

For qPCR analysis of mouse tissue, liver samples were isolated and preserved in Allprotect tissue reagent (Qiagen cat# 76405). RNA was extracted as previously described [85]. cDNA was synthesised using the high capacity RNA-to-cDNA kit (Thermo Fisher Scientific cat# 4387406) and qPCR reactions were performed on a QuantStudio 5 real-time PCR system (Thermo Fisher Scientific) using Taqman FAST advanced master mix and Taqman gene expression assays (all Thermo Scientific) according to the manufacturer’s recommendations and using the assays listed in Supplemental Table 3. Gene expression was quantified relative to the housekeeping gene Beta-galactosidase according to the comparative ΔΔCt method.

Data plotting and statistical analysis

Data were plotted using Prism 7 (Graph Pad). The statistical analysis for each experiment was performed using the test indicated in the relevant figure legend and multiplicity-adjusted p values using the built-in analysis tools of Prism 7. Statistical tests were chosen based on the nature of the comparison being made and the corresponding standard tests utilised in the field. Underlying assumptions for these tests, including sample independence, variance equality, and normality were assumed to be met although not explicitly examined. Figures were prepared using Illustrator (Adobe). Unless otherwise indicated, data are represented as mean ± standard error of the mean (SEM) for error bars. Asterisks denote p value as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding authors upon reasonable request. RNA-seq data discussed in this paper have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession numbers GSE183053 and GSE183082.

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ACKNOWLEDGEMENTS

We would like to thank the Core Facilities and Advanced Technologies at the CRUK Beatson Institute, in particular the animal facilities staff, the histology team, the Molecular Technologies group, and the Bioinformatics services. We thank Catherine Winchester for comments on the manuscript.

AUTHOR CONTRIBUTIONS

TJH and KHV conceived and designed the project. HH analysed the RNA-seq data and liver TCGA-LIHC data. CK performed experiments with Mdm2Ex5/6 mice, for which funding was provided by TGB. CN performed IHC. WC performed RNA sequencing. AH and RS analysed RNA-seq data. TJH performed all other experiments and data analysis with assistance from HH. The work was supervised by TGB, KB and KHV. TJH and KHV wrote the manuscript and all authors discussed the results and revised and approved the manuscript prior to submission.

FUNDING

This work was funded by Cancer Research UK grant CS96/A26855 and supported by The Francis Crick Institute which receives its core funding from Cancer Research UK (FC001557), the United Kingdom Medical Research Council (FC001557), and the Wellcome Trust (FC001557), and the CRUK Beatson Institute which receives its core funding from Cancer Research UK grant CS96/A17196. HH was funded by BBSRC grant BB/N017005/2. Additional funding for the work was provided by Cancer Research UK grant A29799 (KB), TGB was funded by the Welcome Trust (Grant number: WT1074922) and CRUK HUNTER Accelerator Award (Grant number: A26813).

COMPETING INTERESTS

KHV is on the board of directors and a shareholder of Bristol Myers Squibb, and on the science advisory board (with stock options) of PMV Pharma, RAZE Therapeutics and Volastra Therapeutics, Inc. She is also on the SAB of Ludwig Cancer Research. KHV is a co-founder and consultant of Faeth Therapeutics. She has been in receipt of research funding from Astex Pharmaceuticals and AstraZeneca and contributed to CRUK Cancer Research Technology filing of Patent Application WO/2017/144877. TGB is in receipt of research funding from AstraZeneca.

ETHICAL APPROVAL

Procedures involving mice were performed under Home Office licence numbers 70/8645, P66345023, and 70/8891. Experiments were conducted in accordance with the Animals (Scientific Procedures) Act 1986 and the EU Directive 2010 and sanctioned by Local Ethical Review Process (University of Glasgow). No human samples were used in this study.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41418-021-00871-3.

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