c-Jun/AP-1 controls liver regeneration by repressing p53/p21 and p38 MAPK activity

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The AP-1 transcription factor c-Jun is a key regulator of hepatocyte proliferation. Mice lacking c-Jun in the liver (c-jun<sup>-/-</sup>) display impaired liver regeneration after partial hepatectomy (PH). This phenotype correlates with increased protein levels of the cdk-inhibitor p21 in the liver. We performed PH experiments in several double-knockout mouse models to genetically identify the signaling events regulated by c-Jun. Inactivation of p53 in c-jun<sup>-/-</sup> mice abrogated both hepatocyte cell cycle block and increased p21 protein expression. Consistently, liver regeneration was rescued in c-jun<sup>-/-</sup>/p21<sup>−/-</sup> double-mutant mice. This indicated that c-Jun controls hepatocyte proliferation by a p53/p21-dependent mechanism. Analyses of p21 mRNA and protein expression in livers of c-jun<sup>-/-</sup> mice after PH revealed that the accumulation of p21 protein is due to a post-transcriptional/post-translational mechanism. We have investigated several candidate pathways implicated in the regulation of p21 expression, and observed increased activity of the stress kinase p38 in regenerating livers of c-jun<sup>-/-</sup> mice. Importantly, conditional deletion of p38α in livers of c-jun<sup>-/-</sup> mice fully restored hepatocyte proliferation and attenuated increased p21 protein levels after PH. These data demonstrate that c-Jun/AP-1 regulates liver regeneration through a novel molecular pathway that involves p53, p21, and the stress kinase p38α.

[Keywords: c-Jun, p53, p21, p38/liver regeneration; partial hepatectomy]

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Liver regeneration triggered by two-third partial hepatectomy (PH) is a well-established model system in rodents for studying the molecular mechanisms of cell cycle control. Hepatocytes that are normally quiescent and highly differentiated cells enter the S-phase rapidly after surgery and undergo one to two rounds of replication in order to fully restore liver mass [Diehl 2002; Fausto 2004; Taub 2004]. Importantly, abnormal regeneration contributes to the pathogenesis of fulminant liver failure, cirrhosis, and primary liver cancer. Initiation of liver regeneration occurs when hepatocytes are primed to synchronously escape quiescence and enter the prereplicative phase of the cell cycle [G1] after PH [Fausto 2004]. The priming phase is controlled by several cytokines such as tumor necrosis factor α (TNFα) and Interleukin-6 (IL-6) [Akerman et al. 1992; Cressman et al. 1996; Yamada et al. 1997]. Cytokines activate a variety of transcription factors important during the initial stages of liver regeneration, including nuclear factor-κB (NF-κB), signal transducer and activator of transcription 3 (STAT3), CCAAT enhancer-binding protein β (C/EBPβ), and activator protein 1 (AP-1) [Cressman et al. 1995; FitzGerald et al. 1995; Heim et al. 1997; Greenbaum et al. 1998]. At later stages hepatocyte growth factor (HGF), transforming growth factor α (TGFα), and heparin-binding epidermal growth factor (HB-EGF) stimulate S-phase entry of hepatocytes [Mead and Fausto 1989; Borowiak et al. 2004; Huh et al. 2004; Mitchell et al. 2005].

In response to PH, the DNA-binding activity of the dimeric transcription factor AP-1, composed of the products of the Jun and Fos families of genes, is rapidly induced [Heim et al. 1997]. Several reports have demonstrated the requirement for c-Jun in hepatocyte survival and proliferation. Mice lacking c-jun die at mid-gestation and their embryonic lethality is associated with increased apoptosis in fetal liver cells [Hilberg et al. 1993; Johnson et al. 1993; Eferl et al. 1999]. Consistently, an antiapoptotic function for c-Jun has also been found in preneoplastic hepatocytes during liver cancer develop-
Inactivation of p53 rescues the morbidity of c-jun<sup>Δli</sup> mice and restores hepatocyte proliferation after PH

We have addressed the question whether p53 is responsible for impaired liver regeneration in c-jun<sup>Δli</sup> mice after PH. For that purpose we used mice harboring floxed alleles of c-jun<sup>Δli</sup> [c-jun<sup>Δli</sup> mice] (Behrens et al. 2002) and the inducible Mx-cre transgene (Kuhn et al. 1995), and crossed them with p53-deficient mice [p53<sup>−/−</sup> mice] (Donehower et al. 1992) to obtain c-jun<sup>Δli</sup>p53<sup>−/−</sup> double-mutant mice. Since both Alb-cre and Mx-cre combined with the conditional c-jun allele have previously been shown to lead to the same impaired liver regeneration phenotype (Behrens et al. 2002), we used in this study only the Mx promoter-controlled cre expression, which was induced by injection of poly I/C. The efficiency of c-jun deletion in the liver was confirmed by Southern blotting [Fig. 1A] and the lack of c-jun expression in mutant mice after PH was confirmed by RNase protection assay and Western blotting [Fig. 1B,C]. Germline inactivation of p53 was analyzed by PCR [Fig. 1D]. c-jun<sup>Δli</sup>p53<sup>−/−</sup> and corresponding control mice [c-jun<sup>Δli</sup>, p53<sup>−/−</sup>, c-jun<sup>Δli</sup>] did not show any overt phenotype after poly I/C injection [data not shown]. All these mice were subjected to PH in order to trigger liver regeneration. As previously shown, 50% of the c-jun<sup>Δli</sup> mice died 2–4 d after PH due to impaired liver regeneration [Behrens et al. 2002]. In contrast, c-jun<sup>Δli</sup>p53<sup>−/−</sup> mice displayed mortality rates that were similar to controls, indicating a rescue of the liver regeneration defect in the absence of p53 [Fig. 1E].

We next examined the ability of hepatocytes to prolif-
erate after PH by immunohistochemical analysis of the proliferation marker Ki67. Consistent with previously published data (Behrens et al. 2002), hardly any Ki67-positive hepatocytes could be detected in livers of c-jun\textsuperscript{Alm}\textsuperscript{-} mice at the 48-h time point after PH [Fig. 2A]. Moreover, a quantitative time course of Ki67 staining revealed a marked delay of c-jun-deficient hepatocytes in reentering the cell cycle after the surgery [Fig. 2B]. To confirm these results, we measured DNA replication by BrdU incorporation. Consistently, almost no hepatocytes were able to enter S phase in c-jun\textsuperscript{Alm}\textsuperscript{-} mice 48 h after PH [Fig. 2C]. In contrast, cell cycle progression determined by Ki67 staining and BrdU incorporation was completely restored in c-jun\textsuperscript{Alm}\textsuperscript{-}p53\textsuperscript{−/−} double-mutant mice [Fig. 2A,C]. Deletion of p53 alone had no overt ef-}

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\caption{Loss of p53 abrogates hepatocyte cell cycle block in regenerating livers of c-jun\textsuperscript{Alm}\textsuperscript{-} mice. [A] Expression of the proliferation marker Ki67 in regenerating livers was analyzed by immunohistochemistry 48 h after PH. Genotypes are indicated. Arrows indicate Ki67-positive nuclei. [B] A quantitative time course of Ki67 expression at indicated time points following PH. Numbers of mice for each genotype are indicated. [C] Hepatocyte S-phase entry was measured by BrdU incorporation 48 h after PH. Genotypes are indicated. Arrows indicate BrdU-positive nuclei.}
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\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure3.png}
\caption{Impaired cyclin expression in regenerating livers of c-jun\textsuperscript{Alm}\textsuperscript{-} mice correlates with a p53-dependent increase in p21 protein levels. Expression of cell cycle regulators in livers of mice with the indicated genotypes was analyzed by Western blotting at indicated time points after PH. Proteasome was used as a loading control.}
\end{figure}

\textbf{Figure 3.} Impaired cyclin expression in regenerating livers of c-jun\textsuperscript{Alm}\textsuperscript{-} mice correlates with a p53-dependent increase in p21 protein levels. Expression of cell cycle regulators in livers of mice with the indicated genotypes was analyzed by Western blotting at indicated time points after PH. Proteasome was used as a loading control.

\textbf{Inefficient induction of cell cycle regulators in regenerating livers of c-jun\textsuperscript{Alm}\textsuperscript{-} mice correlates with a p53-dependent increase in p21 protein levels}

To investigate potential downstream target genes of c-Jun and p53 in liver regeneration, we have first analyzed the expression of G1, G1/S, and G2 cyclins in liver samples from mice at various time points after the surgery. mRNA expression of different cyclins occurred with similar kinetics after PH irrespective of the mouse genotype [Supplementary Fig. 1]. Cyclin D1 showed a biphasic kinetic with two peaks of expression at 12 and 48 h after PH [Supplementary Fig. 1], and was slightly elevated in livers of c-jun\textsuperscript{Alm}\textsuperscript{-} mice, which is in agreement with previously published data (Behrens et al. 2002). The other cyclins peaked at 48 h after PH even in regeneration-defective livers lacking c-jun [Supplementary Fig. 1]. Overall, we did not observe any major transcriptional alterations of cell cycle regulators in regenerating c-jun-deficient livers after PH. Therefore, we next analyzed protein levels for cyclins and other cell cycle regulators including the p53-regulated cyclin-dependent kinase inhibitor p21. Interestingly, protein expression of cyclin D1 was found to be highly expressed at 12 and 24 h after PH in c-jun\textsuperscript{Alm}\textsuperscript{-} mice, but strongly reduced at the 48-h time point when compared with wild-type mice [Fig. 3]. Moreover, cyclin A was inefficiently induced at the 48-h time point in livers lacking c-jun. In contrast,
levels of cyclin E were strongly elevated 48 h after PH (Fig. 3), presumably due to accumulation at the G1 restriction point and subsequent failure to enter S phase. Importantly, the reduction in cyclin D1 and cyclin A protein levels as well as the elevation of cyclin E protein levels at the 48-h time point were rescued in c-jun<sup>−/−</sup>p53<sup>−/−</sup> livers (Fig. 3). Furthermore, reduced protein levels of the cell cycle regulator PCNA were found in c-jun<sup>−/−</sup> mice 48 h after PH, but not in c-jun<sup>−/−</sup>p53<sup>−/−</sup> double-mutant livers (Fig. 3). We also observed reduced expression of retinoblastoma protein (RB) in c-jun<sup>−/−</sup> mice at 48 h and 72 h after PH, and phosphorylated RB was not detectable at all at the 72-h time point (data not shown). However, RB was expressed at similar levels in control and c-jun<sup>−/−</sup>p53<sup>−/−</sup> double-mutant livers, and phosphorylated RB was readily detectable 72 h after PH (data not shown). Most importantly, p21 protein levels were rapidly induced in c-jun<sup>−/−</sup> mice as early as 6 h after PH and remained elevated until 24 h after PH. However, this induction was abolished in double-mutant livers (Fig. 3), suggesting that the increase in p21 protein levels in c-jun<sup>−/−</sup> mice is due to a p53-dependent mechanism.

Loss of p21 rescues the liver regeneration defect in c-jun<sup>−/−</sup> mice

To test whether impaired liver regeneration in c-jun<sup>−/−</sup> mice is caused by a p53-dependent up-regulation of p21 protein expression, we have generated c-jun<sup>−/−</sup>p21<sup>−/−</sup> double-mutant mice and subjected them to PH. Importantly, morbidity and premature lethality of c-jun<sup>−/−</sup> mice after PH was rescued in c-jun<sup>−/−</sup>p21<sup>−/−</sup> double-mutant mice (data not shown). Moreover, immunohistochemical analysis of Ki67 expression in regenerating livers revealed that loss of p21 fully rescued proliferation of c-jun<sup>−/−</sup> hepatocytes. Ki67 expression peaked 48 h after PH in wild-type, p21<sup>−/−</sup> and c-jun<sup>−/−</sup>p21<sup>−/−</sup> double-knockout livers, but not in c-jun-deficient livers (Fig. 4A,B). Proliferation of hepatocytes lacking p21 alone seemed accelerated when compared with wild-type controls, since p21<sup>−/−</sup> livers showed slightly higher numbers of Ki67-positive hepatocytes at the 48-h time point after PH (Fig. 4B). This observation is in agreement with published data showing that p21<sup>−/−</sup> hepatocytes display higher rate of progression through the G1 phase of the cell cycle after PH (Albrecht et al. 1998). Furthermore, livers lacking p21 alone and c-jun<sup>−/−</sup>p21<sup>−/−</sup> double-knockout livers exhibited higher rates of BrdU incorporation 32–48 h after PH when compared with wild-type controls (data not shown). In contrast, hardly any staining was detected at these time points in mutant c-jun<sup>−/−</sup> livers (data not shown), confirming that hepatocyte S-phase entry is impaired in the absence of c-Jun. Moreover, 10 d after PH, double mutants restored liver mass as efficiently as wild-type littersmates or control p21<sup>−/−</sup> mice (data not shown). These results suggest that increased p21 protein expression is responsible for the delayed G1–S transition of hepatocytes and for impaired liver regeneration in c-jun<sup>−/−</sup> mice.

Figure 4. Deletion of p21 restores hepatocyte proliferation in regenerating livers of c-jun<sup>−/−</sup> mice. (A) Expression of the proliferation marker Ki67 in regenerating livers was analyzed by immunohistochemistry 48 h after PH. Genotypes are indicated. Arrows indicate Ki67-positive nuclei. (B) Quantification of Ki67-positive hepatocytes 48 h after PH. Numbers of mice for each genotype are indicated.

p53 regulates p21 protein levels in livers of c-jun<sup>−/−</sup> mice by a post-transcriptional/post-translational mechanism

At least two principal mechanisms can lead to elevated p21 protein levels in mutant mice: Lack of c-Jun results in increased transcriptional activity of the p53 protein, thereby enhancing p21 mRNA expression, or alternatively, p21 protein levels are regulated by a c-Jun/p53-dependent post-transcriptional mechanism. To test these hypotheses, we first investigated p21 mRNA levels by Northern blotting and semiquantitative RT–PCR analysis. Intriguingly, p21 mRNA expression was induced rapidly and peaked 24 h after PH (Fig. 5A,B) without significant differences between control, p53<sup>−/−</sup>, c-jun<sup>−/−</sup>, and c-jun<sup>−/−</sup>p53<sup>−/−</sup> mice. These results suggested that p21 mRNA induction in regenerating livers is independent of c-Jun and p53. A detailed expression analyses of other p53 target genes such as mdm2, bax, and cyclin G also revealed no significant differences between control, p53<sup>−/−</sup>, c-jun<sup>−/−</sup>, and c-jun<sup>−/−</sup>p53<sup>−/−</sup> mice (Fig. 5B), suggesting that the transcriptional activity of p53 is unchanged in c-jun<sup>−/−</sup> mice after PH. Furthermore, expression of p53 in the absence of c-Jun was affected neither at the RNA level (Fig. 5B) nor at the protein level (data not shown). Analysis of p53 phosphorylation at Ser 15 also revealed no changes in p53 activity.
in livers of c-junΔli* mice (data not shown). However, loss of p53 clearly reduced p21 protein levels in regenerating livers of c-junΔli* mice [Fig. 3]. Overall, these data suggest that the increased abundance of p21 protein in regenerating c-junΔli* livers occurs through a p53-dependent post-transcriptional/post-translational mechanism.

Livers of c-junΔli* mice display increased activity of p38 stress kinase after PH

The regulation of p21 protein expression and stability is rather complex, and many factors are involved. We have studied the expression of several candidate genes implicated in the regulation of p21 protein levels in the context of liver regeneration, including C/EBPα and TGFβ. We did not find any major changes in the expression pattern of C/EBPα and TGFβ, either at the mRNA level or at the protein level (Supplementary Fig. 2; data not shown). Moreover, several kinases like JNK, AKT/PKB, ERK, and p38 MAPK [mitogen-activated protein kinase] have been demonstrated to play important roles in regulating p21 protein expression and stability [Kobayashi and Tsukamoto 2001; Kim et al. 2002; Li et al. 2002]. We have found no significant alterations in the expression of phospho-ERK1/2 [Supplementary Fig. 2; data not shown]. In contrast, phosphorylation of p38 MAPK was found to be consistently up-regulated in livers of c-jun-deficient mice at 6, 12, 24, and 48 h after PH, and correlated with elevated p21 protein expression [Fig. 6A,B; data not shown]. These findings suggested that increased activity of p38 MAPK might be responsible for aberrant p21 protein expression in regenerating livers lacking c-Jun. Induction of p38 activity in wild-type livers occurred only at the 12-h time point and correlated with induction of p21 [Fig. 6B; data not shown]. Most importantly, increased phosphorylation of p38 MAPK was abolished in c-junΔli*p53Δ−/− and c-junΔli*p21Δ−/− double-mutant mice [Figs. 6A,B], implying that both p21 and p53 can positively regulate p38 MAPK activity. Intriguingly, TGFβ1 and MKK3/MKK6 signaling, which is known to influence the activity of p38 stress kinase, was not substantially changed in livers of c-junΔli* mice when compared with wild-type controls [Supplementary Fig. 2]. These results suggest that different, c-Jun-dependent signaling pathways seem to be involved in triggering p38 activation after PH. We therefore asked whether the combined deletion of c-jun and p38α, a major isoform of p38 MAPK, has an impact on hepatocyte proliferation and p21 protein levels after PH. Interestingly, conditional loss of both genes in the liver resulted in hepatocyte proliferation comparable to littermate controls [Fig. 6C]. Most importantly, deletion of both c-jun and p38α attenuated elevated p21 protein levels after PH, since the expression levels were equal in mutant and control livers [Fig. 6D]. These results strongly suggest that in regenerating livers c-Jun prevents p38-mediated accumulation of p21 protein, thereby allowing hepatocyte proliferation.

Discussion

Liver regeneration is a complex process that depends on the precise interplay of several cell types and molecular pathways. In humans, liver regeneration occurs most frequently after liver damage by ischemia or hepatitis, and when impaired, might result in increased morbidity. Therefore, understanding the mechanisms of liver regeneration has an important implication on the pharmacological treatments of conditions that lead to hepatitis, such as toxic damage by alcohol, drug overdose, or viral infections [Taub 2004]. Gene knockouts have been particularly helpful in determining the genes and proteins that are actually required for normal liver regeneration. It has been shown that conditional deletion of c-jun in livers of adult mice leads to impaired hepatocyte proliferation and liver regeneration after PH [Behrens et al. 2002]. However, the molecular events downstream of c-Jun in liver regeneration remained unknown. It has been demonstrated that c-Jun is a repressor of p53 in immortalized fibroblasts and liver cancer cells, and consequently, the levels of p53 protein are increased in both cell types when c-Jun is absent [Schreiber et al. 1999; Eferl et al. 2003]. Increased p53 expression caused a cell-type specific response leading to impaired cell proliferation of c-jun-deficient fibroblasts and increased apoptosis of c-jun-deficient liver cancer cells [Schreiber et al. 1999; Eferl et al. 2003].

We investigated the genetic link between c-Jun and p53 in liver regeneration employing PH in c-junΔli*p53Δ−/− double-mutant mice. These experiments demonstrated that impaired liver regeneration and G1/S transition of hepatocytes in c-junΔli* mice were fully rescued in a p53-
negative background. Therefore, we concluded that the liver regeneration defect in the absence of c-Jun is caused by a cell cycle block imposed by p53. The most prominent p53-target gene implicated in cell cycle progression/growth arrest is the cdk inhibitor p21. Transgenic mice overexpressing p21 specifically in the liver under the control of the transthyretin (TTR) promoter showed impaired hepatocyte cell cycle progression and liver regeneration after PH [Wu et al. 1996]. In contrast, hepatocytes from livers of p21 knockout mice displayed accelerated proliferation after PH due to a higher rate of progression through the G1 phase of the cell cycle (Albrecht et al. 1998). We have observed increased p21 protein levels in livers of c-jun<sup>−/−</sup> mice as early as 6 h after PH. Furthermore, we demonstrated in vivo that the events controlled by c-Jun in hepatocytes from livers of c-jun<sup>−/−</sup> mice might be caused by a p53-dependent effect on p21 mRNA transcription. Intriguingly, analysis of p21 mRNA expression in livers after PH showed that the increase in p21 protein levels in livers of c-jun<sup>−/−</sup> mice was apparently not due to a transcriptional effect of p53. The induction of p21 mRNA was comparable in livers of control, p53<sup>−/−</sup>, c-jun<sup>−/−</sup>, and c-jun<sup>−/−</sup>p21<sup>−/−</sup> mice. These data demonstrated that neither c-jun nor p53 contributed to p21 mRNA expression in the liver after PH. Our results are in agreement with published data which demonstrated that the induction of hepatic p21 mRNA after PH is independent of p53 (Albrecht et al. 1997). Besides p21, the expression of several other p53 target genes was found unchanged in c-jun<sup>−/−</sup> mice. p53 expression and transcriptional activity was not altered in c-jun<sup>−/−</sup> mice, therefore we concluded that increased expression/stability of p21 protein was caused by a post-transcriptional/post-translational, p53-mediated mechanism.

Stability of the p21 protein is largely dependent on the proteasome degradation pathway, which is regulated by interaction with several other proteins such as MDM2, WISp39, and IFI16 [Kwak et al. 2003; Zhang et al. 2004; Jascor et al. 2005]. In addition, C/EBPα was shown to

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**Figure 6.** Conditional inactivation of p38α restores hepatocyte proliferation and rescues aberrant expression of p21 protein in regenerating livers lacking c-jun. [A] Activity of p38 kinase in liver extracts isolated from c-jun<sup>−/−</sup>, c-jun<sup>−/−</sup><sup>p3α<sup>−/−</sup></sup>, and c-jun<sup>−/−</sup><sup>p21<sup>−/−</sup></sup> mice was measured at 24 h (upper panel) and 48 h (lower panel) after PH by Western blotting using an antibody against phosphorylated p38 MAPK. Three lanes represent protein samples from three individual mice of the indicated genotype. (B) Western blot time course analysis of phospho-p38 MAPK expression in regenerating livers. Genotypes are indicated. (C, upper panel) Ki67 immunohistochemistry 48 h after PH in liver sections of c-jun<sup>−/−</sup>p38α<sup>−/−</sup> and c-jun<sup>−/−</sup>p3α<sup>−/−</sup> mice. (Lower panel) Quantification of Ki67 expression in regenerating livers 48 h after PH. Genotypes are indicated (n = 3). (D) Western blot analysis of p21 expression in liver extracts from c-jun<sup>−/−</sup>p38α<sup>−/−</sup> and c-jun<sup>−/−</sup>p3α<sup>−/−</sup> mice at indicated time points following PH.
block proteolytic degradation of p21 by direct protein–protein interaction in livers of newborn mice (Timchenko et al. 1997). However, expression of mdm2 and C/EBPα mRNAs was not changed in c-jun<sup>Δi1</sup> mice after PH (data not shown). Expression and stability of p21 protein may also be influenced by phosphorylation through several kinases such as p38 MAPK, JNK1, and AKT/PKB (Kim et al. 2002, Li et al. 2002). Recently, high activities of p38α and JNKs were detected in primary hepatocytes lacking c-Jun (Eferl et al. 2003), suggesting a role of stress kinases in the expression of p21 protein. We have found high activity of p38 MAPK in livers c-jun<sup>Δi1</sup> mice at various time points following PH. This correlated with impaired hepatocyte proliferation and elevated expression of p21 protein. Importantly, increased phosphorylation of p38 kinase was abolished in c-jun<sup>Δi1</sup><i>p53</i>/<i>−/−</i> and c-jun<sup>Δi1</sup><i>p21</i>/<i>−/−</i> double-mutant mice, implying that both p53 and p21 can activate p38 by a post-transcriptional mechanism.

To gain further insights into the mechanism by which increased p38 activity affected hepatocyte proliferation in c-jun-deficient livers, we performed PH in c-jun<sup>Δi1</sup>p38α<sup>Δi1</sup> mice. Double-mutant livers showed similar rates of proliferating hepatocytes as wild-type controls. Most importantly, similar p21 protein levels were detected in liver extracts from double-mutant and control littermates. These results suggested that increased p38α activity is responsible for altered p21 expression and impaired liver regeneration in c-jun<sup>Δi1</sup> mice.

How p38α affects p21 protein expression is presently unknown. p38 MAPK has been reported to play a key role in stabilizing p21 protein [Alderton et al. 2001; Kim et al. 2002]. It has been demonstrated in a HD3 human colon carcinoma cell line, that stress signals initiated by TGFβ lead to activation of p38α and JNK1, which in turn, phosphorylated p21 at Ser130 leading to increased stability (Kim et al. 2002). However, we did not find any major alterations in the expression pattern of TGFβ in liver extracts from c-jun<sup>Δi1</sup> mice. Therefore, high activity of p38α in regenerating livers lacking c-jun appears to be independent of TGFβ signaling pathway. We propose that p38α is activated by a p53-dependent pathway (Fig. 7), therefore signaling downstream of p53. Alternatively, elevated activity of p38 MAPK might be a result of increased susceptibility of c-jun<sup>Δi1</sup> mice to stress caused by PH and the subsequent p21-mediated cell cycle arrest (Fig. 7).

Several studies have implicated a link between p53 and p38 MAPK in cell cycle regulation. It has been shown that under environmental stress conditions p53 mediates a negative feedback regulation of p38 MAPK signaling by inducing the expression of Wip1, a protein phosphatase that in turn selectively inactivates p38 by dephosphorylation of its conserved threonine residue [Takekawa et al. 2000]. Furthermore, p38α has been shown to enhance p53 activity by phosphorylation, and therefore trigger apoptosis or cell cycle arrest (Bulavin et al. 1999, Huang et al. 1999). It was also shown in the context of colonocyte apoptosis that under stress conditions increased p38α signaling followed by p53 phosphorylation can lead to p21 induction and cell cycle arrest (Kim et al. 2005). Moreover, recent data in T cells revealed that activation of p38 MAPK lead to phosphorylation and accumulation of p53 with subsequent increase of G2/M cell cycle checkpoint [Pedraza-Alva et al. 2006]. These data imply that p38 MAPK can also signal upstream of p53, although the molecular mechanism may be cell context dependent.

In summary, we have demonstrated that c-Jun/AP-1 promotes G1–S transition in hepatocytes in vivo through a p53-dependent pathway. In the context of liver regeneration c-Jun represses a post-transcriptional activity of p53 that imposes an antiproliferative state on hepatocytes by activation of p38α and subsequent p38-dependent increase in p21 protein (Fig. 7). In the absence of c-Jun, this antiproliferative state is maintained even after PH, but can be abolished by additional loss of p53. These data uncover a novel mechanistic link in the complex signaling network involving c-Jun/AP-1, p53/p21, and p38α, which is essential for regulating the restoration of liver mass following stress responses such as PH and liver injury. Future experiments will prove whether this novel molecular pathway is relevant in the control of hepatocyte proliferation during hepatocellular carcinoma formation.

Materials and methods

*Generation of mice and deletion of the floxed alleles in the liver*

All mice used in this study were kept on a mixed background (C57Bl/6,129SV). p53<sup>/−</sup> and p21<sup>/−</sup> mice [Donehower et al. 1992, Deng et al. 1995] were crossed with Mx-cre c-jun<sup>Δi1</sup> [Kuhn et al. 1995, Behrens et al. 2002] mice to obtain mice with the following genotypes: Mx-cre c-jun<sup>Δi1</sup>p53<sup>/−</sup> [c-jun<sup>Δi1</sup>p21<sup>−/−</sup>], Mx-cre c-jun<sup>Δi1</sup><i>p21</i>/<i>−/−</i> [c-jun<sup>Δi1</sup><i>p53</i>/<i>−/−</i>], and c-jun<sup>Δi1</sup><i>p21</i>/<i>−/−</i> [c-jun<sup>Δi1</sup><i>p53</i>/<i>−/−</i>].
c-jun$^{+/+}$, p21$^{+/+}$ (p21$^{+/+}$), Mx-cre c-jun$^{+/+}$p53$^{+/+}$ (c-jun$^{+/+}$), Mx-cre c-jun$^{−/−}$p21$^{+/+}$ (c-jun$^{−/−}$), Mx-cre c-jun$^{−/−}$p53$^{−/−}$ (c-jun$^{−/−}$), and c-jun$^{+/+}$p21$^{+/+}$ (c-jun$^{+/+}$) double-knockout mice were obtained by crossing Mx-cre c-jun$^{+/+}$ with p38$^{αβ}$ mice (Engel et al. 2008). Mx-cre-mediated deletion of the floxed alleles was induced by single intraperitoneal injection of poly I:C (Amersham Pharmacia Biotech, 400 µg in 200 µL PBS) into 8- to 12-wk-old animals 10 d prior to PH.

**PCR genotyping and Southern blotting**

The following primers were used for PCR genotyping of mice: Mx-cre: CRE1, CGGTCCATGCAACGATGAGGC; CRE2, CAAGACGCGAACATTCCGGTCG; FLOX2: LOXP5, CTCA TACCGTTTGCACAGGGC; LOXP6, CGGCTAGCAT CACGTGGTAGGG; FLOX2: CAGGCCGGTGCTGACAGCT; p53: Neo19, CATTCCAGCATAGGTGTT; Xp53, TATATCAGACGGCGGCT; Xs6.5p3, ACACCGTGGTCG TACCTTAT; p21: Eoson2F, GACAGAGCCGGCATGT GGG; Eoson3R, CAATCTGCGCTTGGAGTGAT; PGKF, GCAGCCTCTGTTCACATTACAC. Deletion of c-jun in the liver was determined by Southern blot analysis as described previously (Eferl et al. 2003).

**Partial hepatectomy**

All mice used for liver regeneration experiments were between 8 and 12 wk old. Surgeries were performed between 8 and 12 a.m. under avertin anesthesia and according to a standard procedure. Briefly, the abdominal cavity was opened with a transverse incision below the rib cage and the large left lateral and median lobes were ligated and removed. The abdominal wall was closed by suture and animals were recovered on a 37°C heating block. Liver samples were collected during PH (0-h time point) and at indicated time points following the surgery. For RNA and protein analysis, liver samples were snap-frozen in liquid nitrogen. For histological analysis, livers were fixed with neutral buffered 4% PFA overnight at 4°C and stored in 70% liquid nitrogen. For histological analysis, livers were fixed with neutral buffered 4% PFA overnight at 4°C and stored in 70% ethanol at 4°C until further processed.

**Histology and immunohistochemistry**

Fixed liver tissues were embedded in paraffin, and 5-µm sections were used for immunohistochemistry. For BrdU staining, 100 µg of BrdU per gram of body weight was injected intraperitoneally 2 h before the mice were sacrificed. Positive cells were identified by immunohistochemistry with an anti-BrdU antibody (Becton/Dickinson) according to the manufacturer’s recommendations. Immunohistochemical staining for Ki67 (antibody from Novocastra) was performed using the ABC staining kit (Vector Laboratories) according to the manufacturer’s recommendations. The percentage of Ki67-positive cells was quantified by counting hepatocytes in 10 random fields using a 40x objective. Ki67-stained liver sections from at least three individual animals of each genotype were used for quantification. Data are shown as mean, and the error bars represent the standard deviation.

**RNase protection assay (RPA)**

Total RNA was isolated with the TRIZOL protocol [Invitrogen] according to the manufacturer’s instructions, and 10 µg were used for each RPA reaction. RPA was performed using the RiboQuant multi probe RNase protection assay system mCyc-1 and mFox/Jun [PharMingen] according to the manufacturer’s protocol.

**Semi-quantitative PCR analysis**

Semi-quantitative PCR was performed with 1 µg of total RNA after cDNA synthesis using the “Ready-To-Go You-Prime It First-Strand-Beads” (Amersham Pharmacia Biotech). PCR products were analyzed by agarose gel electrophoresis or alternatively quantified by real-time PCR analysis using an Opticon2 Monitor Fluorescence Thermocycler [MJ Research]. Primers sequences are available upon request.

**Northern and Western blot analyses**

For Northern blot analysis, 20 µg of total RNA was used, and mRNA bands for p21 and fox were detected with labeled PCR products according to standard protocols. Western blot analysis was performed according to standard procedures using the following antibodies: CyclinD1 [Zymed], CyclinE, CyclinA, PCNA, TGFp1, p38 MAP Kinase, and phospho-ERK [Santa Cruz], c-Jun and panERK [Transduction Laboratories], p21 [PharMingen], phospho-p38 MAP Kinase, phospho-MK3/MK6, and MKK3 (Cell Signaling), and Tubulin (Sigma). The antibody against a proteasome subunit was a kind gift of Dr. J.M. Peters [Research Institute of Molecular Pathology, Vienna, Austria].

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**References**

Akerman, P., Cote, P., Yang, S.Q., McClain, C., Nelson, S., Bagby, G.J., and Diehl, A.M. 1992. Antibodies to tumor necrosis factor-α inhibit liver regeneration after partial hepatectomy. *Am. J. Physiol.* 263: G579–G585.

Albrecht, J.H., Meyer, A.H., and Hu, M.Y. 1997. Regulation of cyclin-dependent kinase inhibitor p21[WAF1/Cip1/Sdi1] gene expression in hepatic regeneration. *Hepatology* 25: 557–563.

Albrecht, J.H., Poon, R.Y., Ahonen, C.L., Rieland, B.M., Deng, C., and Crary, G.S. 1998. Involvement of p21 and p27 in the regulation of CDK activity and cell cycle progression in the regenerating liver. *Oncogene* 16: 2141–2150.

Alpertson, F., Humphrey, P.P., and Selman, A. 2001. High-intensity p38 kinase activity is critical for p21[cip1] induction and the antiproliferative function of G[i] protein-coupled receptors. *Mol. Pharmacol.* 59: 1119–1128.

Behrens, A., Sibilia, M., David, J.P., Mohle-Steinlein, U., Tronche, F., Schutz, G., and Wagner, E.F. 2002. Impaired postnatal hepatocyte proliferation and liver regeneration in mice lacking c-jun in the liver. *EMBO J.* 21: 1782–1790.

Borowiak, M., Garratt, A.N., Wustefeld, T., Strehle, M., Trautwein, C., and Birchmeier, C. 2004. Met provides essential growth signals for liver regeneration. *Proc. Natl. Acad. Sci.* 101: 10608–10613.

Bulavin, D.V., Saito, S., Hollandor, M.C., Sakaguchi, K., Anderson, C.W., Appella, E., and Fornace Jr., A.J. 1999. Phosphorylation of human p53 by p38 kinase coordinates N-terminal phosphorylation and apoptosis in response to UV radiation. *EMBO J.* 18: 6845–6854.

Cressman, D.E., Diamond, R.H., and Taub, R. 1995. Rapid ac-
tivation of the Stat3 transcription complex in liver regeneration. *Hepatology* 21: 1443–1449.

Cressman, D.E., Greenbaum, L.E., DeAngelis, R.A., Ciliberto, G., Furth, E.E., Poli, V., and Taub, R. 1996. Liver failure and defective hepatocyte regeneration in interleukin-6-deficient mice. *Science* 274: 1379–1383.

Deng, C., Zhang, P., Harper, J.W., Elledge, S.J., and Leder, P. 1995. Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. *Cell* 82: 675–684.

Diehl, A.M. 2002. Liver regeneration. *Front. Biosci.* 7: e301–e314.

Donohue, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery Jr., C.A., Butel, J.S., and Bradley, A. 1992. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* 356: 215–221.

Eferl, R., Sibilia, M., Hilberg, F., Fuchsbieler, A., Kufferth, I., Guertl, B., Zenz, R., Wagner, E.F., and Zatloukal, K. 1999. Functions of c-Jun in liver and heart development. *J. Cell Biol.* 145: 1049–1061.

Eferl, R., Ricci, R., Kenner, L., Zenz, R., David, J.P., Rath, M., and Wagner, E.F. 2003. Liver tumor development. c-Jun antagonizes the proapoptotic activity of p53. *Cell* 112: 181–192.

Engel, F.B., Schebesta, M., Duong, M.T., Lu, G., Ren, S., Madwed, J.B., Jiang, H., Wang, Y., and Keating, M.T. 2005. p38 MAP kinase inhibition enables proliferation of adult mammalian cardiomyocytes. *Genes & Dev.* 19: 1175–1187.

Fausto, N. 2004. Liver regeneration and repair: Hepatocytes, progenitor cells, and stem cells. *Hepatology* 39: 1477–1487.

FitzGerald, M.J., Webber, E.M., Donovan, J.R., and Fausto, N. 1995. Rapid DNA binding by nuclear factor κB in hepatocytes at the start of liver regeneration. *Cell Growth Differ.* 6: 417–427.

Greenbaum, L.E., Li, W., Cressman, D.E., Peng, Y., Ciliberto, G., Poli, V., and Taub, R. 1998. CCAAT enhancer-binding protein β is required for normal hepatocyte proliferation in mice after partial hepatectomy. *J. Clin. Invest.* 102: 1006–1007.

Heim, M.H., Gamboni, G., Beglinger, C., and Gyr, K. 1997. Specific activation of AP-1 but not Stat3 in regenerating liver in mice. *Eur. J. Clin. Invest.* 27: 948–955.

Hilberg, F., Aguzzi, A., Howells, N., and Wagner, E.F. 1993. c-Jun is essential for normal mouse development and hepatogenesis. *Nature* 365: 179–181.

Huang, C., Ma, W.Y., Maxiner, A., Sun, Y., and Dong, Z. 1999. p38 kinase mediates UV-induced phosphorylation of p53 protein at serine 389. *J. Biol. Chem.* 274: 12229–12235.

Huh, C.G., Factor, V.M., Sanchez, A., Uchina, K., Conner, E.A., and Karin, M. 2000. The mammalian UV response: c-Jun induction is required for exit from p53-imposed growth arrest. *Cell* 103: 897–907.

Takekawa, M., Adachi, M., Nakahata, A., Nakayama, I., Itoh, F., Tsukuda, H., Taya, Y., and Imai, K. 2000. p53-inducible wip1 phosphatase mediates a negative feedback regulation of p38 MAPK–p53 signaling in response to UV radiation. *EMBO J.* 19: 6517–6526.

Taub, R. 2004. Liver regeneration: From myth to mechanism. *Nat. Rev. Mol. Cell Biol.* 5: 836–847.

Kim, G.Y., Mercer, S.E., Ewton, D.Z., Yan, Z., Jin, K., and Friedeman, E. 2002. The stress-activated protein kinases p38α and JNK1 stabilize p21(Cip1) by phosphorylation. *J. Biol. Chem.* 277: 29792–29802.

Kim, H., Kokotou, E., Na, X., Rhee, S.H., Moyer, M.P., Pothoulakis, C., and Lamont, J.T. 2005. *Clostridium difficile* toxin A-induced colonocyte apoptosis involves p53-dependent p21(WAF1/CIP1) induction via p38 mitogen-activated protein kinase. *Gastroenterology* 129: 1875–1888.

Kobayashi, K. and Tsukamoto, I. 2001. Prolonged Jun N-terminal kinase (JNK) activation and the upregulation of p38 and p21(WAF1/CIP1) preceded apoptosis in hepatocytes after partial hepatectomy and cisplatin. *Biochim. Biophys. Acta* 1537: 79–88.

Kuhn, R., Schwenk, F., Aguet, M., and Rajewsky, K. 1995. Inducible gene targeting in mice. *Science* 269: 1427–1429.

Kwak, J.C., Ongusaha, P.P., Ouchi, T., and Lee, S.W. 2003. IFI16 as a negative regulator in the regulation of p53 and p21(Waf1). *J. Biol. Chem.* 278: 40899–40904.

Li, Y., Dowbenko, D., and Lasky, L.A. 2002. AKT/PKB phosphorylation of p21Cip1/WAF1 and promotes cell survival. *J. Biol. Chem.* 277: 11352–11361.

Mead, J.E. and Fausto, N. 1989. Transforming growth factor α may be a physiological regulator of liver regeneration by means of an autocrine mechanism. *Proc. Natl. Acad. Sci.* 86: 1558–1562.

Mitchell, C., Nivison, M., Jackson, L.F., Fox, R., Lee, D.C., Campbell, J.S., and Fausto, N. 2005. Heparin-binding epidermal growth factor-like growth factor links hepatocyte priming with cell cycle progression during liver regeneration. *J. Biol. Chem.* 280: 2562–2568.

Passegue, E., Jochum, W., Behrens, A., Ricci, R., and Wagner, E.F. 2002. Junβ can substitute for Jun in mouse development and cell proliferation. *Nat. Genet.* 30: 158–166.

Bedraza-Alva, G., Koulmies, M., Charland, C., Thornton, T., Clements, J.L., Schlissel, M.S., and Rincon, M. 2006. Activation of p38 MAP kinase by DNA double-strand breaks in V(D)J recombination induces a G2/M cell cycle checkpoint. *EMBO J.* 25: 763–773.

Schreiber, M., Kolbus, A., Piu, F., Szabowski, A., Mohle-Steinlein, U., Tian, J., Karin, M., Angel, P., and Wagner, E.F. 1999. Control of cell cycle progression by c-Jun is p53 dependent. *Genes & Dev.* 13: 607–619.

Shaullian, E., Schreiber, M., Piu, F., Beeche, M., Wagner, E.F., and Karin, M. 2000. The mammalian UV response: c-Jun induction is required for exit from p53-imposed growth arrest. *Cell* 103: 897–907.
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