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*^{Δli}) 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*^{Δli} mice abrogated both hepatocyte cell cycle block and increased p21 protein expression. Consistently, liver regeneration was rescued in *c-jun*^{Δli} p21^{-/-} 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*^{Δli} 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*^{Δli} mice. Importantly, conditional deletion of p38α in livers of *c-jun*^{Δli} 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.

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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) (Aker-

man 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-

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ment (Eferl et al. 2003). In contrast, conditional inactivation of *c-jun* in normal hepatocytes after birth had no effect on hepatocyte survival, but negatively affected hepatocyte proliferation during liver development and in stress response after PH (Behrens et al. 2002).

The molecular mechanism by which c-Jun controls hepatocyte proliferation in vivo is still unknown. Several reports have established a correlation between loss of *c-jun* and p53 activity. For example, the proliferation defect in MEFs lacking *c-jun* is abrogated after additional inactivation of *p53* (Schreiber et al. 1999). Moreover, it has been shown that c-Jun regulates the exit from p53-imposed growth arrest after UV treatment in MEFs by controlling the association of p53 with the *p21* promoter (Shaulian et al. 2000). In liver cancer, c-Jun seems to inhibit apoptosis of tumor cells through a p53-dependent mechanism. Tumors lacking *c-jun* displayed increased p53 levels and up-regulation of the proapoptotic p53 target gene *noxa* (Eferl et al. 2003). We therefore asked whether c-Jun controls hepatocyte proliferation in vivo via a p53-dependent molecular mechanism. For this purpose we used PH in double-knockout mouse models as an experimental system.

As shown previously, conditional inactivation of *c-jun* in the liver by employing either constitutively active, hepatocyte-specific *Alb-cre* line (*c-jun^{Alb}*) or an inducible *Mx-cre* line deleting in hepatocytes and hematopoietic cells (*c-jun^{Alb}*) caused a severe liver regeneration defect (Behrens et al. 2002). At the molecular level, impaired hepatocyte proliferation correlated with increased protein levels of p21, a p53-dependent inhibitor of cyclin-dependent kinases. Here we show that impaired liver regeneration of *c-jun^{Alb}* mice is completely rescued in a *p53* or *p21*-negative genetic background. We demonstrate that the increase in p21 protein levels in *c-jun* deficient livers is p53-dependent and sufficient to block hepatocyte proliferation. Furthermore, we show that the up-regulation of p21 protein is not caused by a direct transcriptional activation of the *p21* promoter by p53, since no alterations in *p21* mRNA expression were found after PH in *c-jun^{Alb}* mice. Intriguingly, up-regulation of p21 protein in regenerating livers of *c-jun^{Alb}* mice correlated with increased phosphorylation of p38, a stress kinase that is known to stabilize p21. The aberrant activity of p38 was abolished by loss of *p53* or *p21*, suggesting a signaling cross-talk between these proteins. p38 stress kinase is most likely responsible for increased p21 levels and impaired liver regeneration in *c-jun^{Alb}* mice, since combined conditional deletion of *c-jun* and *p38 α* in the liver abrogated both elevated expression of p21 protein and impaired hepatocyte proliferation in vivo.

Results

Inactivation of *p53* rescues the morbidity of *c-jun^{Alb}* mice and restores hepatocyte proliferation after PH

We have addressed the question whether p53 is responsible for impaired liver regeneration in *c-jun^{Alb}* mice

after PH. For that purpose we used mice harboring floxed alleles of *c-jun* (*c-jun^{f/f}* mice) (Behrens et al. 2002) and the inducible *Mx-cre* transgene (Kuhn et al. 1995), and crossed them with *p53*-deficient mice (*p53^{-/-}* mice) (Donehower et al. 1992) to obtain *c-jun^{Alb}**p53^{-/-}* 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^{Alb}**p53^{-/-}* and corresponding control mice (*c-jun^{Alb}*, *p53^{-/-}*, *c-jun^{f/f}*) 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^{Alb}* mice died 2–4 d after PH due to impaired liver regeneration (Behrens et al. 2002). In contrast, *c-jun^{Alb}**p53^{-/-}* 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 proliferate

Figure 1. Inactivation of *p53* rescues the morbidity of *c-jun^{Alb}* mice after PH. (A) Southern blot analysis confirms a complete deletion of *c-jun* in the liver after poly I/C injection. The floxed (f) and the deleted (Δ) alleles of *c-jun* in the liver of Mx-cre-negative (control) and Mx-cre-positive (mutant) mice are indicated. RNase protection assay (B) and Western blot (C) analyses confirm lack of *c-jun* expression 24 h after PH in the livers of mutant mice. (*) Nonspecific band. (D) Absence and presence of *p53* was tested by PCR using tail DNA of respective mice. Knockout (ko) and wild-type (wt) bands are indicated. (*) Nonspecific band. (E) Lethality curve after PH. Numbers of mice for each genotype are indicated.

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*^{Δli} 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*^{Δli} 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*^{Δli}*p53*^{-/-} double-mutant mice (Fig. 2A,C). Deletion of *p53* alone had no overt ef-

Figure 2. Loss of *p53* abrogates hepatocyte cell cycle block in regenerating livers of *c-jun*^{Δli} 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.

Figure 3. Impaired cyclin expression in regenerating livers of *c-jun*^{Δli} 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.

fect on hepatocyte proliferation, since the proliferation rate was comparable to *c-jun*^{f/f} control livers (Fig. 2A). These data suggest that impaired liver regeneration in *c-jun*^{Δli} mice is due to a cell cycle block imposed by p53 activity.

Inefficient induction of cell cycle regulators in regenerating livers of *c-jun*^{Δli} 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*^{Δli} 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*^{Δli} 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*^{Δli}*p53*^{-/-} livers (Fig. 3). Furthermore, reduced protein levels of the cell cycle regulator PCNA were found in *c-jun*^{Δli} mice 48 h after PH, but not in *c-jun*^{Δli}*p53*^{-/-} double-mutant livers (Fig. 3). We also observed reduced expression of retinoblastoma protein (RB) in *c-jun*^{Δli} 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*^{Δli}*p53*^{-/-} 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*^{Δli} 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*^{Δli} mice is due to a p53-dependent mechanism.

Loss of p21 rescues the liver regeneration defect in *c-jun*^{Δli} mice

To test whether impaired liver regeneration in *c-jun*^{Δli} mice is caused by a p53-dependent up-regulation of p21 protein expression, we have generated *c-jun*^{Δli}*p21*^{-/-} double-mutant mice and subjected them to PH. Importantly, morbidity and premature lethality of *c-jun*^{Δli} mice after PH was rescued in *c-jun*^{Δli}*p21*^{-/-} 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*^{Δli} hepatocytes. Ki67 expression peaked 48 h after PH in wild-type, *p21*^{-/-} and *c-jun*^{Δli}*p21*^{-/-} 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*^{-/-} 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*^{-/-} 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*^{Δli}*p21*^{-/-} 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*^{Δli} 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 littermates or control *p21*^{-/-} 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*^{Δli} mice.

Figure 4. Deletion of p21 restores hepatocyte proliferation in regenerating livers of *c-jun*^{Δli} 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*^{Δli} 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*^{-/-}, *c-jun*^{Δli}, and *c-jun*^{Δli}*p53*^{-/-} 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 difference between control, *p53*^{-/-}, *c-jun*^{Δli}, and *c-jun*^{Δli}*p53*^{-/-} mice (Fig. 5B), suggesting that the transcriptional activity of p53 is unchanged in *c-jun*^{Δli} 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

Figure 5. *c-jun*-deficient livers show no transcriptional changes of known p53 cell cycle targets including *p21*. (A) Northern blot analysis of *p21* mRNA expression in the livers of *c-jun^{fl/fl}*, *c-jun^{Δli}**, *c-jun^{Δli}* p53^{-/-}*, and *p53^{-/-}* mice at indicated time points after PH. The *fox* transcript was used as a loading control. (B) Semiquantitative PCR analysis of mRNA expression of several indicated p53-target genes. (*mdm2*) Murine double minute 2; (*bax*) *Bcl2* (B-cell leukemia/lymphoma 2)-associated X protein; (*cylG*) cyclin G; (*hprt*) hypoxanthine guanine phosphoribosyl transferase. Expression of *hprt* was used as a loading control.

in livers of *c-jun^{Δli}** mice (data not shown). However, loss of *p53* clearly reduced p21 protein levels in regenerating livers of *c-jun^{Δli}** mice (Fig. 3). Overall, these data suggest that the increased abundance of p21 protein in regenerating *c-jun^{Δli}** livers occurs through a p53-dependent post-transcriptional/post-translational mechanism.

Livers of *c-jun^{Δli}** 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 pro-

tein 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-jun^{Δli}* p53^{-/-}* and *c-jun^{Δli}* p21^{-/-}* double-mutant mice (Fig. 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-jun^{Δli}** 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-jun^{Δli}* p53^{-/-}* double-mutant mice. These experiments demonstrated that impaired liver regeneration and G1/S transition of hepatocytes in *c-jun^{Δli}** mice were fully rescued in a *p53*-

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*^{fl/fl}, *c-jun*^{Δli*}, and *c-jun*^{Δli*}*p21*^{-/-} 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*^{Δli*}*p38α*^{Δli*} and *c-jun*^{fl/fl}*p38α*^{fl/fl} 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*^{fl/fl}*p38α*^{fl/fl} and *c-jun*^{Δli*}*p38α*^{Δli*} mice at indicated time points following PH.

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*^{Δli*} mice as early as 6 h after PH. Furthermore, we demonstrated in vivo that this molecular event is sufficient to cause impaired liver regeneration in *c-jun*^{Δli*} mice, since hepatocyte proliferation was completely rescued in *c-jun*^{Δli*}*p21*^{-/-} double-mutant mice. It is worth mentioning that germline deletion of neither *p53* nor *p21* was able to rescue the lethality of *c-jun* knockout embryos (Schreiber et al. 1999; Passegue et al. 2002) implying that the events controlled by c-Jun in developmental processes and in hepatocyte proliferation after PH seem to be fundamentally different.

Our results indicated that liver regeneration is impaired in *c-jun*^{Δli*} mice due to a cell cycle block imposed by a p53-dependent up-regulation of p21. Since it has

been shown that c-Jun negatively regulates p53 association with the *p21* promoter (Shaulian et al. 2000), we assumed that increased abundance of p21 protein in the livers of *c-jun*^{Δli*} 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*^{Δli*} mice was apparently not due to a transcriptional effect of p53. The induction of *p21* mRNA was comparable in livers of control, *p53*^{-/-}, *c-jun*^{Δli*}, and *c-jun*^{Δli*}*p53*^{-/-} 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*^{Δli*} mice. p53 expression and transcriptional activity was not altered in *c-jun*^{Δli*} 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; Jascur et al. 2005). In addition, C/EBPα was shown to

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/EBP α* mRNAs was not changed in *c-jun^{Δli}* 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 p38 α 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^{Δli}* 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^{Δli}p53^{-/-}* and *c-jun^{Δli}p21^{-/-}* 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^{Δli}p38^{Δli}* 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 p38 α activity is responsible for altered p21 expression and impaired liver regeneration in *c-jun^{Δli}* mice.

How p38 α 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 TGF β lead to activation of p38 α 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 TGF β in liver extracts from *c-jun^{Δli}* mice. Therefore, high activity of p38 α in regenerating livers lacking *c-jun* appears to be independent of TGF β signaling pathway. We propose that p38 α 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^{Δli}* 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, p38 α 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 condi-

Figure 7. Schematic model for c-Jun/AP-1-dependent control of hepatocyte proliferation in regenerating livers. During liver regeneration, p53 and p38 MAPK are kept at low basal activities by c-Jun/AP-1, therefore preventing p21 protein accumulation and allowing hepatocyte proliferation. In the absence of c-Jun, a p53-dependent and p21-mediated G1/S cell cycle block inhibits liver regeneration. p38 MAPK is activated by a p53-dependent post-transcriptional mechanism and contributes to p21 protein stabilization. The stress condition imposed by PH in *c-jun^{Δli}* mice might also contribute to p38 activation, thereby establishing a positive feedback loop.

tions increased p38 α 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 induction of G2/M cell cycle checkpoint (Pedraza-Alva et al. 2006). These data imply that p38 MAPK can also signal upstream of 53, 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 p38 α 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 p38 α , 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^{-/-}* and *p21^{-/-}* mice (Donehower et al. 1992; Deng et al. 1995) were crossed with *Mx-cre c-jun^{fl/fl}* (Kuhn et al. 1995; Behrens et al. 2002) mice to obtain mice with the following genotypes: *Mx-cre c-jun^{fl/fl} p53^{-/-}* (*c-jun^{Δli} p53^{-/-}*), *Mx-cre c-jun^{fl/fl} p21^{-/-}* (*c-jun^{Δli} p21^{-/-}*), *c-jun^{fl/fl} p53^{-/-}* (*p53^{-/-}*),

c-jun^{fl/fl}*p21*^{-/-} (*p21*^{-/-}), *Mx-cre c-jun*^{fl/fl}*p53*^{+/+} (*c-jun*^{Δli/+}), *Mx-cre c-jun*^{fl/fl}*p21*^{+/+} (*c-jun*^{Δli/+}), *c-jun*^{fl/fl}*p53*^{+/+} (*c-jun*^{fl/fl}), and *c-jun*^{fl/fl}*p21*^{+/+} (*c-jun*^{fl/fl}). *c-jun*^{Δli/+}*p38*^{Δli/+} double-knockout mice were obtained by crossing *Mx-cre c-jun*^{fl/fl} with *p38*^{Δli/+} mice (Engel et al. 2005). *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, CGGTCGATGCAACGAGTGATGAGG; CRE2, CAAGAGACGGAAATCCATCGCTCG; *c-jun*: LOXP5, CTCA TACCAGTTCGCACAGCGCGG; LOXP6, CCGCTAGCACT CACGTTGGTAGGC; FLOX2: CAGGCCGTTGTGTCACTG AGCT; p53: Neo19, CATTAGGACATAGCGTTGG; X7p53, TATACTCAGAGCCCGCCT; X6.5p53, ACACGCTGGTGG TACCTTAT; p21: Exon2F, GACAAGAGGCCAGTACTTCC TC; Exon3R, CAATCTGCGCTTGGAGTGATAG; PGKF, GCAGCCTCTGTTCCACATACAC. 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% 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 40× 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 Ribo-Quant multiprobe RNase protection assay system mCyc-1 and mFos/jun (PharMingen) according to the manufacturer's protocol.

Semiquantitative PCR analysis

Semiquantitative 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, TGFβ1, p38 MAP Kinase, and phospho-ERK (Santa Cruz), c-Jun and panERK (Transduction Laboratories), p21 (PharMingen), phospho-p38 MAP Kinase, phospho-MKK3/MKK6, 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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