Published in final edited form as:
Cancer Cell. 2008 August 12; 14(2): 156–165. doi:10.1016/j.ccr.2008.06.016.

Hepatocyte necrosis induced by oxidative stress and IL-1α release mediate carcinogen-induced compensatory proliferation and liver tumorigenesis

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SUMMARY

Hepatocyte IκB kinase β (IKKβ) inhibits hepatocarcinogenesis by suppressing accumulation of reactive oxygen species (ROS) and liver damage, whereas JNK1 activation promotes ROS accumulation, liver damage and carcinogenesis. We examined whether hepatocyte p38α, found to inhibit liver carcinogenesis, acts similarly to IKKβ in control of ROS metabolism and cell death. Hepatocyte-specific p38α ablation enhanced ROS accumulation and liver damage, which were prevented upon administration of an antioxidant. In addition to elevated ROS accumulation, hepatocyte death, augmented by loss of either IKKβ or p38α, was associated with release of IL-1α. Inhibition of IL-1α action or ablation of its receptor inhibited carcinogen-induced compensatory proliferation and liver tumorigenesis. IL-1α release by necrotic hepatocytes is therefore an important mediator of liver tumorigenesis.

SIGNIFICANCE

Chronic liver injury and inflammation increase the risk of hepatocellular carcinoma (HCC), the third leading cause of cancer deaths worldwide. How chronic liver injury enhances tumor development is not known. Previously, we found that loss of hepatocyte IKKβ markedly enhanced carcinogen-induced liver injury and HCC development. Increased HCC development was also seen in mice lacking hepatocyte p38α. We now show that loss of hepatocyte p38α or IKKβ results in increased accumulation of ROS and hepatocyte necrosis. We found that hepatocyte necrosis triggers the release of IL-1α, which acts as a critical mediator of carcinogen-induced compensatory proliferation and HCC development. IL-1α may be a common tumor promoter produced in different forms of chronic liver injury and could be a target for tumor prevention.

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INTRODUCTION

The mammalian liver is the major drug detoxifying organ, responsible for metabolic activation and elimination of toxic chemicals and metabolic intermediates (Liska, 1998). Many chemicals metabolized in the liver also induce liver damage (Park et al., 2005a), and increase the risk of hepatocellular carcinoma (HCC), the most common type of liver cancer and the third leading cause of cancer deaths worldwide (Thorgeirsson and Grisham, 2002). In addition to toxic chemicals, major HCC risk factors include hepatitis B or C viruses (HBV, HCV), all of which cause chronic liver injury and inflammation (Bosch et al., 2004). The US incidence of HCC has been rapidly increasing due to the current HCV epidemic, which together with ethanol consumption dramatically increases HCC risk (Yuan et al., 2004). HCC usually develops in the setting of chronic hepatitis or cirrhosis, conditions that result in hepatocyte death and activation of resident liver macrophages (Kupffer cells; KC) and newly recruited inflammatory cells. These conditions stimulate compensatory hepatocyte proliferation, a response that maintains liver mass but may also be the main driver of hepatocarcinogenesis (Fausto, 1999). Although the precise carcinogenic function of chronic liver inflammation remains to be elucidated, results obtained in a mouse model in which HCC is induced by the chemical procarcinogen diethylnitrosamine (DEN) suggest that inflammation promotes hepatocarcinogenesis through production of cytokines that stimulate compensatory proliferation (Maeda et al., 2005; Naugler et al., 2007). Enhanced hepatocyte turnover was also seen in HCV-linked HCC (Ikeda et al., 1998; Ghany et al., 2003). Such results suggest that mechanisms that maintain hepatocyte viability and prevent liver damage may reduce the risk of HCC. Indeed, hepatocyte-specific expression of anti-apoptotic Bcl-2 proteins prevents HCC development (Pierce et al., 2002), although Bcl-2 promotes oncogenesis elsewhere (Korsmeyer, 1992).

Conversely, hepatocyte-specific ablation of IKKβ, the catalytic subunit of the IκB kinase (IKK) complex required for NF-κB activation (Rothwarf and Karin, 1999; Ghosh and Karin, 2002) and prevention of hepatocyte death (Maeda et al., 2003), greatly enhances DEN-induced hepatocarcinogenesis (Maeda et al., 2005). Accelerated HCC development was also seen after hepatocyte-specific deletion of IKKγ/NEMO, the regulatory subunit of the IKK complex that is required for IKKβ activation (Rothwarf and Karin, 1999; Makris et al., 2000), even without DEN exposure (Luedde et al., 2007). Increased HCC development was also seen upon hepatocyte specific ablation of p38α (Hui et al., 2007). Like IKKβ, p38α has anti-apoptotic activity (Park et al., 2002; Park et al., 2005b). By contrast, inactivation of IKKβ in myeloid cells inhibited compensatory proliferation and development of DEN-induced HCC, even in mice lacking hepatocyte IKKβ (Maeda et al., 2005).

Important for induction of hepatocyte death are reactive oxygen species (ROS), whose accumulation is prevented by NF-κB-induced antioxidant proteins (Pham et al., 2004; Kamata et al., 2005). Administration of the chemical antioxidant butylated hydroxyanisole (BHA) to $Ikk\beta^{\Delta hep}$ mice, lacking hepatocyte IKKβ, prevents DEN-induced ROS accumulation and liver damage, thereby attenuating HCC development (Maeda et al., 2005). The hepatocyte IKKγ/NEMO deficiency also increases ROS accumulation and its adverse effects are also reversed by BHA (Luedde et al., 2007). Although it has not been examined whether loss of hepatic p38α results in increased ROS accumulation after carcinogen exposure, loss of p38α in fibroblasts augments oxidative stress (Dolado et al., 2007). Exactly how hepatocyte death promotes HCC development is not clear, but it was proposed that necrotic hepatocytes release factors (damage signals or alarmins) that activate KC which in turn produce cytokines, such as interleukin 6 (IL-6), that promote compensatory hepatocyte proliferation (Naugler et al., 2007). The identity of the alarmins released by necrotic hepatocytes is not fully known but it was proposed that one such factor is IL-1α (Chen et al., 2007).

We now show that similar to *Ikkβ*$^{\Delta hep}$ mice, mice that lack p38α in hepatocytes, *p38α*$^{\Delta hep}$ mice, also exhibit elevated ROS accumulation after DEN exposure. Although p38α and IKKβ control different anti-oxidant genes to prevent ROS accumulation, in both cases BHA administration prevents hepatocyte damage and carcinogen-induced compensatory proliferation. Furthermore, we demonstrate that the critical mediator that is released by necrotic hepatocytes to stimulate compensatory proliferation and release of pro-carcinogenic IL-6 is IL-1α. Interference with IL-1α signaling or ablation of its receptor inhibit compensatory proliferation and HCC induction. We suggest that IL-1α is a general pro-tumorigenic mediator that is released upon chronic liver damage.

RESULTS

Enhanced hepatocarcinogenesis, liver damage and compensatory proliferation in *p38α*$^{\Delta hep}$ mice

We generated *p38α*$^{\Delta hep}$ or *p38α*$^{\Delta L+H}$ mice by crossing *p38α*$^{F/F}$ mice (Nishida et al., 2004) with either *Alb*-cre or *Mx1*-cre mice, respectively. *p38α*$^{\Delta hep}$ and *p38α*$^{\Delta L+H}$ progeny were obtained in the expected Mendelian ratio, were healthy and did not show any apparent liver dysfunction, based on histomorphology and serum levels of alanine aminotransferase (ALT) (data not shown). Neither strain exhibited spontaneous liver tumors up to one year of age, despite efficient ablation of p38α only in hepatocytes of *p38α*$^{\Delta hep}$ mice or in both hepatocytes and KC of *p38α*$^{\Delta L+H}$ mice (Figure S1A, B). However, upon DEN injection on postnatal day 14 (Maeda et al., 2005), *p38α*$^{\Delta hep}$ mice exhibited elevated HCC multiplicity and size relative to similarly treated *p38α*$^{F/F}$ controls (Figure 1A, B), as previously reported for mice given DEN plus phenobarbital (Hui et al., 2007). HCCs isolated from *p38α*$^{\Delta hep}$ mice retained the p38α deficiency but exhibited elevated c-Jun expression and increased JNK activity (Figure 1C). In contrast, there was no significant difference in HCC multiplicity between *p38α*$^{\Delta L+H}$ and *p38α*$^{\Delta F/F}$ mice (Figure S1C). To rule out a contribution of Cre-induced hepatocyte toxicity to the observed increase in tumor load, we examined HCC induction in *p38α*$^{+/F}$*/Alb-Cre* and found no difference from *p38α*$^{\Delta F/F}$ controls (Figure S1D).

Cytokine-driven compensatory proliferation was suggested to promote DEN-induced hepatocarcinogenesis (Maeda et al., 2005; Naugler et al., 2007). Of the different cytokines induced by DEN administration, a tumor promoting role was shown for IL-6 (Naugler et al., 2007). Whereas deletion of p38α only in hepatocytes augmented expression of IL-6 mRNA, deletion of p38α in both hepatocytes and KC inhibited IL-6 production (Figure 1D). A similar effect was seen for IL-1β mRNA at 4 hrs and HGF mRNA at 24 hrs after DEN administration. No obvious differences in TNFα and IL-1α mRNA amounts were seen. Thus, in agreement with its previously documented role in macrophages (Park et al., 2005b), p38α is also required for induction of IL-6 mRNA in KC, its main site of expression in the DEN-treated liver (Maeda et al., 2005). Given the important role of IL-6 in hepatocarcinogenesis (Naugler et al., 2007), its reduced production in *p38α*$^{\Delta L+H}$ mice can explain why these mice do not show the elevated hepatocarcinogenesis seen in *p38α*$^{\Delta hep}$ mice, despite the absence of p38α in their hepatocytes. It should be noted, however, that using a different liver carcinogenesis protocol that involves co-administration of phenobarbital, Hui et al. found that absence of KC p38α had little further impact on tumor load beyond the effect of hepatocyte p38α deficiency (Hui et al., 2007). This may be due to replacement of IL-6 produced by KC by other tumor promoters induced by phenobarbital.

The substantial increase in tumor load in *p38α*$^{\Delta hep}$ mice is similar to what was seen in *Ikkβ*$^{\Delta hep}$ mice (Maeda et al., 2005). Indeed, like *Ikkβ*$^{\Delta hep}$ mice (Maeda et al., 2005), *p38α*$^{\Delta hep}$ mice exhibited more DEN-induced liver damage assessed by ALT release, and hepatocyte apoptosis measured by a TUNEL assay, relative to controls (Figure 2A, B). Only a fraction of all hepatocytes undergoes cell death in response to a carcinogenic dose of DEN

and the location of the dead cells parallels that of hepatocytes involved in DEN metabolism (Yang et al., 1990). Histological analysis confirmed more zone 3 hepatocyte necrosis in $p38\alpha^{\Delta hep}$ mice 2 days after DEN administration than in $p38\alpha^{F/F}$ counterparts (data not shown). $p38\alpha^{\Delta hep}$ mice also exhibited elevated neutrophil infiltration measured by myeloperoxidase (MPO) activity after DEN administration (Figure 2C). Due to their high regenerative capacity surviving hepatocytes undergo compensatory proliferation and thereby maintain liver mass after liver damage (Fausto et al., 2006). Labeling with bromodeoxyuridine (BrdU) revealed more proliferating hepatocytes in $p38\alpha^{\Delta hep}$ mice after DEN exposure (Figure 2D), located mainly around clusters of apoptotic cells in centrilobular lesions (data not shown). No differences in DEN-induced liver damage and compensatory proliferation between $p38\alpha^{F/F}$ and $p38\alpha^{+/F}$*/Alb-Cre* mice were found (Figure S1E), ruling out the possible contribution of Cre toxicity to the observed phenotype.

Deletion of IKKβ in hepatocytes augments DEN-induced JNK activation (Maeda et al., 2005), which contributes to enhanced hepatocyte death, compensatory proliferation and hepatocarcinogenesis (Sakurai et al., 2006). Absence of p38α also enhanced and prolonged JNK activation (Hui et al., 2007; Figure 2E), but had no substantial effect on IKK activation (Figure S2A). The increase in JNK activity in DEN-injected $p38\alpha^{\Delta hep}$ mice was of higher magnitude than in $Ikk\beta^{\Delta hep}$ mice (Figure 2E, Figure 2F). Furthermore, $p38\alpha^{\Delta hep}$ mice exhibited increased activation of the JNK kinases MKK4 and MKK7, an effect not seen in $Ikk\beta^{\Delta hep}$ mice (Figure 2E). Immunohistochemical analysis revealed that JNK activation detected by phosphorylation of c-Jun, a specific JNK substrate (Hibi et al., 1993), mostly occurred in zone 3 hepatocytes, the cells involved in DEN metabolism and ROS production (Figure 2G). In both $p38\alpha^{\Delta hep}$ and $Ikk\beta^{\Delta hep}$ mice, administration of a JNK inhibitor inhibited DEN-induced liver damage and compensatory proliferation (Figure S2B, C). Loss of neither p38α nor IKKβ had a significant effect on ERK activity after DEN treatment (Figure S2D). p38 phosphorylation also did not differ between $Ikk\beta^{F/F}$ and $Ikk\beta^{\Delta hep}$ mice (Figure S2E).

Enhanced ROS accumulation in $p38\alpha^{\Delta hep}$ mice accounts for increased liver injury and compensatory proliferation

A causal link between oxidative stress and cancer was proposed (Ames, 1983). Ablation of hepatocyte IKKβ enhances ROS accumulation after DEN injection (Kamata et al., 2005; Maeda et al., 2005) and similar observations were made in unchallenged mice lacking hepatic IKKγ/NEMO (Luedde et al., 2007). We assessed accumulation of hepatocyte superoxides by staining freshly frozen liver sections with dihydroethidine (DHE), whose oxidation gives rise to the fluorescent derivative ethidine (Veerman et al., 2004). More extensive fluorescence was seen in centrilobular areas (zone 3), the site of DEN metabolism, 12 hrs after DEN administration in $p38\alpha^{\Delta hep}$ and $Ikk\beta^{\Delta hep}$ mice than in matched controls (Figure 3A, 3B). We also detected increased accumulation of H_2O_2 in livers of DEN-treated $p38\alpha^{\Delta hep}$ and $Ikk\beta^{\Delta hep}$ mice using the ROS indicator 5-[and-6]-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) (Figure S3). To evaluate the contribution of oxidative stress to DEN-induced liver damage, we placed a group of mice on chow diet supplemented with the antioxidant BHA 2 days before DEN treatment. Like $Ikk\beta^{\Delta hep}$ mice (Maeda et al., 2005), $p38\alpha^{\Delta hep}$ mice kept on BHA-supplemented diet showed a marked reduction in DEN-induced liver injury (Figure 3C) and compensatory proliferation (Figure 3D). Thus, loss of either p38α or IKKβ enhances DEN-induced cell death and compensatory proliferation through mechanisms that may depend on ROS accumulation.

IKKβ and p38α control different anti-oxidant genes

In macrophages, p38α is required for induction of a subset of NF-κB target genes, including the survival genes *Pai2* and *Bfl1* (Park et al., 2005b). To investigate whether hepatic p38α and IKKβ co-regulate genes that inhibit ROS accumulation and maintain cell survival, we

conducted expression profiling of $p38\alpha^{\Delta hep}$, $Ikk\beta^{\Delta hep}$, and control livers 4 hrs after DEN administration using whole genome arrays (Supplementary Table and Figure S4). Data analysis revealed changes in expression of several genes relevant to regulation of ROS accumulation, cell death or cell proliferation. Curiously, very few of these genes were equally dependent on both kinases. We confirmed increased expression of *Fas*, *E2F1*, *Gadd45β* and *cyclin D1* and decreased expression of $p21^{waf1/cip1}$ and *MKP2* by quantitative RT-PCR in $p38\alpha^{\Delta hep}$ livers relative to controls (data not shown). In $Ikk\beta^{\Delta hep}$ livers, *cyclin D1* was also increased and *MKP2* was decreased relative to control livers (Supplementary Table).

Interestingly, $p38\alpha^{\Delta hep}$ livers exhibited a marked decrease (2.5-fold) in expression of Hsp25 (heat shock protein 1) mRNA relative to controls, a change not observed in $Ikk\beta^{\Delta hep}$ livers (Supplementary Table). We therefore examined the involvement of Hsp25 in ROS accumulation in $p38\alpha^{\Delta hep}$ mice. DEN injection led to considerable Hsp25 protein accumulation by 8 hrs in $p38\alpha^{F/F}$, but not in $p38\alpha^{\Delta hep}$, livers (Figure 4A). Defective Hsp25 mRNA induction was specific to $p38\alpha^{\Delta hep}$ mice and was not seen in $Ikk\beta^{\Delta hep}$ mice (Figure 4B). In contrast, IKKβ, but not p38α, contributed to DEN-induced SOD2 expression (data not shown; Maeda et al., 2005). These experiments also confirmed that p38α ablation enhanced expression of IRF1 mRNA and protein (Figure 4A, Figure 4B). We also found a difference in expression of heme oxygenase 1 (HO-1, also known as Hsp32), the inducible HO isoform that provides protection against oxidative stress and inflammation (Poss and Tonegawa, 1997). In $p38\alpha^{\Delta hep}$, but not $Ikk\beta^{\Delta hep}$ (data not shown), mice HO-1 induction was attenuated after DEN injection relative to $p38\alpha^{F/F}$ mice (Figure 4C).

Hsp25 was reported to inhibit ROS accumulation (Escobedo et al., 2004; Garrido et al., 2006). To examine whether Hsp25 also controls ROS accumulation in DEN treated mice, we constructed an adenovirus expressing Hsp25 and injected it via the tail vein into $p38\alpha^{\Delta hep}$ mice. This restored liver Hsp25 expression (Figure 4D), lowered ROS accumulation (Figure 4E) and reduced liver damage (Figure 4F) and compensatory proliferation (Figure 4G) in DEN-injected mice. By contrast, adenoviral mediated expression of IRF1 in liver enhanced ROS accumulation and liver damage (Figure S5A). Hsp25 was proposed to inhibit ROS accumulation by increasing reduced glutathione (GSH) concentration (Escobedo et al., 2004; Garrido et al., 2006). Indeed, liver GSH content, which was lower in $p38\alpha^{\Delta hep}$ relative to $p38\alpha^{F/F}$ mice (Figure 4H), was restored upon Hsp25 re-expression (Figure S5B).

ROS accumulation results in oxidative inhibition of MKPs, leading to enhanced JNK activation that contributes to liver failure but can be prevented by BHA administration (Kamata et al., 2005; Maeda et al., 2005). BHA administration partially inhibited DEN-induced JNK activation in $p38\alpha^{\Delta hep}$ mice, but the effect was less pronounced than in $Ikk\beta^{\Delta hep}$ mice, where BHA feeding almost completely inhibited JNK activation (Figure 5A). Thus, enhanced ROS accumulation makes only a partial contribution to JNK activation in $p38\alpha^{\Delta hep}$ mice. Consistent with this notion, BHA had little effect, if any, on MKK4 activity (Figure 5B), which was enhanced by loss of p38α but not IKKβ (Figure 2E).

The microarray analysis revealed elevated Chop mRNA in DEN-treated $p38\alpha^{\Delta hep}$ liver (Supplementary Table). Chop (C/EBP homologous protein) is a leucine-zipper transcription factor, also known as growth arrest and DNA damage-inducible gene 153 (Gadd153), that activates an apoptotic response downstream of ROS (Lai and Wong, 2005). DEN injection into $p38\alpha^{F/F}$ mice induced Chop protein within 12 hrs, and this response was augmented in $p38\alpha^{\Delta hep}$ mice (Figure 5C). The amount of Chop mRNA was significantly higher in DEN-treated $p38\alpha^{\Delta hep}$ livers than in $p38\alpha^{F/F}$ or $Ikk\beta^{\Delta hep}$ livers (Figure 5D). As expected for a gene whose expression is induced upon ROS accumulation, induction of Chop mRNA in DEN-treated $p38\alpha^{\Delta hep}$ mice was suppressed by BHA, which had no effect on expression of Hsp25 mRNA (Figure 5E), which acts upstream to ROS by inhibiting their accumulation.

Correspondingly, Hsp25 re-expression in $p38\alpha^{\Delta hep}$ liver suppressed Chop expression (Figure 5F), whereas Chop expression was increased in livers of IRF1 adenovirus infected mice (Figure 5G).

IL-1α release and signaling promote IL-6 production, compensatory proliferation and hepatocarcinogenesis after hepatocyte death

We have proposed that an inflammatory response triggered by hepatocyte death promotes compensatory proliferation and HCC development by inducing KC production of IL-6 (Naugler et al., 2007). Indeed, $p38\alpha^{\Delta hep}$ and $Ikk\beta^{\Delta hep}$ mice, which exhibit more liver damage after DEN administration, produce more IL-6 than control mice (Figure 1D; Maeda et al., 2005). DEN-induced IL-6 production was shown to depend on the adaptor protein MyD88, which is also required for liver carcinogenesis (Naugler et al., 2007). However, the receptor responsible to DEN-induced MyD88 signaling was not identified. Recently, it was shown that liver inflammation and failure caused by acetaminophen administration are mediated by IL-1α release from necrotic cells and IL-1R activation (Chen et al., 2007). We therefore examined whether DEN-induced liver damage also results in release of IL-1R ligands. In vitro, hepatocyte necrosis resulted in extensive IL-1α, but not IL-1β or IL-6 release (Figure 6A and data not shown). We collected venous blood through reverse perfusion of the portal vein 4 hrs after DEN administration and found higher levels of IL-1α in $p38\alpha^{\Delta hep}$ and $Ikk\beta^{\Delta hep}$ mice than in control mice (Figure 6B). Administration of BHA, which prevents hepatocyte death (Figure 3C), inhibited DEN-induced IL-1α release and IL-6 production (Figure 6C), suggesting they are both indeed linked to oxidative stress and liver injury. Next we examined the role of IL-1R using $Il1r^{-/-}$ mice (Abcouwer et al., 1996). IL-1R deficiency reduced DEN-induced IL-6 production to almost the same extent as the MyD88 deficiency (Figure 6D). $Il1r^{-/-}$ mice also exhibited less DEN-induced neutrophilic inflammation (Figure 6E) and lower compensatory proliferation relative to WT mice (Figure 6F). Reduced DEN-induced compensatory proliferation in $p38\alpha^{\Delta hep}$ and $Ikk\beta^{\Delta hep}$ mice was also seen upon administration of IL-1R antagonist (IL-1Ra or Anakinra), which also reduced IL-6 production (Figure 6G). This experiment provides further evidence that IL-6 production is dependent on IL-1 signaling. Most importantly, $Il1r^{-/-}$ mice exhibited a marked reduction in DEN-induced hepatocarcinogenesis (Figure 6H). Consistent with the absence of IL-1β release by necrotic hepatocytes, mice lacking caspase-1, the enzyme required for production of mature IL-1β, but not IL-1α, by macrophages (Greten et al., 2007), did not exhibit any defect in DEN-induced compensatory proliferation (Figure S6).

DISCUSSION

Oxidative stress was suggested to be a major contributor to cancer development (Ames, 1983) because it can exert many pro-tumorigenic effects, including altered gene expression (Allen and Tresini, 2000), enhanced cell proliferation and higher DNA-mutation rates (Toyokuni, 2006), as well as genomic instability (Woo and Poon, 2004). However, the exact impact of oxidative stress and anti-oxidant responses on tumor development needs to be better understood. NF-κB activation was found to play a critical role in preventing ROS accumulation through induction of the anti-oxidants FHC and SOD2 (Pham et al., 2004; Kamata et al., 2005; Sakon et al., 2003). Correspondingly, hepatocyte-specific IKKβ ablation, which prevents NF-κB activation (Maeda et al., 2003), augments ROS accumulation in livers of mice exposed to DEN and potentiates HCC development (Maeda et al., 2005). Consequently, the antioxidant BHA prevents the increase in HCC induction seen in $Ikk\beta^{\Delta hep}$ mice (Maeda et al., 2005). BHA administration also prevents HCC formation in mice lacking the IKKγ/NEMO regulatory subunit (Luedde et al., 2007). We now describe that the p38α MAPK pathway also prevents ROS accumulation and DEN-induced hepatocyte death. As found for $Ikk\beta^{\Delta hep}$ mice, elevated susceptibility to liver damage and increased carcinogen-induced compensatory proliferation

in $p38\alpha^{\Delta hep}$ mice are reversed by BHA administration. Thus increased ROS accumulation may be the main cause of hepatocyte death in IKKβ-, IKKγ/NEMO- and p38α-deficient mice. Importantly, we found that DEN-induced hepatocyte death results in the release of IL-1α and activation of IL-1R signaling, leading to IL-6 induction and compensatory proliferation, processes that are critical for hepatocarcinogenesis (Sakurai et al., 2006; Naugler et al., 2007).

In the case of DEN, oxidative stress mainly affects centrilobular (zone 3) hepatocytes, the cells in which DEN is metabolized via a ROS generating reaction (Yang et al., 1990). Both $Ikk\beta^{\Delta hep}$ (Maeda et al., 2005) and $p38\alpha^{\Delta hep}$ mice accumulate more ROS in these cells after DEN administration and exhibit more hepatocyte death than control mice. Yet, neither $Ikk\beta^{\Delta hep}$ nor $p38\alpha^{\Delta hep}$ mice show spontaneous liver damage or HCC formation unless challenged with a carcinogen. Although liver damage has been known to cause compensatory proliferation (Fausto et al., 2006), which is enhanced in both $Ikk\beta^{\Delta hep}$ and $p38\alpha^{\Delta hep}$ mice, the mechanism accounting for this response was not fully understood. We now show that DEN-induced liver injury results in rapid release of IL-1α, whose concentration in venous blood of $p38\alpha^{\Delta hep}$ and $Ikk\beta^{\Delta hep}$ livers is significantly higher than in controls. Most importantly, inhibition of IL-1R activation or its ablation inhibit DEN-induced IL-6 production, compensatory proliferation and/or hepatocarcinogenesis, processes that also depend on MyD88 (Naugler et al., 2007), the adaptor protein that connects IL-1R to downstream effector pathways (Akira et al., 2006). The pathway initiated by hepatocyte death leading to compensatory proliferation and liver tumor promotion is summarized in Figure 7.

Despite similar effects on liver damage and hepatocellular carcinogenesis, IKKβ and p38α use distinct mechanisms to prevent ROS accumulation. In the case of IKKβ and NF-κB, the most critical anti-oxidants are FHC (Pham et al., 2004) and SOD2 (Kamata et al., 2005), but p38α mainly acts via Hsp25, whose expression is only marginally dependent on IKKβ. Hsp25 is the mouse homologue of human Hsp27, which is phosphorylated by MAPKAPK2/MK2 (Stokoe et al., 1992), a substrate for p38 MAPK (Freshney et al., 1994). Curiously, in human HCCs, attenuated Hsp27 phosphorylation correlates with tumor progression (Yasuda et al., 2005), but it is not clear whether this is due to decreased p38 activity. Hsp27 possesses anti-oxidant properties associated with its ability to maintain reduced GSH and neutralize the toxic effects of oxidized proteins (Garrido et al., 2006). Importantly, Hsp25 re-expression in $p38\alpha^{\Delta hep}$ livers increased GSH concentration and reduced ROS accumulation, resulting in less DEN-induced damage and compensatory proliferation. DEN-challenged $p38\alpha^{\Delta hep}$ mice express more Chop than $p38\alpha^{F/F}$ mice. Chop was described as a ROS-induced regulator of apoptosis (Lai and Wong, 2005) and its expression is induced by different stresses, including oxidative stress (Oyadomari and Mori, 2004). Oxidative stress was suggested to induce *Chop* gene transcription through an AP-1 binding site (Guyton et al., 1996) and the absence of p38α augments JNK activation (Hui et al., 2007), which stimulates AP-1 transcriptional activity (Karin, 1995) and also results in upregulation of c-Jun, a critical component of AP-1 (Shaulian and Karin, 2002).

ROS accumulation also results in oxidative inhibition of MKPs, the phosphatases responsible for termination of JNK activation, and this is the major cause of enhanced JNK activation in IKKβ-deficient cells and mice (Kamata et al., 2005). Indeed, there is little change in activation of the JNK kinases MKK4/7 in $Ikk\beta^{\Delta hep}$ livers. However, MKP inactivation accounts for only a part of the increase in JNK activity in $p38\alpha^{\Delta hep}$ mice, which exhibit elevated MKK4/7 activity, an observation also made by Hui et al. (Hui et al., 2007). These findings suggest that p38α is involved in negative regulation of a yet-to-be identified MKK kinase (MAP3K or MKKK) that activates MKK4/7 (Figure 7). Both JNK1 activation (Sakurai et al., 2006) and c-Jun expression (Eferl et al., 2003) are important contributors to hepatocyte proliferation and HCC development. Notably, sustained JNK activation also contributes to ROS accumulation

(Ventura et al., 2004), thus explaining why BHA although not fully inhibiting excess JNK activation in $p38\alpha^{\Delta hep}$ mice still provides effective protection against liver damage. In summary, p38α and IKKβ negatively regulate ROS accumulation and JNK activity through different mechanisms, all of which maintain hepatocyte viability and suppress liver carcinogenesis (Figure 7). Enhanced hepatocyte death caused by the absence of either kinase results in increased IL-1α release, IL-1R activation, IL-6 production and compensatory proliferation, which eventually augments HCC development. Our results suggest that the role of IL-1α release in chronic liver injury in patients suffering from different forms of persistent hepatitis needs to be evaluated. If IL-1α is also found to be present in the injured human liver, interference with IL-1R signaling may provide a highly specific and effective mean to interfere with the most dreadful consequence of chronic hepatitis, HCC development.

EXPERIMENTAL PROCEDURES

Animals, tumor induction and analysis

$Ikk\beta^{F/F}$, $Ikk\beta^{F/F}$:*Alb-Cre* (referred to as $Ikk\beta^{\Delta hep}$), and $p38\alpha^{F/F}$ mice were described (Maeda et al., 2003; Nishida et al., 2004). $p38\alpha^{\Delta hep}$ mice were generated by crossing $p38\alpha^{F/F}$ and *Alb-Cre* mice. $p38\alpha^{\Delta L+H}$ mice were generated by treating $p38\alpha^{F/F}$:*Mx1-Cre* mice with poly(IC) as described (Maeda et al., 2005). All mice were maintained in filter-topped cages on autoclaved food and water at UCSD according to NIH guidelines and all experiments were performed in accordance with UCSD and NIH guidelines and regulations. Fourteen-day-old mice and littermates on a C57BL/6 background were injected with 25 mg/kg DEN (Sigma, Taufkirchen, Germany). After 8 months on normal chow, mice were sacrificed and their livers removed, separated into individual lobes, analyzed for presence of HCCs, and subjected to histological and immunochemical parameters as described (Sakurai et al., 2006). Hepatocytes were isolated and plated as described (Leffert et al., 1979). Hepatocytes were cultured for 24 hr in arginine-free medium containing 10% dialyzed serum to eliminate other cell types (Leffert et al., 1979) and no KC contamination was detected by immunostaining with antibodies against F4/80.

Collection of venous blood and IL-1α measurement

We collected venous blood through reverse perfusion of the portal vein. After clamping the inferior vena cava above the confluence of the hepatic vein, livers were perfused with PBS containing heparin (200 U/mouse) from the inferior vena cava and portal blood samples were used to measure cytokine concentration. IL-1α and IL-1β were quantified by ELISA (R&D systems, Minneapolis, MN) according to manufacturer's instructions.

Biochemical and immunochemical analyses

JNK assays, real time Q-PCR, immunoblotting, and immunohistochemistry were described (Sakurai et al., 2006). Antibodies used were: anti-phospho-MKK4, anti-phospho-MKK7, anti-phospho-ERK, anti-ERK1/2, anti-phospho-p38, anti-phospho-c-Jun (Cell Signaling Technology, Beverly, MA); anti-Hsp27/25, anti-IRF-1, anti-Chop, anti-c-Jun, anti-MKK4, anti-MKK7, anti-HO-1, anti-p38 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-JNK1, (Pharmingen, San Diego, CA); anti-actin (Sigma); and anti-IKKβ (Upstate, Charlottesville, VA). Immunohistochemistry was performed using ABC staining kit (Vector Laboratory, Burlingame, CA) according to manufacturer's recommendations. To examine accumulation of superoxide anions or H_2O_2, freshly prepared frozen sections were incubated with 2 μM dihydroethidine hydrochloride (Invitrogen, Carlsbad, CA) or 5 μM 5-[and-6]-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate (Invitrogen), respectively for 30 min at 37°C, after which they were observed by fluorescent microscopy and quantified with Metamorph software. Myeloperoxidase (MPO) activity was measured using MPO activity assay kit (Invitrogen). Livers were homogenized in MPO buffer (0.5% hexadecyl trimethyl ammonium bromide, 10

mM EDTA, 50 mM Na_2HPO_4, pH 5.4). GSH concentration was measured as described (Kamata et al., 2005).

Microarray analysis

Livers from *p38α*$^{\Delta hep}$, *Ikkβ*$^{\Delta hep}$, and corresponding "floxed" mice were removed 4 hrs after DEN injection and lysed in TRIzol reagent (Invitrogen). Total RNA was extracted from livers using an RNeasy kit (Qiagen Inc., Valencia, CA). Biotinylated cRNA was prepared using Illumina RNA Amplification Kit, Catalog #1L1791 (Ambion, Inc., Austin, TX) according to manufacturer's directions. For microarray analysis, the Illumina Mouse 6 Sentrix Expression BeadChip was used (Illumina, San Diego, CA). Data analysis and quality control were carried out using BeadStudio software (Illumina). Array data has been deposited in the EBI Array Express Database (accession number E-TABM-351).

Adenoviral transduction

Adenoviruses expressing IRF-1 and Hsp25 were prepared as described (Iimuro et al., 1998). Adenovirus stocks were injected via the tail vein at 1×10^9 plaque-forming units (PFU)/mouse. Before infection, virus stocks were dialyzed against PBS containing 10% glycerol.

Statistical analysis

Data are presented as means ± S.E. Differences were analyzed by Student's t test. p values < 0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We thank J. Feramisco for help with image capture and analysis and J. Lapira and R. Šášik for Illumina Beadarray processing and bioinformatics analysis. T.S. was supported by the Japan Society for the Promotion of Science and Takeda Science Foundation. G.H. was supported by NIH/NIDDK - Award 1 P30 DK063491-03. Research was supported by grants from the National Institute of Health (ES004151, ES006376 and CA118165) and the Superfund Basic Research Program (ES0100337). M.K. is an American Cancer Society Research Professor.

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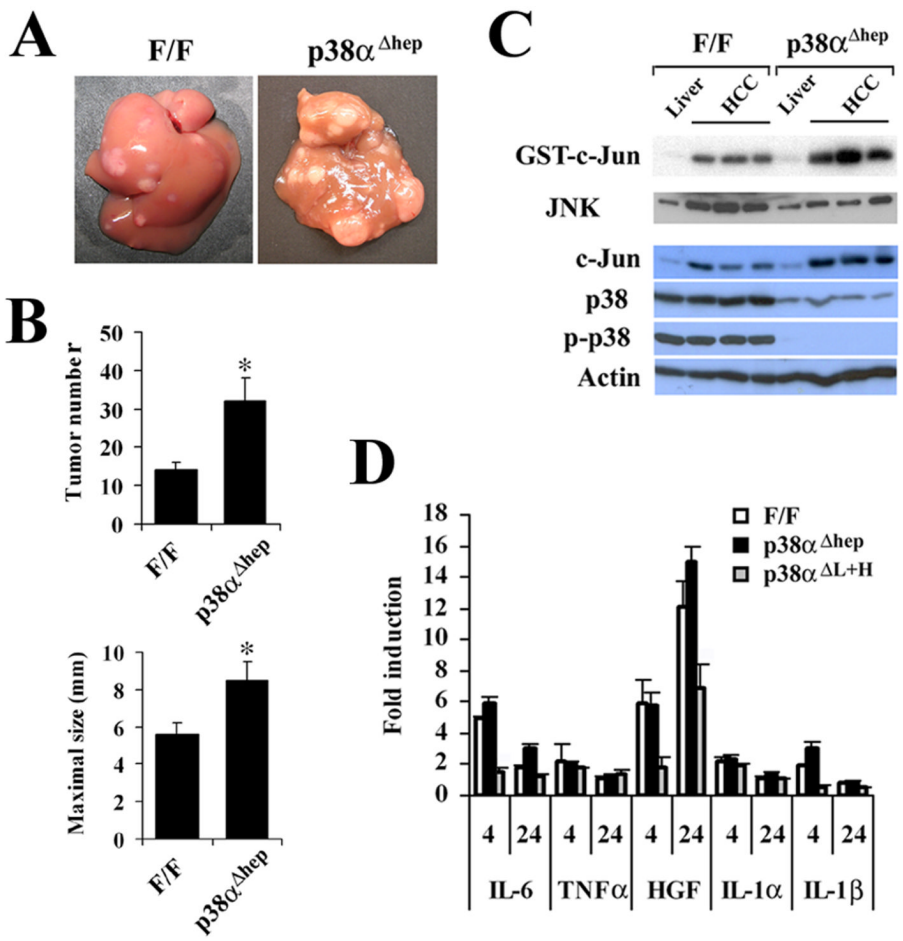

Figure 1. Hepatocellular carcinogenesis in *p38α*$^{\Delta hep}$ mice

(A) Livers of male *p38α*$^{\Delta hep}$ and *p38α*$^{F/F}$ mice 8 months after DEN injection (25 mg/kg). (B) Tumor multiplicity (>0.5mm) and maximal tumor sizes (diameters) in livers of male *p38α*$^{F/F}$ (F/F, n=15) and *p38α*$^{\Delta hep}$ (n=14) mice. Results are means ± SEM. *, p<0.05 vs. control mice. (C) Loss of p38α enhances JNK activity and c-Jun expression in HCCs. Lysates of microdissected HCCs (three separate samples) or non-tumor liver tissue (Liver) from DEN-treated mice were analyzed for JNK activity by immunecomplex kinase assay with GST-c-Jun as a substrate. Lysates were also gel-separated and analyzed by immunoblotting with antibodies to the indicated proteins. (D) Effects of p38α in hepatocytes and Kupffer cells (KC) on cytokine gene expression. Mice of the indicated genotypes were injected with DEN and liver RNA was extracted at the indicated times. Relative amounts of cytokine mRNAs were determined by real time Q-PCR and normalized to the amount of actin mRNA. The amount of each cytokine mRNA in untreated liver was given an arbitrary value of 1.0. Results are means ± SEM (n=4).

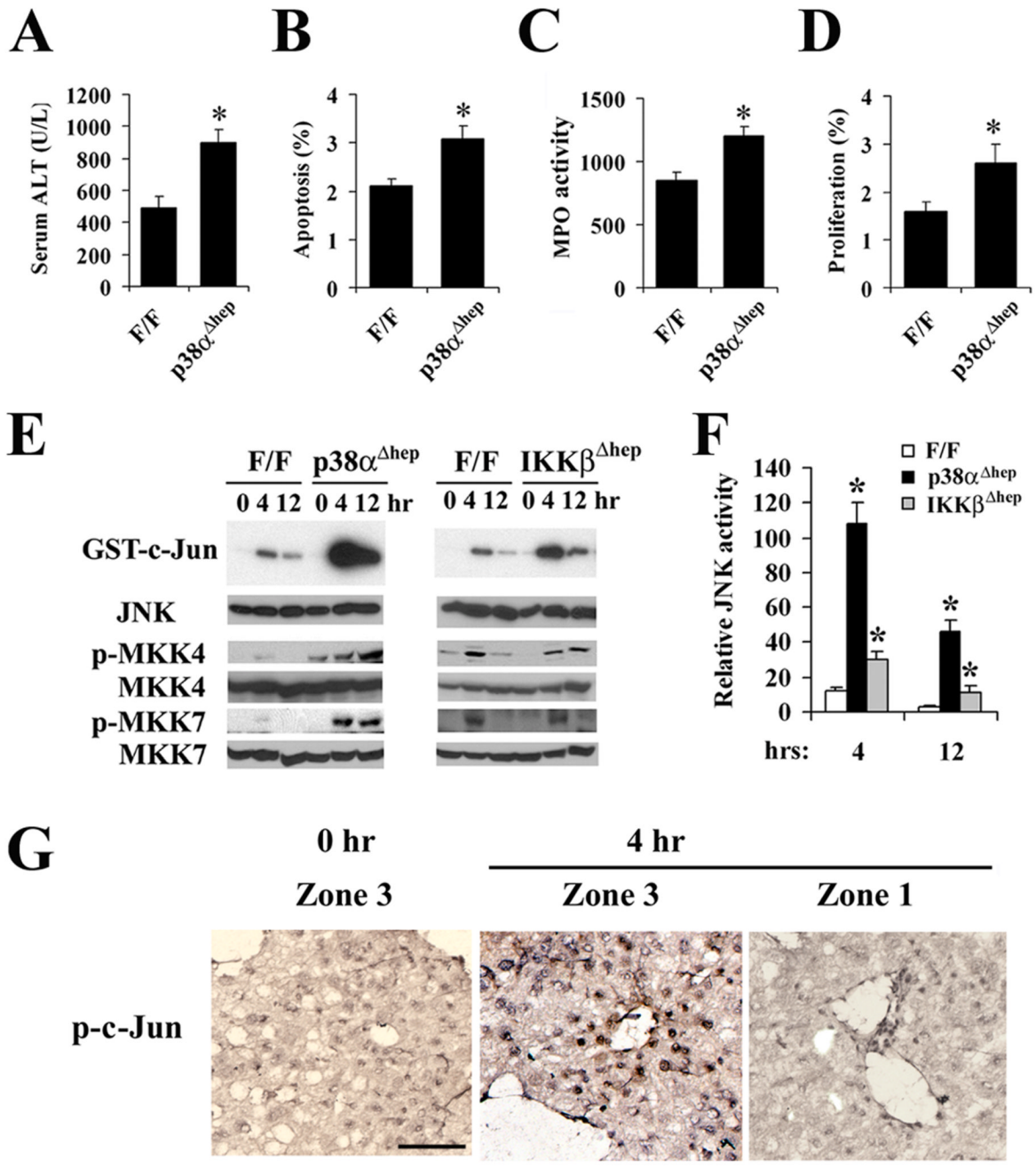

Figure 2. Enhanced DEN-induced cell death, compensatory proliferation and JNK activation in *p38α*$^{\Delta hep}$ mice

(A) ALT levels in serum were determined 48 hrs after DEN injection. Extent of hepatocyte apoptosis (B), neutrophil infiltration (C) and compensatory proliferation (D) were determined by TUNEL staining, MPO assay (results show fluorescent intensity) or BrdU labeling, respectively. Results are means ± SEM (n=4). *, $p<0.05$ vs. control mice (F/F). (E) Mice were given DEN as above and their livers isolated at the indicated times, homogenized and JNK activity was determined by immunecomplex kinase assay. Protein recovery was determined by immunoblotting with JNK1 antibody. MKK4 and MKK7 phosphorylation was analyzed by immunoblotting. (F) Relative JNK activity in *p38α*$^{F/F}$, *p38α*$^{\Delta hep}$ and *Ikk*β$^{\Delta hep}$ mice after DEN

injection. JNK activity in untreated *p38α*$^{F/F}$ mice was given an arbitrary value of 1.0. Results are means ± SEM (n=3). *, p<0.05 vs. control mice (F/F). (G) Expression of phospho-c-Jun in DEN-treated livers. Cryosections of the indicated liver regions before and after DEN injection were immunostained with polyclonal phospho-c-Jun antibody. Scale bar, 50 μm.

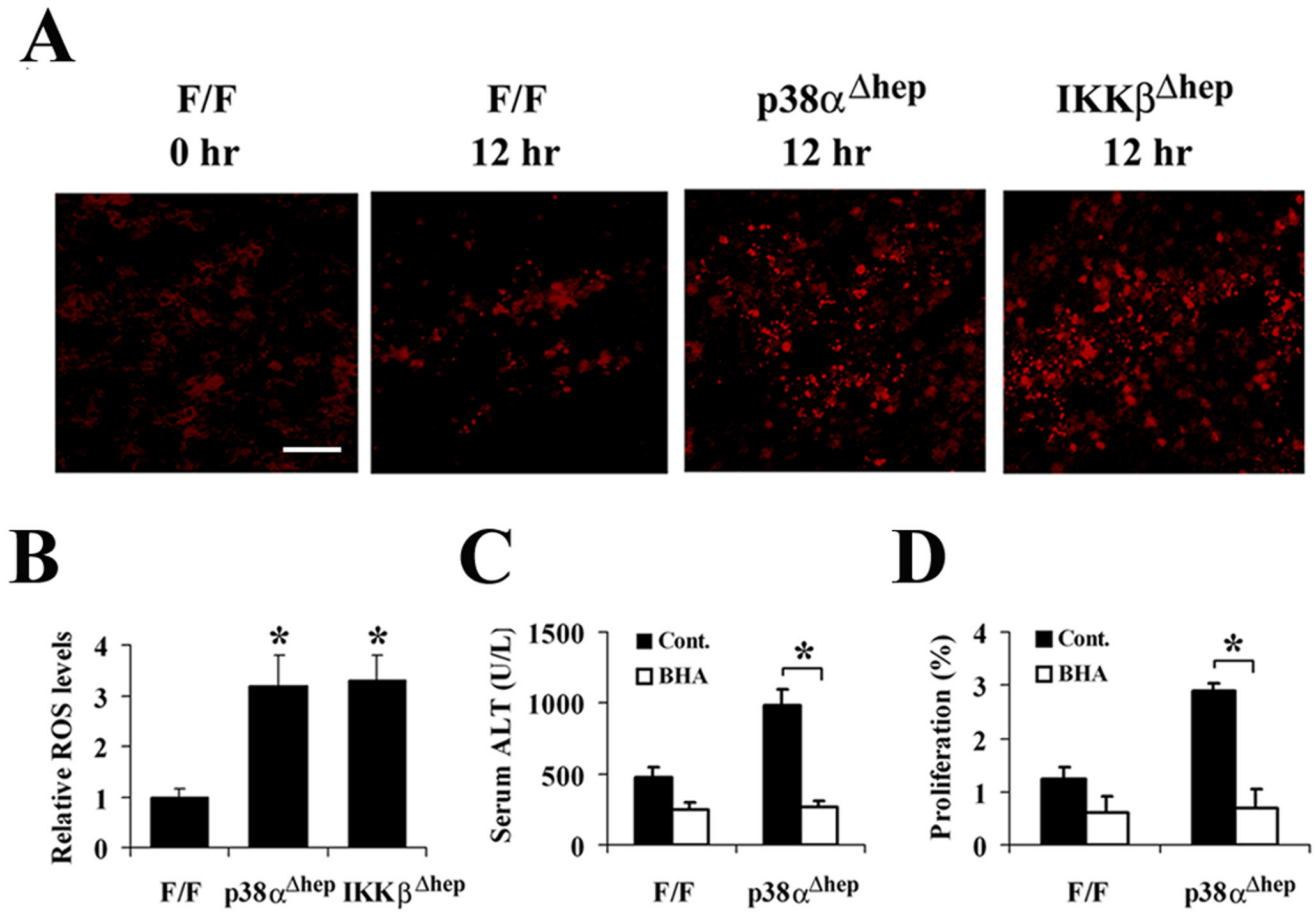

Figure 3. Enhanced ROS accumulation in *p38α*$^{\Delta hep}$ and *Ikk*β$^{\Delta hep}$ mice

(A, B) Liver cryosections prepared 12 hrs after DEN injection were incubated with 2 μM dihydroethidine hydrochloride for 30 min at 37°C. Cells staining positively for the oxidized dye were identified by fluorescent microscopy (A) and quantified (B) by image analysis software. Scale bar, 50 μm. Results are means ± SEM (n=4). *, $p<0.05$ vs. control mice (F/F). (C, D) Mice were fed either BHA-supplemented (0.7%) or regular chow for 2 days prior to DEN injection. After 48 hrs, serum ALT was measured (C) and the mice were pulsed with BrdU and proliferating cells were identified by immunostaining (D). Results are means ± SEM (n=4). *, $p<0.05$.

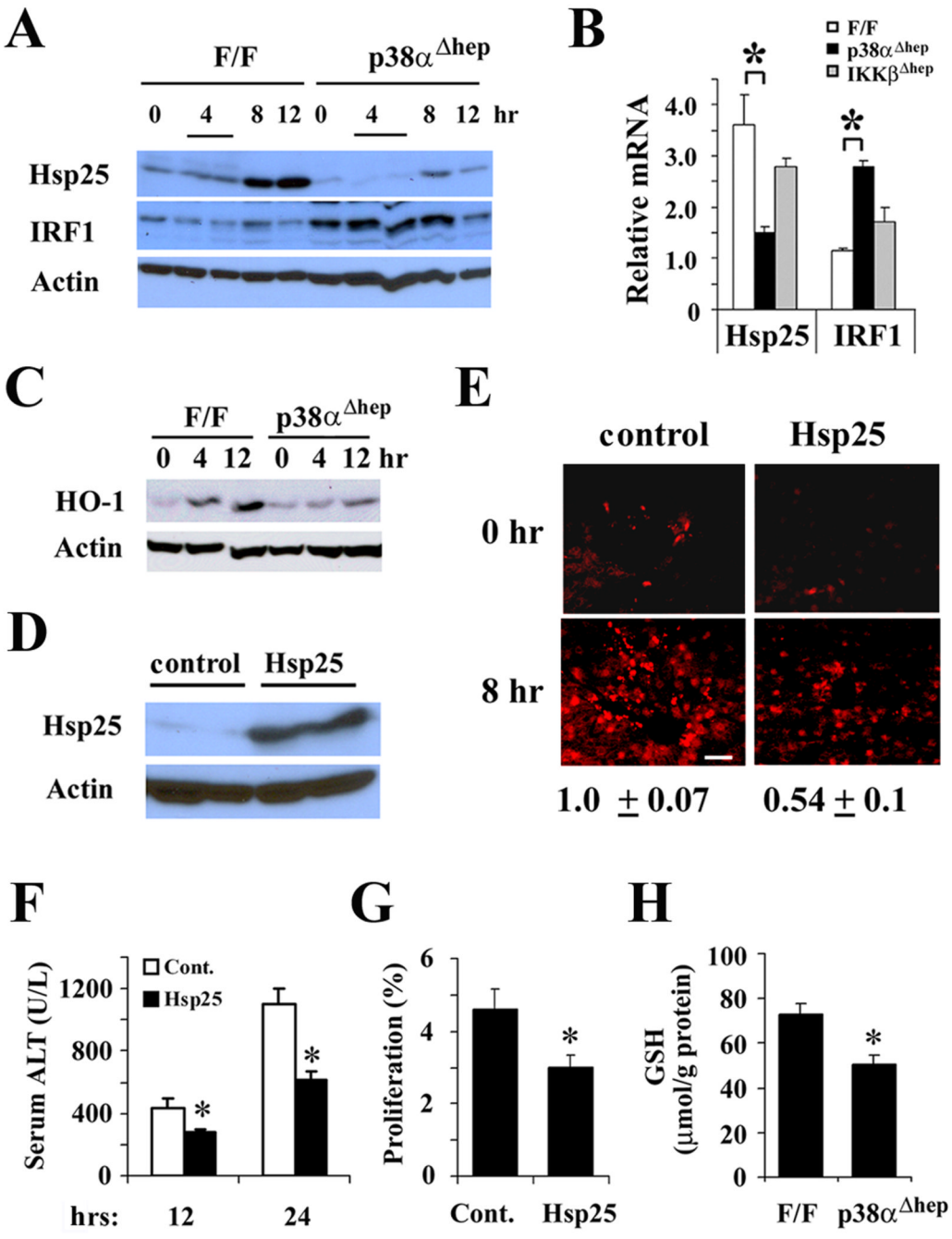

Figure 4. Requirement of p38α for Hsp25 expression

(A) Mice were injected with DEN and their livers isolated at the indicated times and homogenized. Homogenates were gel-separated and immunoblotted with the indicated antibodies. (B) Mice were treated as above and total liver RNA was extracted 4 hrs after DEN injection. Amounts of mRNA relative to those in untreated $p38\alpha^{F/F}$ livers were determined by real time Q-PCR. Results are means ± SEM (n=4). *, $p<0.05$. (C) Mice were injected with DEN and their livers isolated at the indicated times, homogenized and analyzed by immunoblotting as above. (D–G) $p38\alpha^{\Delta hep}$ mice were infected with adenovirus expressing Hsp25 or a control adenovirus 20 hrs before DEN injection. (D) Liver homogenates prepared 8 hrs after DEN injection were analyzed by immunoblotting (results from 2 different mice are

shown). (E) Liver cryosections prepared before and after DEN injection were incubated with 2 mM dihydroethidine hydrochloride for 30 min at 37°C. Cells staining positively for the oxidized dye were identified by fluorescent microscopy. Scale bar, 50 μm. The numbers at the bottom are mean fluorescent intensity ± SEM (n=3). (F) ALT levels in serum were determined at the indicated times after DEN injection. Results are means ± SEM (n=4). (G) Compensatory proliferation was determined by BrdU labeling. Results are means ± SEM (n=4). *, $p<0.05$ vs. control mice (F/F). (H) Liver homogenates prepared at 48 hrs after DEN injection were analyzed for GSH content. Results are means ± SEM (n=4). *, $p<0.05$ vs. control mice (F/F).

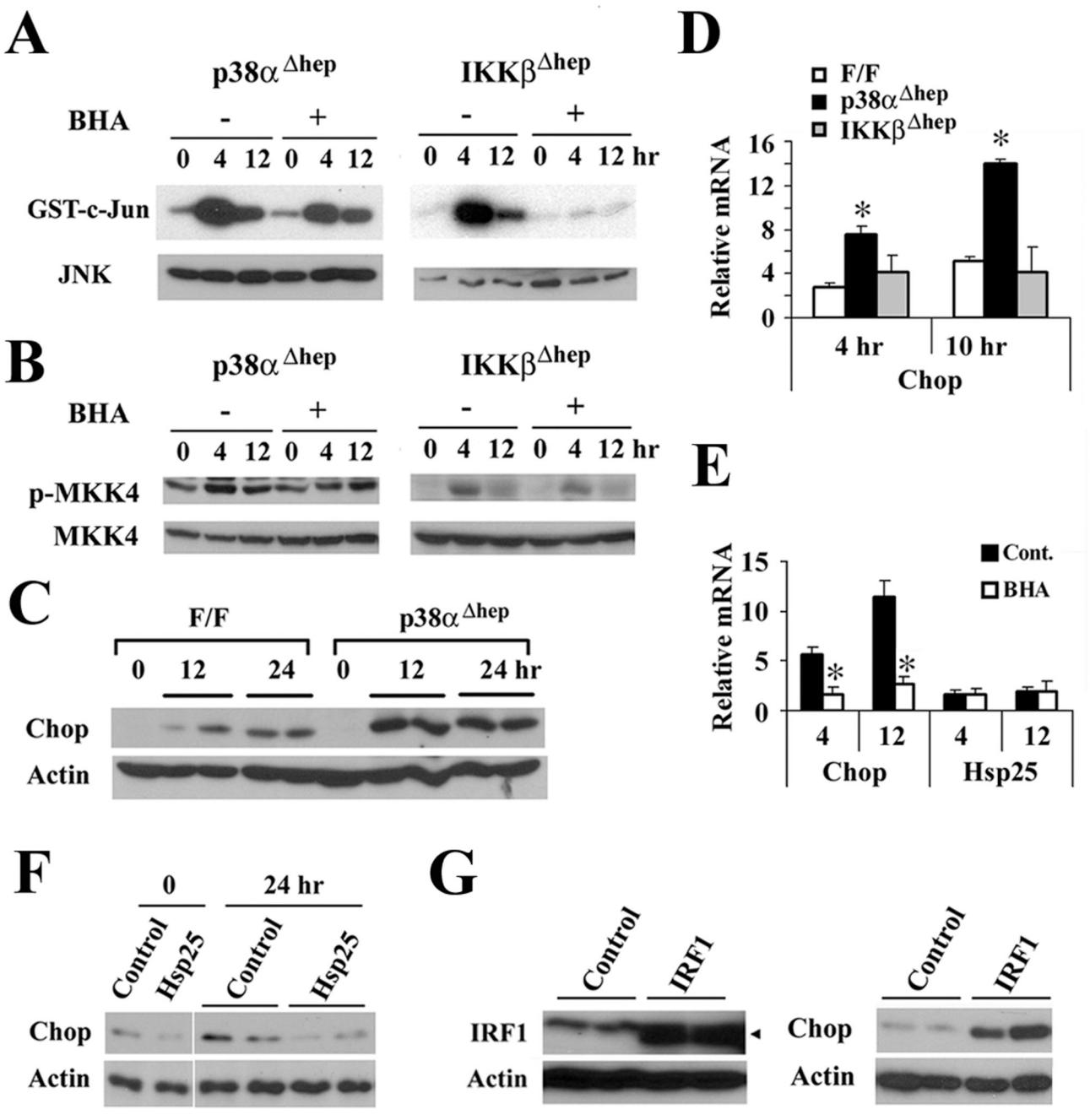

Figure 5. Regulation of JNK and Chop by ROS in *p38α*$^{\Delta hep}$ and *Ikk*β$^{\Delta hep}$ mice

(A, B) Mice of the indicated genotypes were fed either BHA-containing (0.7%) or regular chow for 2 days, and then injected with DEN. JNK activity was determined by immunecomplex kinase assays of liver lysates prepared at the indicated times after DEN injection (A). MKK4 phosphorylation was analyzed by immunoblotting (B). (C) Mice were injected with DEN and their livers isolated at the indicated times, homogenized and Chop expression was examined by immunoblotting. (D) Mice of the indicated genotypes were injected with DEN, total liver RNA was extracted 4 or 10 hrs later, and expression of Chop mRNA was quantified. Results are means ± SEM (n=4). *, $p<0.05$ vs. control mice (F/F). (E) *p38α*$^{\Delta hep}$ mice were fed either BHA-supplemented (0.7%) or regular chow (Cont.) for 2 days, and then injected with DEN.

Total liver RNA was extracted 4 and 12 hrs later and expression of the indicated genes was measured as above. Results are means ± SEM (n=4). *, $p<0.05$. (F) $p38\alpha^{\Delta hep}$ mice were infected with adenovirus expressing Hsp25 or a control adenovirus 20 hrs before DEN injection. Liver lysates prepared at the indicated times after DEN injection, were analyzed for Chop expression. (G) $p38\alpha^{F/F}$ mice were infected with adenovirus expressing IRF1 or a control adenovirus 20 hrs before DEN injection. Liver lysates prepared 8 hrs after DEN injection were analyzed for IRF1 and Chop expression.

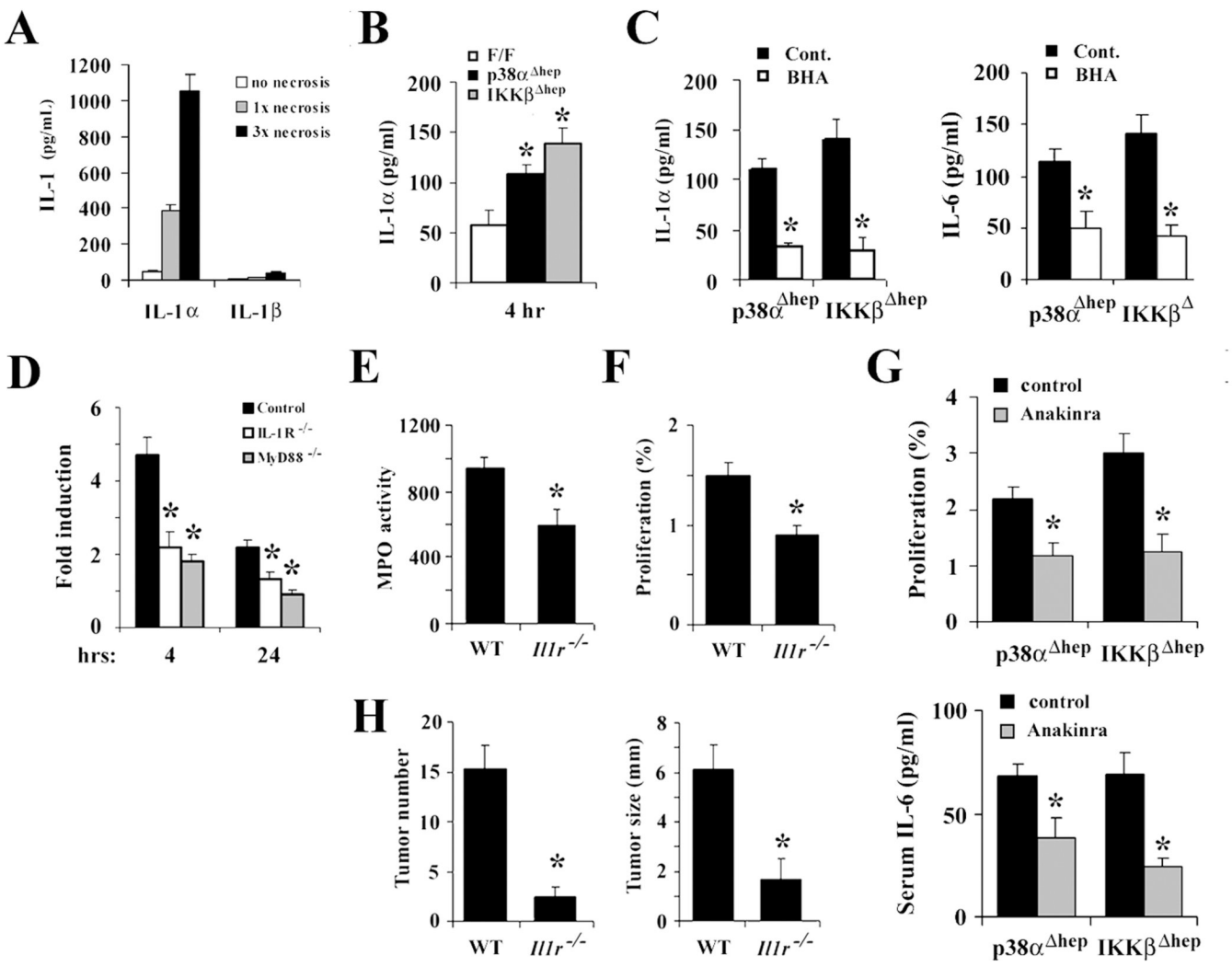

Figure 6. IL-1α release by necrotic hepatocytes and IL-1R signaling promote IL-6 production, compensatory proliferation and hepatocarcinogenesis

(A) Concentrations of IL-1α and IL-1β in supernatants of live hepatocytes (no necrosis), necrotic hepatocytes (1×10^6 cells per ml) lysed by one (1× necrosis) or three (3× necrosis) cycles of freezing and thawing were measured by ELISA. Results are means ± SEM (n=3). (B) IL-1α in venous blood collected 4 hrs after DEN injection was determined by ELISA. Results are means ± SEM (n=4). *, $p<0.05$ vs. control mice (F/F). (C) Mice were fed either BHA-supplemented (0.7%) or regular chow for 2 days prior to DEN injection. IL-1α and IL-6 in venous blood collected 4 hrs after DEN injection were determined by ELISA. Results are means ± SEM (n=3). *, $p<0.05$ vs. control. (D) Roles of IL-1R and MyD88 in IL-6 induction. Mice were injected with DEN and liver RNA was extracted at the indicated times. IL-6 mRNA was quantified by real time Q-PCR. Results are means ± SEM (n=4). *, $p<0.05$ vs. control mice. (E, F) Effects of IL-1R ablation on inflammation and compensatory proliferation. Extent of neutrophil infiltration (E) and compensatory proliferation (F) were determined by MPO assay (results show fluorescent intensity) and BrdU labeling, respectively, 48 hrs after DEN injection. Results are means ± SEM (n=4). *, $p<0.05$ vs. control mice (WT). (G) Anakinra inhibits compensatory proliferation and IL-6 production. PBS or Anakinra (1 g/kg/day) were given for 2 days starting at the time of DEN injection. Hepatocyte proliferation was measured

by BrdU incorporation at 48 hrs while serum IL-6 was measured at 6 hrs after DEN administration. Results are means ± SEM (n=3). *, $p<0.05$ vs. control. (H) Tumor multiplicity (>0.5mm) and maximal tumor sizes (diameters) in livers of male *Il1r*$^{-/-}$ (n=12) and WT (n=14) mice. Results are means ± SEM. *, $p<0.05$ vs. control mice (WT).

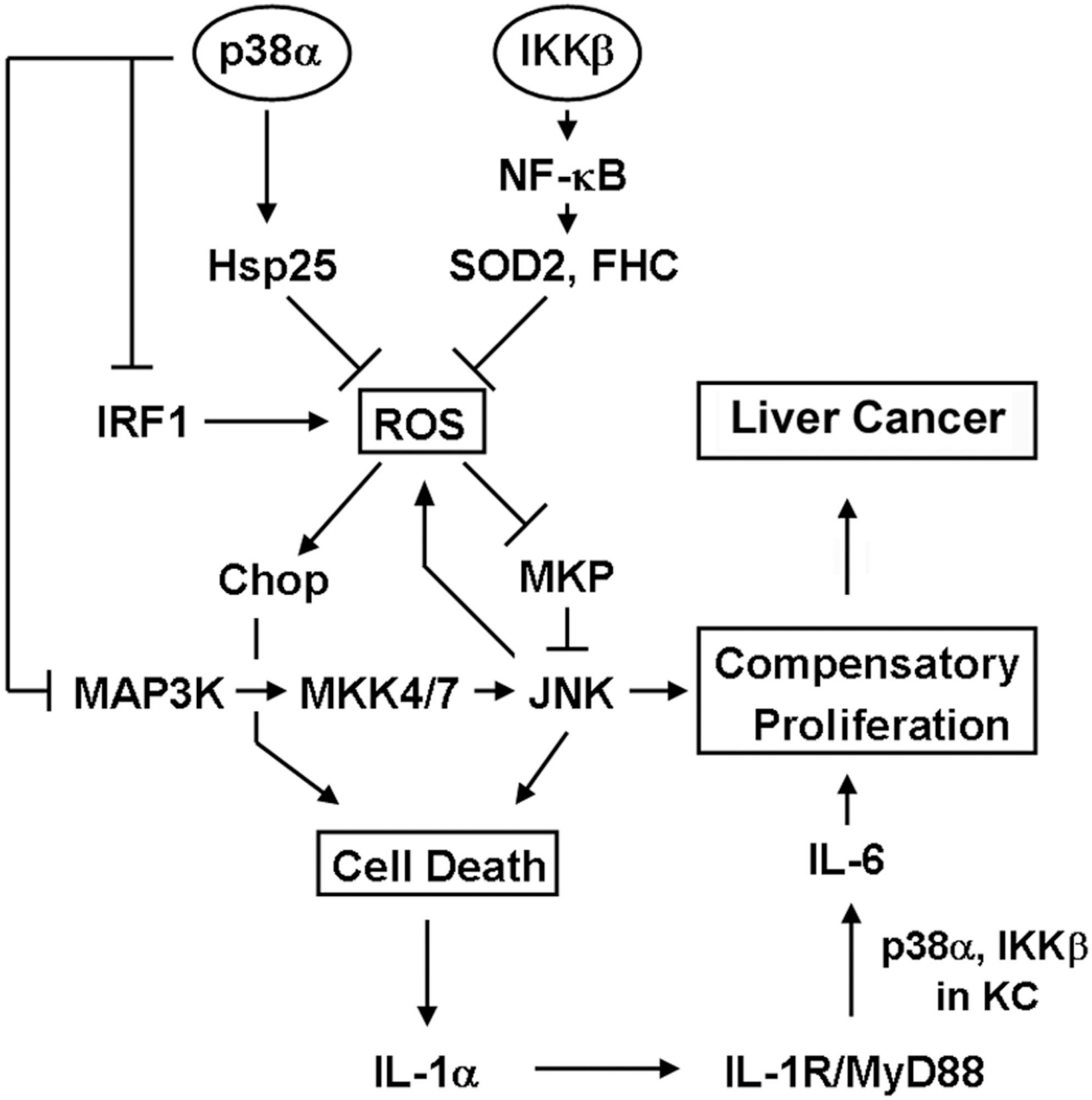

Figure 7. Control of ROS accumulation, IL-1α release, hepatocyte death, compensatory proliferation and their role in DEN-induced hepatocarcinogenesis

IKKβ and p38α use different mechanisms to prevent ROS accumulation and excessive JNK activation and thereby maintain hepatocyte survival and suppress liver injury. Whereas IKKβ acts via NF-κB which induces expression of the antioxidants SOD2 and FHC, p38α upregulates expression of Hsp25, which also prevents ROS accumulation and subsequent Chop induction. Another consequence of ROS accumulation is inhibition of MKPs, resulting in prolonged JNK activation, which contributes to ROS accumulation and hepatocyte death. p38α is also involved in the negative regulation of IRF1 expression and a yet-to-be identified MKK4/7 kinase (MAP3K or MKKK). Increased MKK4/7 activity contributes to elevated JNK

activity which promotes ROS accumulation. All of these pathways control hepatocyte death, which results in the release of IL-1α and activation of IL-1R/MyD88 signaling in Kupffer cells (KC), leading to activation of IKKβ and p38α, induction of IL-6 production, stimulation of compensatory proliferation and hepatocarcinogenesis.