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IKKβ Couples Hepatocyte Death to Cytokine-Driven Compensatory Proliferation that Promotes Chemical Hepatocarcinogenesis

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Summary

IκB kinase β (IKKβ), required for NF-κB activation, links chronic inflammation with carcinogenesis. We investigated whether IKKβ is involved in chemically induced liver cancer, a model not involving overt inflammation. Surprisingly, mice lacking IKKβ only in hepatocytes (*Ikkβ*$^{\Delta hep}$ mice) exhibited a marked increase in hepatocarcinogenesis caused by diethylnitrosamine (DEN). This correlated with enhanced reactive oxygen species (ROS) production, increased JNK activation, and hepatocyte death, giving rise to augmented compensatory proliferation of surviving hepatocytes. Brief oral administration of an antioxidant around the time of DEN exposure blocked prolonged JNK activation and compensatory proliferation and prevented excessive DEN-induced carcinogenesis in *Ikkβ*$^{\Delta hep}$ mice. Decreased hepatocarcinogenesis was also found in mice lacking IKKβ in both hepatocytes and hematopoietic-derived Kupffer cells. These mice exhibited reduced hepatocyte regeneration and diminished induction of hepatomitogens, which were unaltered in *Ikkβ*$^{\Delta hep}$ mice. IKKβ, therefore, orchestrates inflammatory crosstalk between hepatocytes and hematopoietic-derived cells that promotes chemical hepatocarcinogenesis.

Introduction

Carcinogenesis involves alterations such as insensitivity to growth inhibition, evasion of apoptosis, immortalization, angiogenesis, and metastasis (Hanahan and Weinberg, 2000). Some of these alterations are genetic and provided by activation of protooncogenes or inactivation of tumor suppressors. However, other alterations involve epigenetic events and environmental factors extrinsic to the cancer cell. Early on, a major insight to cancer etiology was provided by the realization that carcinogenesis occurs through sequential stages termed initiation, promotion, and progression (Berenblum, 1954). These stages were characterized in model systems as well as human neoplasms (Klein and Klein, 1985). Whereas tumor initiation depends on somatic mutations, the mechanisms underlying tumor promotion are poorly understood and likely to involve epigenetic factors. For instance, in liver cancer, different substances that trigger either cell death, chronic inflammation, or peroxisome proliferation all act as tumor promoters (Sarma et al., 1986). One common tumor-promoting mechanism may involve inflammation (Philip et al., 2004). However, it was estimated that inflammation may play a role in the etiology of only 15% of human cancers, mostly acting as a tumor promoter (Coussens and Werb, 2002).

A major link between inflammation and carcinogenesis may depend on NF-κB transcription factors, critical regulators of innate immune responses and inflammation (Karin et al., 2002). Many proinflammatory stimuli activate NF-κB, mostly through IκB kinase (IKK)-dependent phosphorylation and degradation of inhibitor of κB (IκB) proteins. Once activated, NF-κB dimers stimulate transcription of genes encoding cytokines, growth factors, chemokines, and antiapoptotic factors (Ghosh and Karin, 2002). IKK consists of two catalytic subunits, IKKα and IKKβ, and a regulatory component, IKKγ/NEMO. IKK and NF-κB activation by most stimuli largely depends on IKKβ (Ghosh and Karin, 2002), whose absence increases susceptibility to tumor necrosis factor α (TNFα)-induced apoptosis and causes loss of innate immunity (Li et al., 1999). NF-κB was shown to be instrumental for tumor promotion in a colitis-associated cancer (CAC) model (Greten et al., 2004) and inflammation-associated liver cancer (Pikarsky et al., 2004). Yet, inhibition of NF-κB in keratinocytes promotes squamous cell carcinoma (Dajee et al., 2003). Such results suggest that the impact of NF-κB on cancer development may be cell type or model specific. It is likely that NF-κB activation is most critical in those cancers in which inflammation acts as a tumor promoter, but a more general role for NF-κB and the inflammation driven by it, in cancers that are not associated with chronic inflammation, remains to be established.

Hepatocellular carcinoma (HCC) is the third largest cause of cancer deaths worldwide, particularly in Africa and Asia. Major HCC risk factors include dietary exposure to the fungal contaminant aflatoxin B_1 (AFB_1), infection with hepatitis B (HBV) or C viruses (HCV), and cirrhosis associated with chronic inflammation. Additional risk factors are environmental pollutants, such as aromatic amines, vinyl chloride, polycyclic aromatic hydrocarbons, and nitrosamines (Bosch et al., 2004). However, the causal and mechanistic relationships between HCC risk factors and carcinogenic mechanisms have been difficult to elucidate in either humans (Thorgeirsson and Grisham, 2002) or in animal models (Sell and Leffert, 1982).

Studies with genetically altered mice have revealed many genes associated with HCC. Such genes fall into two categories: those that augment spontaneous HCC and those that increase susceptibility to chemically induced HCC. HCC develops spontaneously in transgenic mice overexpressing HBV surface antigen (HBsAg), the hepatomitogen transforming growth factor α (TGFα), the c-*Myc* protooncogene, or *SV40* T antigen (Fausto, 1999). HCC development is also augmented in mice deficient in type II TGFβ receptor (Im et al., 2001) or the

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Mdr2 P-glycoprotein transporter (Mauad et al., 1994). Most of these mouse strains display enhanced susceptibility to chemical carcinogens, yet the underlying molecular and cellular mechanisms are not well understood.

Certain chemical carcinogens are efficient inducers of HCC. A single postnatal injection of the tumor initiator diethylnitrosamine (DEN), which induces hepatocyte DNA damage, can result in HCC, whose course of development is similar to human HCC (Verna et al., 1996). In very young mice, DEN is a complete carcinogen, but when given to older mice it requires assistance from tumor promoters, acting through diverse mechanisms (Sarma et al., 1986). As the ability of DEN to form DNA adducts should not be age dependent (Dyroff et al., 1986), it is not clear why it is so effective as a single agent in neonates but not in adults. Recently, it was shown that a hepatocyte-specific deletion of either the *c-Jun* or the *Foxm1b* genes, which encode transcription factors needed for hepatocyte proliferation, inhibit DEN-induced hepatocarcinogenesis (Eferl et al., 2003; Kalinichenko et al., 2004). Thus, hepatocyte proliferation is required for DEN-induced carcinogenesis.

To determine whether inflammation and NF-κB activation in hepatocytes are involved in chemical hepatocarcinogenesis, we used the DEN-induced HCC model. Surprisingly, hepatocyte-specific deletion of IKKβ, which prevents NF-κB activation (Maeda et al., 2003), markedly increased DEN-induced hepatocarcinogenesis. IKKβ deletion also augmented hepatocyte death mediated by enhanced ROS production and prolonged JNK activation (Kamata et al., 2005). This death response was accompanied by augmented compensatory proliferation. A deletion of IKKβ in both hepatocytes and hematopoietic-derived cells, however, had the opposite effect, decreasing compensatory proliferation and carcinogenesis. The death of DEN-exposed hepatocytes was found to activate adjacent myeloid cells (Kupffer cells) to produce hepatomitogens that promote compensatory proliferation of surviving hepatocytes. Production of these mitogens depends on NF-κB in hematopoietic-derived Kupffer cells. These results, which differ from a previous suggestion that the tumor-promoting function of NF-κB is excreted in hepatocytes (Pikarsky et al., 2004), indicate that even chemical carcinogenesis depends on inflammation and raise the prospect of anti-inflammatory intervention targeting Kupffer cells in chemoprevention of HCC. Our results also suggest that chemicals or viruses that interfere with NF-κB activation in hepatocytes may promote HCC development.

Results

Ikkβ$^{\Delta hep}$ Mice Exhibit Increased Susceptibility to Chemical Hepatocarcinogenesis

A single DEN injection to 15-day-old male mice results in efficient HCC induction (Sarma et al., 1986). To determine the role of IKKβ in this process, male mice homozygous for either a floxed *Ikkβ* allele (*Ikkβ*$^{F/F}$) or an hepatocyte-specific deletion thereof (*Ikkβ*$^{\Delta hep}$) (Maeda et al., 2003) were injected with DEN (5 mg/kg) on day 15 postnatally when IKKβ was absent from hepatocytes but present in nonparenchymal cells (Figure 1A). Previous studies revealed no spontaneous liver dysfunction or HCC in *Ikkβ*$^{\Delta hep}$ mice for up to 2 years (Maeda et al., 2003). All males given DEN developed typical HCCs within 8 months (Figures 1B–1E). Strikingly, the number of detectable HCCs was 3-fold higher in *Ikkβ*$^{\Delta hep}$ mice than in *Ikkβ*$^{F/F}$ controls (Figure 1F). The tumor-occupied area was also 3-fold larger in *Ikkβ*$^{\Delta hep}$ mice (Figure 1G), and so were maximal tumor diameters (Figure 1H). Notably, many HCCs in *Ikkβ*$^{\Delta hep}$ mice, but none in the controls, exhibited signs of neovascularization (Figures 1C and 1E, see Figure S1 in the Supplemental Data available with this article online). Congruently, some *Ikkβ*$^{\Delta hep}$ mice but none of the *Ikkβ*$^{F/F}$ controls had human-like HCC metastases (random cord structure, liver plates two to four cells thick) in their lungs (Figures 1I and 1J) or peritoneal cavity (data not shown) at 8 months. DEN-injected *Ikkβ*$^{\Delta hep}$ mice also exhibited significantly higher and accelerated mortality than similarly treated *Ikkβ*$^{F/F}$ mice (Figure 1K). A high dose (25 mg/kg) of DEN also induced higher HCC load in *Ikkβ*$^{\Delta hep}$ mice than *Ikkβ*$^{F/F}$ controls (Figure S2A).

Female mice are less sensitive than males to DEN-induced carcinogenesis due to hormonal factors (Sarma et al., 1986). As expected, HCCs were observed only in 10% of DEN-treated *Ikkβ*$^{F/F}$ females, but tumor incidence was 4-fold higher in *Ikkβ*$^{\Delta hep}$ females (Figure 1L).

In older mice, DEN is a less potent carcinogen, and it requires assistance from tumor promoters such as phenobarbital (Sarma et al., 1986). To examine the effect of IKKβ on this initiation-promotion model, we injected 4-week-old mice with 100 mg/kg DEN and administered 0.07% phenobarbital in the drinking water for 6 months starting 1 month after DEN injection. This led to HCCs in 55% of *Ikkβ*$^{F/F}$ mice and 100% of *Ikkβ*$^{\Delta hep}$ mice. Tumor number was also higher in *Ikkβ*$^{\Delta hep}$ mice (Figure S2B).

Hence, loss of IKKβ in hepatocytes increases susceptibility to DEN-induced carcinogenesis. This stands in marked contrast to IKKβ ablation in intestinal epithelial cells, which prevents chemically induced CAC (Greten et al., 2004).

Ikkβ$^{\Delta hep}$ Tumors Retain IKKβ Deficiency

Given these surprising findings, we wished to confirm that HCCs in *Ikkβ*$^{\Delta hep}$ mice are IKKβ deficient. Fine-needle biopsies of control nontreated liver, DEN-exposed normal liver, and tumor nodules were analyzed by immunoblotting. IKKβ was present in *Ikkβ*$^{F/F}$ nontumor and tumor samples but barely detectable in *Ikkβ*$^{\Delta hep}$ samples (Figure 2A). Since isolated *Ikkβ*$^{\Delta hep}$ hepatocytes do not express IKKβ (Maeda et al., 2003), the trace amounts of IKKβ in *Ikkβ*$^{\Delta hep}$ samples probably originated from nonparenchymal cell types, all of which express IKKβ. Consistently, IKKβ was absent in isolated cancer cells (Figure 2B). Presence of two *Ikkβ*$^{\Delta}$ alleles in *Ikkβ*$^{\Delta hep}$ tumors was confirmed by PCR (Figure 2C). No significant changes in expression of IKKα, RelA (p65), cRel, RelB, or IκBα were found (Figure 2A). Levels of NF-κB binding activity in all samples were very low and required long exposure times for detection, but LPS injection into tumor-bearing mice increased NF-κB

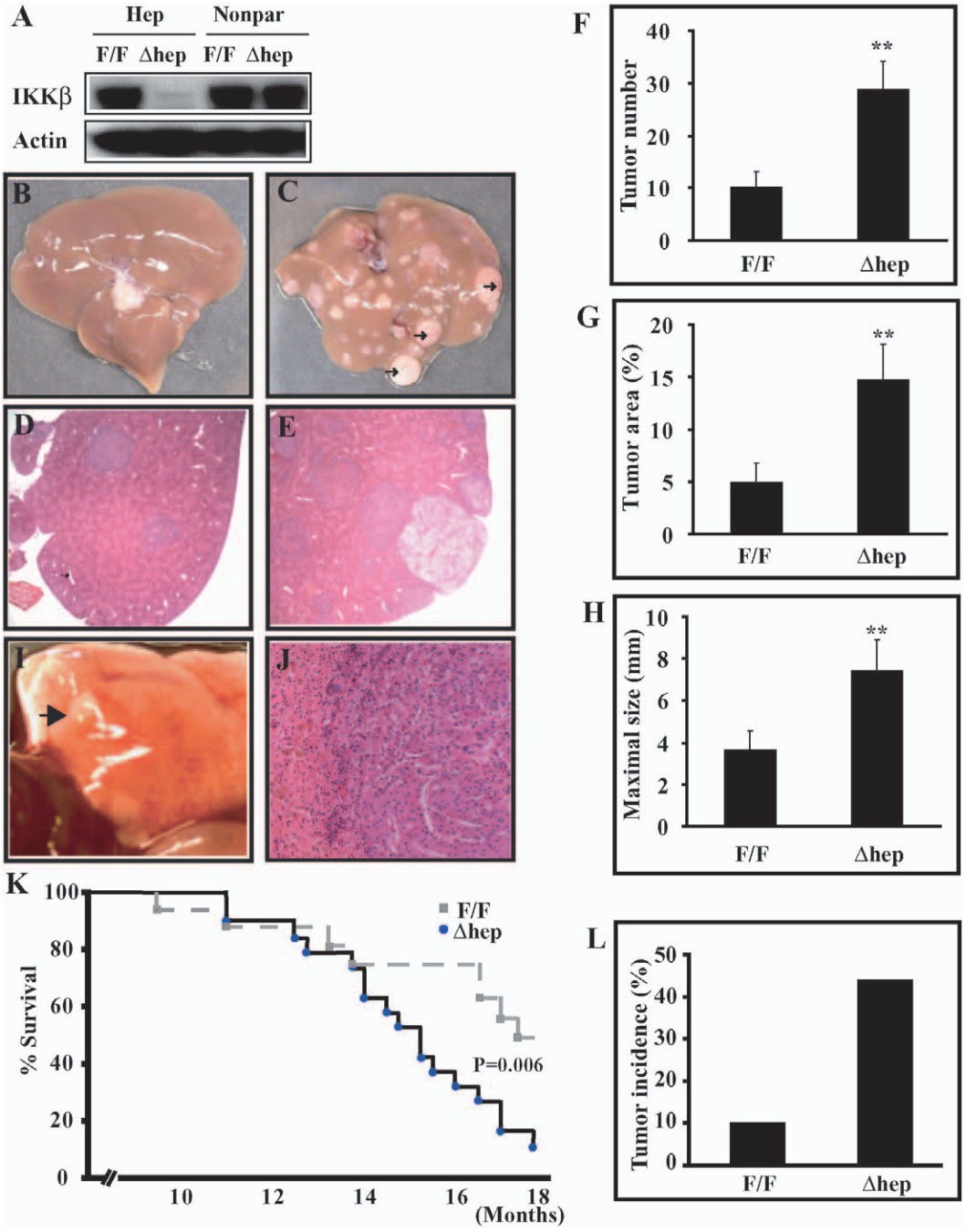


Figure 1. Increased Tumor Development in *Ikkβ*$^{\Delta hep}$ Mice

(A) Expression of IKKβ in hepatocytes (Hep) and nonparenchymal cells (nonpar) of 15-day-old *Ikkβ*$^{\Delta hep}$ mice.
(B and C) Livers of 8-month-old DEN-treated male *Ikkβ*$^{F/F}$ (B) and *Ikkβ*$^{\Delta hep}$ (C) mice. Arrows, neovascularization.
(D and E) Typical liver histology (2.5× magnification; H&E stain) in 8-month-old DEN-treated male *Ikkβ*$^{F/F}$ (D) and *Ikkβ*$^{\Delta hep}$ (E) mice.
(F) Numbers of tumors (≥0.5 mm) in livers of male *Ikkβ*$^{F/F}$ (n = 13) and *Ikkβ*$^{\Delta hep}$ (n = 14) mice 8 months after DEN (5 mg/kg) injection.
(G) Fractions of tumor occupied area in the large lobe of the liver (mouse number and treatment as above).
(H) Maximum tumor sizes (diameters).
(I and J) Gross ([I], arrowhead) and histological ([J], 200× magnification) appearance of lung metastases in *Ikkβ*$^{\Delta hep}$ mice.
(K) Survival curves of *Ikkβ*$^{F/F}$ (n = 16) and *Ikkβ*$^{\Delta hep}$ (n = 19) mice injected with DEN (5 mg/kg) at 15 days of age (p = 0.006).
(L) Incidence of HCCs (≥0.5 mm) in female *Ikkβ*$^{F/F}$ (n = 10) and *Ikkβ*$^{\Delta hep}$ (n = 11) mice 8 months after DEN (5 mg/kg) injection. Results in (F) and (H) are averages ±S.E. Asterisks, $p < 0.05$ by Student's t test.

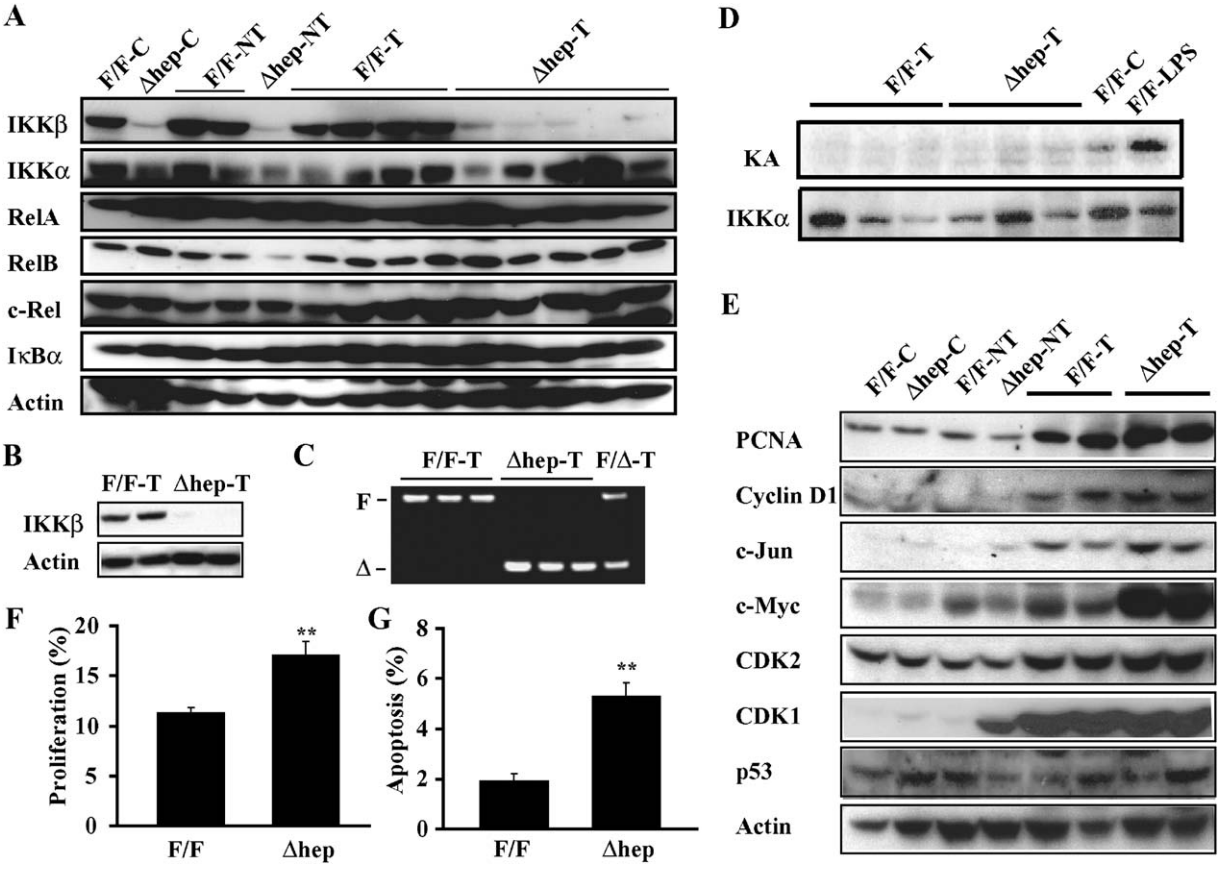


Figure 2. Biochemical and Cellular Characteristics of HCCs

(A) Expression of IKK and NF-κB proteins in tumor (T) and nontumor (NT) tissues 8 months after DEN treatment. Lysates of microdissected HCCs or nontumor liver tissue from untreated (C) or DEN-treated mice were gel separated and analyzed by immunoblotting with antibodies to the indicated proteins.

(B) Expression of IKKβ in isolated HCC cells.

(C) HCCs were genotyped by PCR. F, floxed allele, Δ, deleted allele.

(D) IKK activities (KA) were determined by immunecomplex (anti-IKKα) kinase assays of tumor lysates. F/F-C and F/F-LPS are lysates of untreated and LPS-treated mouse liver, respectively, used as controls. Protein recovery was determined by immunoblotting with anti-IKKα antibody.

(E) Expression of cell-cycle-associated proteins. The lysates described in (A) were gel separated and immunoblotted with antibodies to the indicated proteins.

(F and G) Frequencies of proliferating (F) and apoptotic (G) cells in $Ikk\beta^{F/F}$ (n = 10) and $Ikk\beta^{\Delta hep}$ (n = 10) HCCs determined by PCNA nuclear staining or a TUNEL assay, respectively. Results are averages ±S.E. Asterisks, $p < 0.05$.

activity in $Ikk\beta^{F/F}$ tumors but not $Ikk\beta^{\Delta hep}$ tumors (Figure S3). Consistent with these results, neither $Ikk\beta^{F/F}$ nor $Ikk\beta^{\Delta hep}$ tumors from untreated mice exhibited detectable IKK activity (Figure 2D).

$Ikk\beta^{\Delta hep}$ Tumors Exhibit Increased Cellular Turnover

Cell-cycle regulators and tumor suppressors in tumor and nontumor samples were also analyzed. PCNA, cyclin D1, c-Jun, c-Myc, CDK2, and CDK1 were elevated in HCCs compared to nontumor tissues (Figure 2E). PCNA and c-Jun were slightly higher and c-Myc was vastly higher in $Ikk\beta^{\Delta hep}$ than $Ikk\beta^{F/F}$ tumors (Figure 2E). No differences in p53 were detected between genotypes or tumors versus normal tissue (Figure 2E). IKKβ-deficient HCCs contained more proliferating cells than $Ikk\beta^{F/F}$ HCCs (Figure 2F) but also contained more apoptotic cells (Figure 2G), indicating enhanced turnover.

Loss of IKKβ Augments Hepatocellular Death and Compensatory Proliferation

To reveal the basis for the increased susceptibility of $Ikk\beta^{\Delta hep}$ mice to chemical carcinogenesis, we examined early effects of DEN on signal transduction and cell behavior. DEN administration activated IKK and JNK in livers of $Ikk\beta^{F/F}$ mice (Figure 3A). Loss of IKKβ diminished IKK activation but enhanced the magnitude and duration of JNK activation (Figure 3A and data not shown). Immunohistochemistry revealed nuclear RelA only in nonparenchymal cells in DEN-treated $Ikk\beta^{\Delta hep}$ liver, whereas both hepatocytes and nonhepatocytes showed nuclear RelA in $Ikk\beta^{F/F}$ liver (Figure S4A). The increase in JNK activity suggested that deletion of IKKβ may result in more DEN-induced cell death (Kamata et al., 2005; Maeda et al., 2003). Indeed, appearance of circulating liver enzymes (indicative of necrotic liver in-

jury) and TUNEL assays revealed more hepatocyte death in centrilobular lesions in DEN-treated *Ikkβ^{Δhep}* mice relative to similarly-treated *Ikkβ$^{F/F}$* mice (Figures 3B, 3C, and 3E). Electron microscopy and histological analysis revealed more apoptotic and necrotic cells after DEN administration in *Ikkβ^{Δhep}* than *Ikkβ$^{F/F}$* mice (Figure 3C and Figures S4B and S4C). It should be noted, however, that only a fraction of the total hepatocytes undergoes cell death in response to a carcinogenic dose of DEN, even in *Ikkβ^{Δhep}* mice.

Due to their high regenerative capacity, surviving hepatocytes should undergo a compensatory proliferative response. Bromodeoxyuridine (BrdU) labeling revealed higher numbers of proliferating hepatocytes in *Ikkβ^{Δhep}* than *Ikkβ$^{F/F}$* mice after DEN exposure (Figures 3D and 3E). The proliferating cells were most common around clusters of apoptotic cells in centrilobular lesions, confirming that DEN-induced cell loss triggers compensatory hepatocyte proliferation. This response is augmented in *Ikkβ^{Δhep}* mice.

TNFα is a potent inducer of liver cell death but is also involved in liver regeneration (Fausto, 2000). As shown below, TNFα mRNA was induced after DEN injection, but no differences were observed between *Ikkβ^{Δhep}* and *Ikkβ$^{F/F}$* mice. To investigate the involvement of TNFα in either DEN-induced cell death and/or compensatory regeneration in IKKβ-deficient mice, *Ikkβ^{Δhep}* and *Tnfr1$^{-/-}$* mice were crossed, their progeny were given DEN, and circulating liver enzymes were measured. TNFR1 ablation reduced the amount of circulating liver enzymes at 48 hr but not at 24 hr after DEN exposure by approximately 50% (Figure 3F). *Ikkβ^{Δhep}/Tnfr1$^{-/-}$* mice exhibited an even greater reduction in the number of proliferating hepatocytes at 48 hr after DEN exposure relative to *Ikkβ^{Δhep}* mice (Figure 3G).

Prolonged JNK activation in NF-κB-deficient cells contributes to TNFα-induced cell death (Kamata et al., 2005; Maeda et al., 2003; Tang et al., 2001). To investigate the role of JNK in the response to DEN, *Ikkβ^{Δhep}* and *Jnk1$^{-/-}$* mice were crossed, their progeny were injected with DEN, and liver damage and compensatory proliferation were assessed. JNK1 deficiency reduced the amount of circulating liver enzymes at 48 hr after DEN administration (Figure 3F), the extent of cell death (data not shown), and the number of proliferating cells (Figure 3G).

Ikkβ^{Δhep} Mice Exhibit Increased ROS Production in Response to DEN

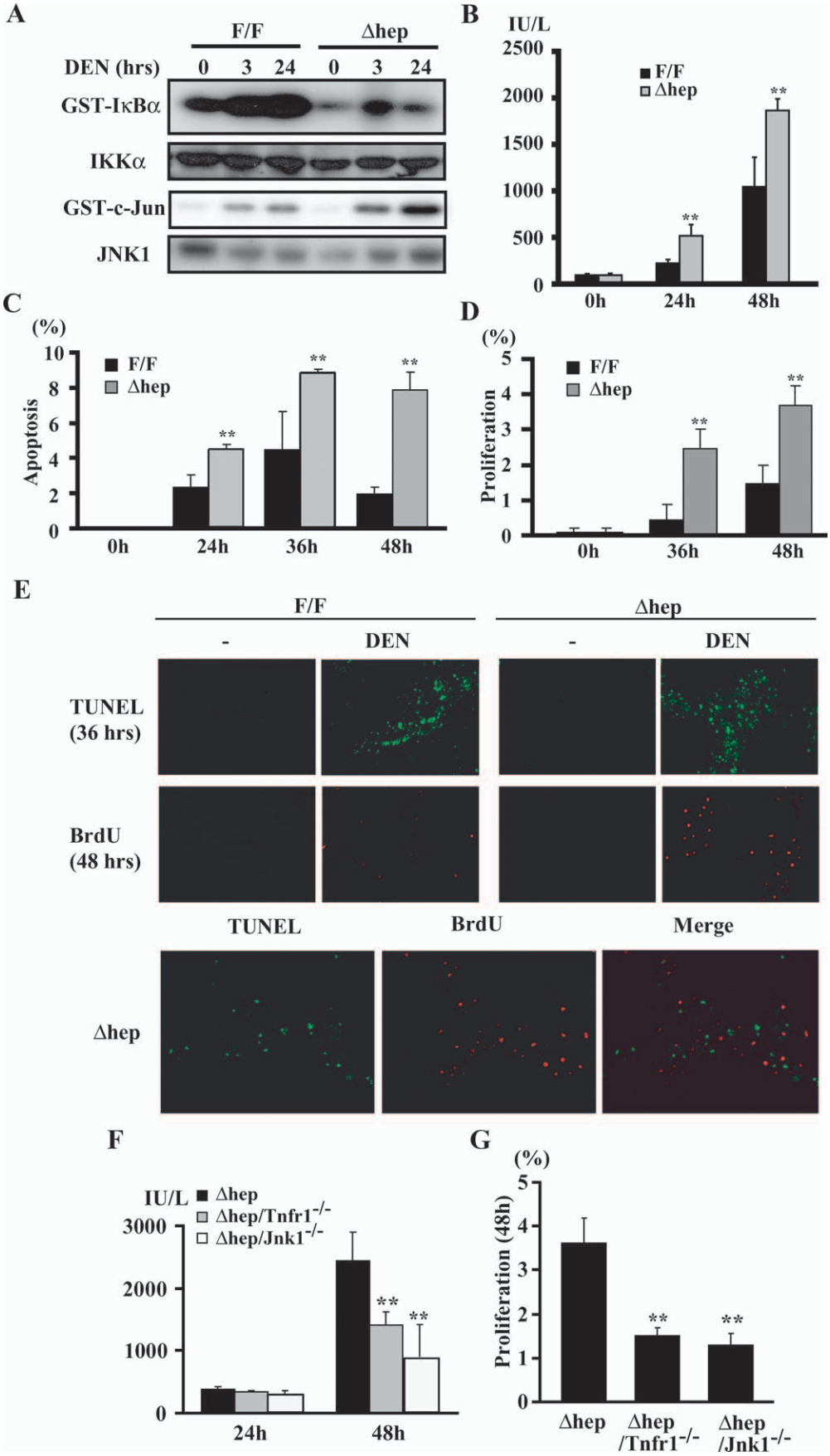
NF-κB, by controlling expression of Mn-superoxide dismutase (MnSOD), a mitochondrial enzyme that detoxifies superoxide anions, inhibits oxidative stress (Wong and Goeddel, 1988). As expected, MnSOD but not CuZnSOD (the cytoplasmic SOD) mRNA was decreased in hepatocytes from untreated or DEN-treated *Ikkβ^{Δhep}* mice (Figure 4A). Increased ROS accumulation following TNFα stimulation causes prolonged JNK activation in NF-κB- or IKKβ-deficient cells (Kamata et al., 2005; Sakon et al., 2003). Accordingly, *Ikkβ^{Δhep}* mice display increased oxidative stress after DEN administration. The levels of reduced glutathione (GSH), a major cellular antioxidant, were much lower in the livers of DEN-treated *Ikkβ^{Δhep}* mice than in similarly treated *Ikkβ$^{F/F}$* mice (Figure 4B). Conversely, DEN-induced lipid peroxidation, assessed by malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) content (Esterbauer and Cheeseman, 1990), was substantially higher in *Ikkβ^{Δhep}* than *Ikkβ$^{F/F}$* mice (Figure 4C). To assess accumulation of superoxide anions, we stained freshly frozen tissue sections with dihydroethidine (Zhou et al., 2004). More extensive staining in centrilobular lesions was seen after DEN administration in *Ikkβ^{Δhep}* than *Ikkβ$^{F/F}$* mice (Figure 4D). ROS production may cause oxidative DNA damage, which can be detected with antibodies specific to 8-hydroxydeoxyguanosine (8-OHdG). One day after DEN administration, livers of *Ikkβ^{Δhep}* mice exhibited a much higher level of anti-8-OHdG reactivity than *Ikkβ$^{F/F}$* livers (Figure 4E).

Increased ROS production is a major contributor to JNK activation and acute liver failure, both of which can be prevented by feeding mice with the antioxidant butylated hydroxyanisole (BHA) (Kamata et al., 2005). To investigate the role of ROS in the response to DEN, mice were given BHA-containing (0.7%) or control chow for 2 days and then challenged with DEN. As found previously, consumption of BHA-containing diet prevented the sustained phase of JNK activation in *Ikkβ^{Δhep}* mice but had no effect on the initial response (Figure 4F). Furthermore, BHA administration reduced DEN-induced liver injury in *Ikkβ^{Δhep}* mice to a level similar to that in *Ikkβ$^{F/F}$* mice (Figure 4G). Histological analysis revealed that BHA reduced both apoptosis and necrosis (data not shown). Strikingly, BHA consumption almost completely blocked compensatory proliferation (Figure 4H) and appearance of 8-OHdG positive hepatocytes (data not shown). Thus, loss of hepatocyte IKKβ enhances DEN-induced cell death and compensatory proliferation through a mechanism likely to depend on ROS accumulation.

BHA Consumption Decreases Hepatocarcinogenesis in *Ikkβ^{Δhep}* Mice

If augmented ROS accumulation, the ensuing increases in JNK activation, and compensatory hepatocyte proliferation account for the elevated susceptibility of *Ikkβ^{Δhep}* mice to DEN-induced hepatocarcinogenesis, consumption of BHA around the time of DEN exposure should reduce HCC development in these mice to the level seen in *Ikkβ$^{F/F}$* mice. To test this prediction, we kept a cohort of *Ikkβ^{Δhep}* mice on normal diet, whereas another cohort was fed a BHA-containing (0.7%) diet for 4 days starting 2 days before DEN administration at 18 days of age (Figure 5A). Mice in both groups were kept for 8 additional months on normal diet, after which they were sacrificed and analyzed to determine tumor load. Strikingly, the numbers of HCCs in BHA-fed *Ikkβ^{Δhep}* mice were reduced 2.5-fold (Figure 5B). Tumor-occupied areas and maximal tumor sizes were also reduced to levels similar to those in *Ikkβ$^{F/F}$* mice (Figure 5C and data not shown).

To exclude the possibility that BHA prevents metabolic activation of DEN or accelerates its detoxification, we examined the expression of p53-regulated genes, which provide an indirect measure of DEN-induced DNA damage. We also examined expression of enzymes involved in DEN metabolism. No significant ef-


A
F/F
Δhep
DEN (hrs)
0 3 24 0 3 24
GST-IκBα
IKKα
GST-c-Jun
JNK1
B
IU/L
2500
2000
1500
1000
500
0
F/F
Δhep
**
0h
24h
48h
C
(%)
Apoptosis
10
8
6
4
2
0
F/F
Δhep
**
0h
24h
36h
48h
D
(%)
Proliferation
5
4
3
2
1
0
F/F
Δhep
**
0h
36h
48h
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F/F
Δhep
-
DEN
TUNEL
(36 hrs)
BrdU
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TUNEL
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F
IU/L
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Δhep/Tnfr1-/-
Δhep/Jnk1-/-
3000
2000
1000
0
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24h
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(%)
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5
4
3
2
1
0
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Δhep
Δhep /Tnfr1-/-
Δhep /Jnk1-/-

fects of BHA on expression and induction of any of these genes were observed (Figure 5D). BHA also did not affect DEN-induced IKK activity (Figure S5). Thus, a brief treatment with an antioxidant at the time of DEN exposure prevented most of the elevated susceptibility to DEN-induced liver carcinogenesis in *Ikkβ$^{\Delta hep}$* mice, most likely via its ability to inhibit ROS accumulation.

Deletion of IKKβ in Hematopoietic-Derived Cells Reduces Susceptibility to DEN-Induced Carcinogenesis

The major cell type responsible for production of mitogens that stimulate compensatory hepatocyte proliferation during liver injury is the Kupffer cell, a type of macrophage endogenous to the liver (Fausto, 2000). One possible explanation for the difference between the results presented above and those reported by Pikarsky et al. (2004), who found that inhibition of NF-κB decreases hepatocarcinogenesis, is that the IκB superrepressor used by these investigators to inhibit NF-κB activation was also expressed in Kupffer cells. To test this possibility, we wished to delete IKKβ in these cells. Unfortunately, however, the *LysM-Cre* transgene, which efficiently deletes the *Ikkβ^{F}* allele in circulating and bone-marrow-derived macrophages and neutrophils (Greten et al., 2004), is not useful in Kupffer cells (Figure S6). As an alternative approach, we crossed *Ikkβ$^{F/F}$* mice with *Mx1-Cre* transgenic mice, which express CRE recombinase from the interferon-inducible *Mx1* promoter. Injection of *Ikkβ$^{F/F}$:Mx1-Cre* mice with poly(IC), which induces interferon production, efficiently deletes IKKβ in liver and spleen but not in most other tissues (Figure 6A). In addition to macrophages (Hsu et al., 2004), the *Mx1-Cre* mediated deletion is very effective in B cells, Kupffer cells (Figure S6A), and hepatocytes (hence the nearly complete absence of IKKβ in intact liver). We therefore refer to these mice as *Ikkβ$^{\Delta L+H}$*, for deletion in liver + hematopoietic cells. *Ikkβ$^{F/F}$:Mx1-Cre* and *Ikkβ$^{F/F}$* mice were injected with poly(IC) on days 9, 11, and 13, followed by DEN injection at day 15. After 8 months, HCC development was analyzed. HCC load was 4-fold lower in *Ikkβ$^{\Delta L+H}$* than in *Ikkβ$^{F/F}$* mice (Figure 6B). Furthermore, the tumor-occupied area (data not shown) and maximal tumor size (Figure 6C) were also reduced in *Ikkβ$^{\Delta L+H}$* mice.

These results were completely opposite to those obtained with *Ikkβ$^{\Delta hep}$* mice, in which IKKβ is missing only in hepatocytes. One likely contributor to this difference is the absence of IKKβ in Kupffer cells, where it may be required for induction of hepatomitogens such as TNFα and IL-6 (Yamada et al., 1997). To examine this possibility, we compared the effect of DEN on expression of these factors in livers of *Ikkβ$^{F/F}$*, *Ikkβ$^{\Delta hep}$*, and *Ikkβ$^{\Delta L+H}$* mice. Whereas deletion of IKKβ only in hepatocytes did not inhibit induction of any cytokine mRNA, deletion of IKKβ in liver and hematopoietic cells attenuated induction of the mRNAs for TNFα (especially at the 4 hr time point), IL-6, and HGF but had no effect on expression of TGFα mRNA (Figure 7A). Furthermore, absence of IKKβ solely from hepatocytes augmented expression of TNFα, IL-6, and HGF mRNAs at the 24 hr time point, most likely by increasing the extent of liver injury. Deletion of IKKβ in macrophages and neutrophils but not in Kupffer cells did not affect the induction of any of these mRNAs (Figure S6B), underscoring the importance of Kupffer cells rather than other myeloid cells for production of hepatomitogens. To further establish the importance of Kupffer cells, *Ikkβ$^{\Delta hep}$* mice were treated with gadolinium chloride ($GdCl_3$), which selectively depletes or inactivates Kupffer cells (Hardonk et al., 1992), 24 hr before DEN injection. This resulted in diminished induction of TNFα (especially at the 24 hr time point), IL-6, and HGF mRNAs but had no effect on TGFα mRNA (Figure 7B). These results are similar to those found in *Ikkβ$^{\Delta L+H}$* mice (Figure 7A). We isolated Kupffer cells from DEN-injected *Ikkβ$^{\Delta hep}$* mice and measured mRNA levels. Whereas TNFα and IL-6 mRNAs were abundant, HGF and TGFα mRNAs were scarce (Figure S7A). Immunohistochemistry confirmed that IL-6 is expressed mainly in Kupffer cells after DEN challenge of *Ikkβ$^{F/F}$* mice, but no IL-6 was detected in *Ikkβ$^{\Delta L+H}$* mice (Figure 7C). Correspondingly, the proliferative response to DEN was reduced in *Ikkβ$^{\Delta L+H}$* mice (Figure 7D) or after $GdCl_3$ treatment (Figure S7B). *Ikkβ$^{\Delta L+H}$* mice also exhibited higher levels of liver injury and cell death mostly at 24 hr after DEN administration relative to *Ikkβ$^{F/F}$* mice (Figure S8). This difference, however, was smaller at 48 hr after DEN administration, suggesting that liver injury at later stages might depend on an IKKβ-mediated inflammatory response. Indeed, mice lacking TNFR1 (Figure 3F) or IL-6 (Figure S9) exhibited reduced liver injury but only at 48 hr after DEN injection. Nonetheless, given the marked elevation in liver injury seen in *Ikkβ$^{\Delta L+H}$* mice at 24 hr after DEN administration, the reduction of the proliferative response (Figure 7D) is quite remarkable and likely to account for the lower susceptibility to DEN-induced carcinogenesis. Reduced growth factor expression in *Ikkβ$^{\Delta L+H}$* mice was

Figure 3. Loss of IKKβ Enhances both Cell Death and Compensatory Hepatocyte Proliferation after DEN Exposure

(A) Mice of indicated genotypes were injected with DEN and their livers isolated at indicated times and homogenized. IKK and JNK activities were determined by immunecomplex kinase assays. Protein recovery was determined by immunoblotting with anti-IKKα and anti-JNK1 antibodies.

(B) Mice were treated as above, and ALT levels in serum were determined when indicated.

(C and D) The extent of apoptosis (C) and cell proliferation (D) in livers of DEN-injected mice was determined by TUNEL staining or BrdU labeling, respectively.

(E) Liver sections from untreated and DEN-injected mice were analyzed by indirect immunoperoxidase staining for BrdU (red)- and TUNEL (green)-positive cells at the indicated times after DEN injection.

(F) ALT levels in mice of different genotypes were determined at indicated times after DEN injection.

(G) Frequencies of BrdU-positive proliferating cells at 48 hr after DEN injection. Results in (B)–(D) and (F) and (G) are averages ±S.E. Asterisks, $p < 0.05$ by Student's t test.

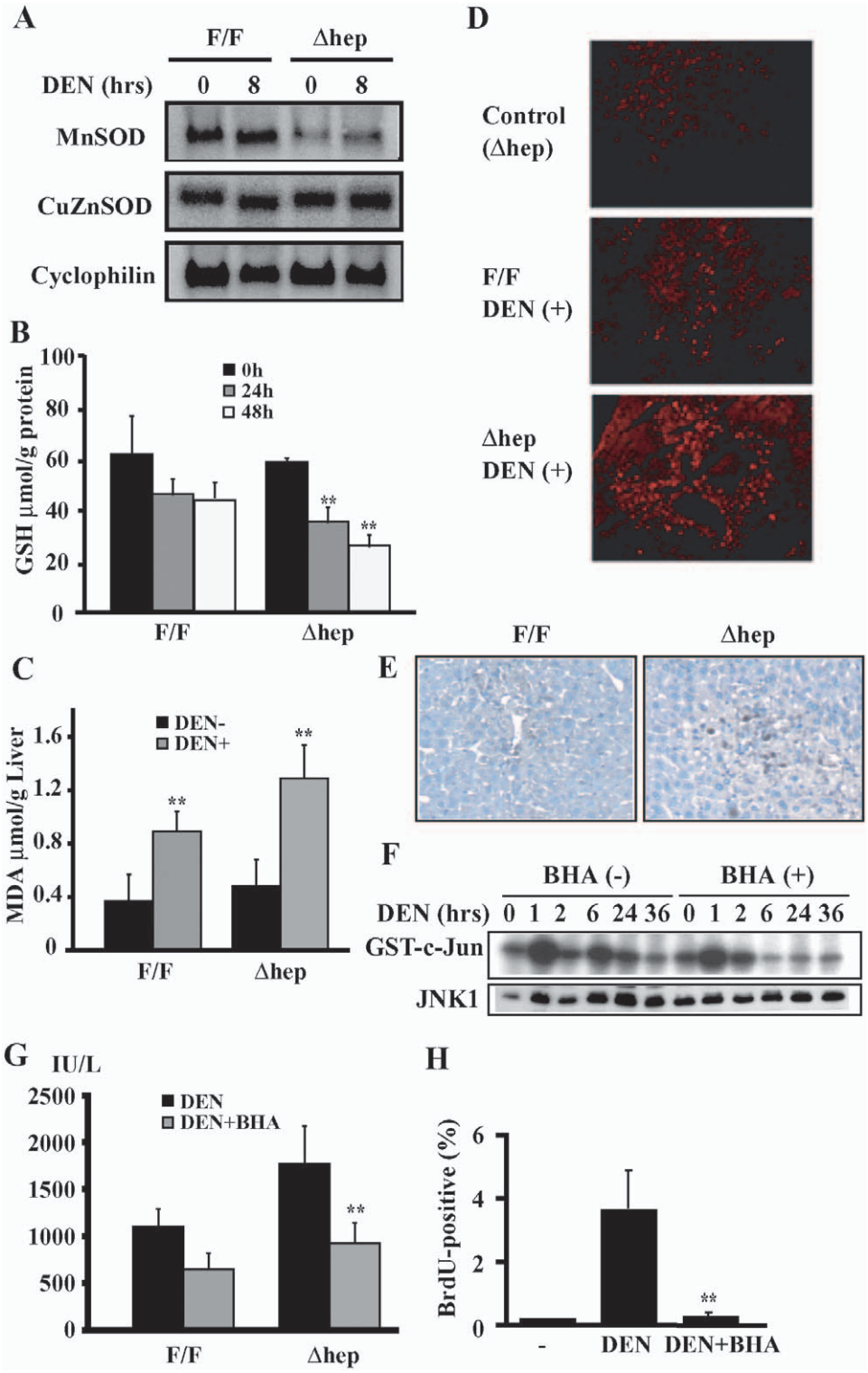

Figure 4. Loss of IKKβ Enhances DEN-Induced Oxidative Stress

(A) RNA was isolated from *Ikkβ*$^{F/F}$ or *Ikkβ*$^{\Delta hep}$ hepatocytes as indicated after DEN injection and Northern blotted for MnSOD, CuZnSOD, and cyclophilin mRNAs.

(B) Liver lysates prepared at indicated times after DEN injection were analyzed for GSH content.

(C) Lipid peroxidation was examined at 0 and 24 hr after DEN injection by measuring malondialdehyde (MDA) in liver homogenates.

(D) Frozen liver sections prepared 24 hr after DEN or PBS (control) injection were stained with 2 μM dihydroethidine hydrochloride for 30 min at 37°C. Cells staining positively for the oxidized dye were identified by fluorescent microscopy.

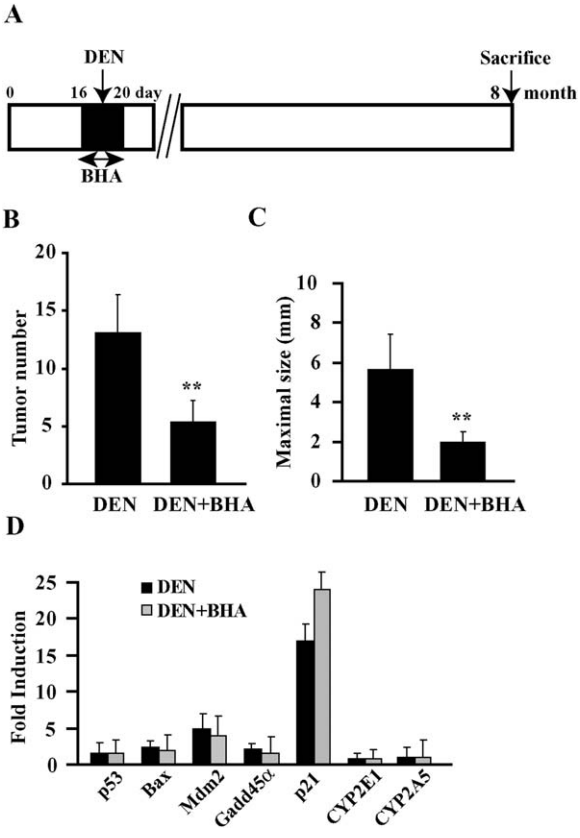

Figure 5. Consumption of BHA-Containing Diet Prevents Augmented Hepatocarcinogenesis in *Ikkβ*$^{\Delta hep}$ Mice

(A) Outline of the experimental protocol. Mice were kept on either regular or BHA-containing (0.7%) chow for 4 days, starting 2 days before DEN (25 mg/kg) injection at postnatal day 18. On day 20, mice were placed on normal diet and analyzed 8 months later.

(B and C) Numbers (B) and maximal sizes (C) of HCCs (≥0.5 mm) in livers of male *Ikkβ*$^{\Delta hep}$ mice treated as indicated (n = 15 for each group). Results are averages ±S.E. Asterisks, $p < 0.05$ by Student's t test.

(D) Mice (*Ikkβ*$^{\Delta hep}$) were treated with or without BHA for 48 hr before DEN injection. Their livers were removed at 4 hr after DEN injection for RNA extraction. Expression of the indicated genes was determined by real-time PCR. Results are averages ±S.E. (n = 3).

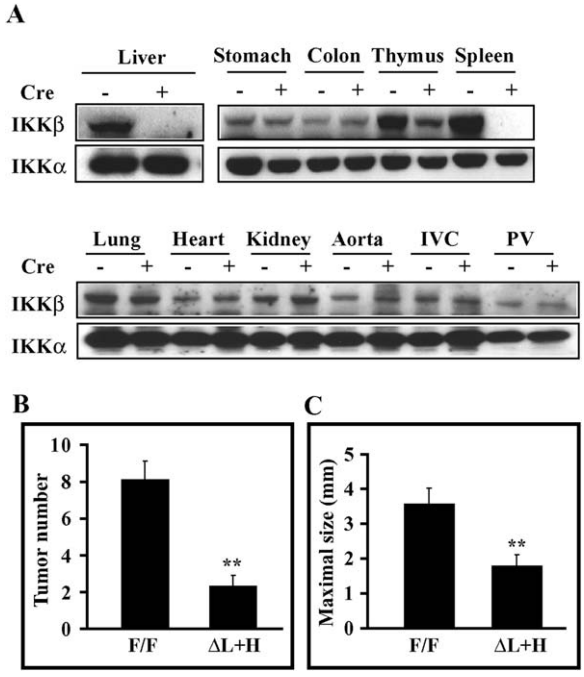

Figure 6. *Ikkβ*$^{\Delta L+H}$ Mice Exhibit Lower Levels of DEN-Induced Hepatocarcinogenesis

(A) IKKβ is absent in livers of *Ikkβ*$^{\Delta L+H}$ mice. Lysates from various organs of 6-week-old *Ikkβ*$^{F/F}$ (–Cre) and *Ikkβ*$^{F/F}$*:Mx1-Cre* (+Cre) mice injected with poly(IC) were immunoblotted for IKKα and IKKβ expression.

(B and C) Numbers (B) and maximal sizes (C) of HCCs (≥0.5 mm) in male *Ikkβ*$^{F/F}$ (n = 9) and *Ikkβ*$^{\Delta L+H}$ (n = 9) mice 8 months after DEN (5 mg/kg) injection. Results are averages ±S.E. Asterisks, $p < 0.05$ by Student's t test.

also seen after partial (70%) hepatectomy (Figure S10). Correspondingly, the proliferative response after partial hepatectomy was reduced in *Ikkβ*$^{\Delta L+H}$ but not *Ikkβ*$^{\Delta hep}$ mice (Figure 7E).

Discussion

Although chemically induced hepatocarcinogenesis was not expected to depend on inflammation, the results described above indicate that IKKβ, a major activator of inflammatory processes, plays a key role in DEN-induced hepatocarcinogenesis. Although IKKβ was previously shown to be required for development of CAC (Greten et al., 2004), its role in DEN-induced hepatocarcinogenesis is different and more complex, antitumorigenic in hepatocytes, the presumptive tumor progenitors, and procarcinogenic in Kupffer cells, which do not undergo malignant transformation. The prototypic carcinogen used in this study, DEN, is metabolized into an alkylating agent that induces DNA damage and mutations (Verna et al., 1996) as well as hepatocyte death (Farber and Gerson, 1984). As expected, loss of IKKβ in hepatocytes augments DEN-induced death, but in this particular situation more cell death potentiates, rather than attenuates, carcinogenesis. By contrast, in the CAC model, ablation of IKKβ in enterocytes inhibits carcinogenesis by accelerating apoptotic elimination of initiated cells (Greten et al., 2004). In the DEN model, however, an anticarcinogenic

(E) Paraffin-embedded liver sections prepared 24 hr after DEN injection were immunostained with anti-8-OHdG antibody.

(F) *Ikkβ*$^{\Delta hep}$ mice 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 assay of liver lysates prepared when indicated after DEN injection.

(G) Mice were treated as above. After DEN injection (48 hr), ALT in serum was measured.

(H) *Ikkβ*$^{\Delta hep}$ mice were treated as above or left untreated (–). Frequencies of BrdU-positive proliferating cells were determined 48 hr after DEN injection. Results in (B), (C), (G), and (H) are averages ±S.E. Asterisks, $p < 0.05$ by Student's t test.

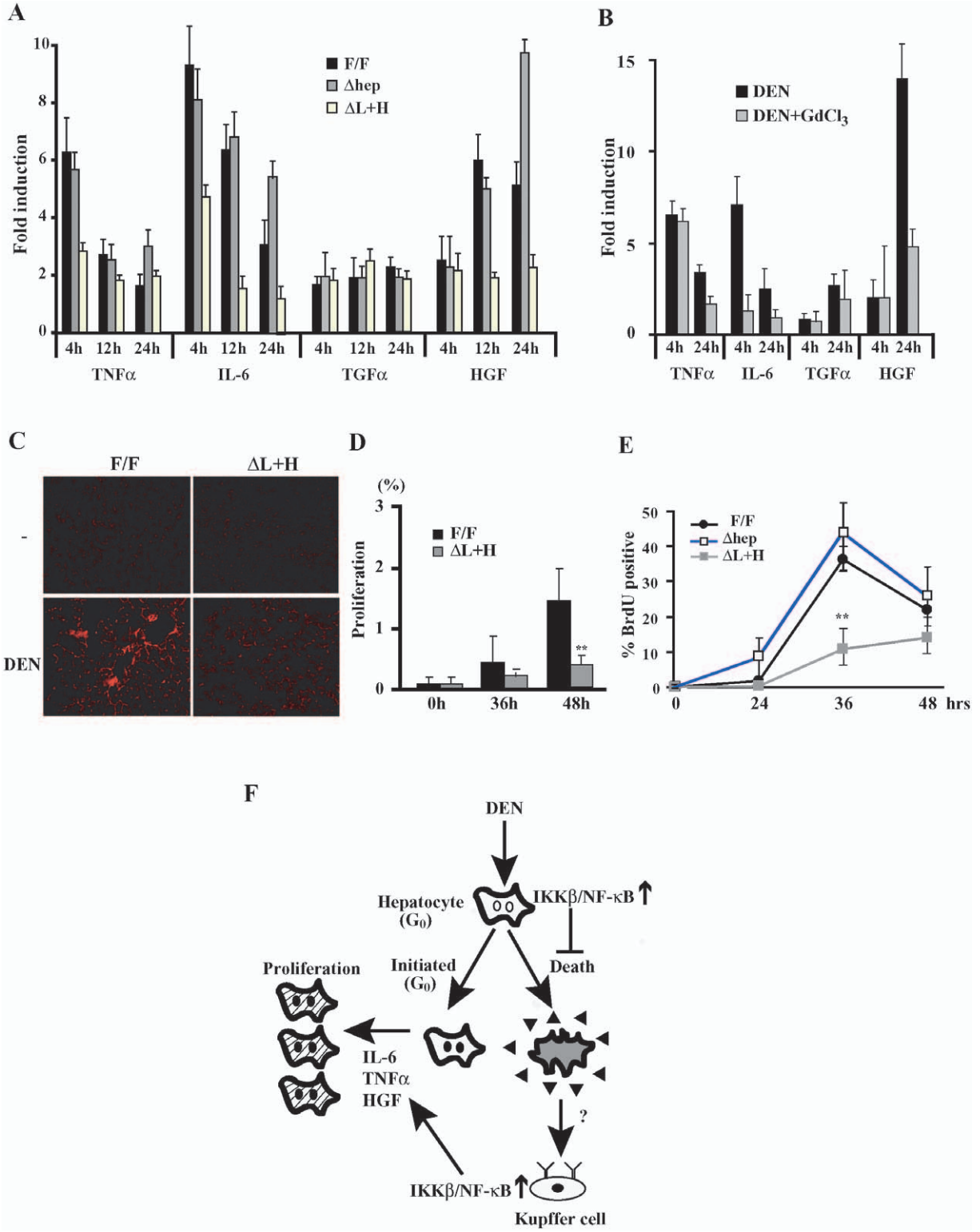

Figure 7. *Ikkβ*$^{\Delta L+H}$ Mice Express Lower Levels of Liver Growth Factors and Exhibit Decreased Compensatory Proliferation and a Model

(A) Mice of indicated genotypes were injected with DEN and at the indicated times liver RNA was extracted. The levels of various growth factor mRNAs were determined by real-time PCR. Results are averages ±S.E. (n = 3).

(B) Mice were treated with or without $GdCl_3$ 24 hr before DEN administration at time = 0. The levels of growth factor mRNAs were determined as above.

(C) Frozen liver sections were prepared at 0 or 12 hr after DEN injection and immunostained for IL-6 expression. IL-6-positive cells were also positive for F4/80 (data not shown).

effect was achieved only when IKKβ was eliminated in Kupffer cells and other types of myeloid cells, in addition to hepatocytes. As demonstrated, Kupffer cells are critical for production of hepatomitogens. Unlike the colon, which is subject to continuous renewal and thus has a high cell turnover rate, the rate of cell turnover in mature liver is extremely low and, without compensatory proliferation, DEN-exposed hepatocytes do not yield genetically transformed progeny. IKKβ plays a critical role in this response by orchestrating oncogenic crosstalk between dying hepatocytes and Kupffer cells that is likely to depend on release of cellular constituents from the former that activate NF-κB in the latter, where it induces expression of hepatomitogens that act on surviving DEN-initiated hepatocytes (Figure 7F).

IKKβ and Chemical Hepatocarcinogenesis

Once activated by cytochrome P-450 enzymes, DEN yields alkylating agents that modify DNA and induce mutations (Verna et al., 1996). However, induction of mutations is not sufficient for hepatocarcinogenesis. In adult mice, due to its reduced toxicity (S.M., unpublished data), DEN is a weak carcinogen that requires assistance from tumor promoters. Some promoters, such as carbon tetrachloride (Sarma et al., 1986) or agonistic anti-Fas antibody (Hara et al., 2000), are cytotoxic. Others, such as phenobarbital and peroxisome proliferators, act through various mechanisms, mostly obscure, that eventually induce hepatocyte proliferation (Sarma et al., 1986). Our results provide strong genetic support to the notion that critical for tumor promotion is compensatory proliferation, causing initiated hepatocytes to enter the cell cycle and transmit oncogenic mutations to their progeny (Fausto, 1999). By facilitating hepatocyte killing, the loss of IKKβ results in a paradoxical increase in DEN-induced hepatocarcinogenesis. In other words, IKKβ is a tumor suppressor in hepatocytes because it promotes their survival. A similar effect was seen upon hepatocyte-specific overexpression of *Bcl-2* (Pierce et al., 2002). Like IKKβ (Greten et al., 2004), *Bcl-2* is tumor promoting in other cell types (Korsmeyer, 1992). Yet, in hepatocytes, *Bcl-2* and IKKβ suppress HCC by inhibiting compensatory proliferation. Conversely, ablation of positive regulators of hepatocyte proliferation, c-Jun (Eferl et al., 2003) and Foxm1b (Kalinichenko et al., 2004), inhibits hepatocarcinogenesis.

Implicit to the conclusion that hepatocyte proliferation is essential to DEN-induced hepatocarcinogenesis is the assumption that it is the mature hepatocyte that is being transformed by DEN (Fausto, 1999; Fausto, 2000). This assumption is strongly supported by the finding that every HCC in *Ikkβ*$^{\Delta hep}$ mice that was analyzed was deleted for *Ikkβ*. This deletion was caused by CRE recombinase expressed from an *albumin* gene promoter/enhancer that is specifically and strongly activated in neonatal hepatocytes (Liao et al., 1980). Thus, although it is formally possible that HCCs arise from intrahepatic progenitors, such as oval cells (Sell and Leffert, 1982), in which the *albumin* promoter had become activated upon differentiation, this will delete *Ikkβ* only after acquisition of DEN-induced mutations. Therefore, the most parsimonious interpretation of our results is that DEN transforms differentiated hepatocytes, an interpretation consistent with the strong correlation between hepatocyte proliferation in the different mouse strains used in this study and HCC formation.

Hepatocyte proliferation in response to DEN exposure depends on cytokine growth factors. Important sources of these factors are Kupffer cells, which produce TNFα and IL-6 in an IKKβ-dependent manner. Interestingly, HGF is produced by stellate cells that are of mesothelial origin rather than by Kupffer cells (Schirmacher et al., 1993). Yet HGF production depends on factors produced by Kupffer cells (Figure 7B). The role of these mitogens in hepatocyte proliferation after partial hepatectomy is well established (Fausto, 2000; Yamada et al., 1997), and HGF is required for DEN-induced hepatocarcinogenesis (Horiguchi et al., 2002). Another growth factor involved in liver regeneration is TGFα, whose overexpression promotes spontaneous and DEN-induced HCCs (Fausto, 1999). Unlike TNFα, IL-6, and HGF, TGFα expression is IKKβ independent and takes place in hepatocytes (Fausto, 2000).

How does DEN induce expression of hepatocyte growth factors? Although DEN is a relatively potent IKK activator (Figure 3), it is unlikely that this effect is direct or mediated through a specific receptor. Furthermore, activation of IKK and NF-κB in response to DEN occurs mainly in differentiated hepatocytes (Figure S4), whereas the IKKβ-dependent mitogens are produced by other cells. One likely mechanism may involve an inflammatory signal transduction causing activation of Kupffer cells by substances released by necrotic hepatocytes such as HMGB1 (Scaffidi et al., 2002). Not surprisingly, a supernatant of necrotic hepatocytes activates NF-κB in primary macrophages (S.M., unpublished data). Given the high rate of cell death in *Ikkβ*$^{\Delta hep}$ tumors, this response may occur even at later stages of the carcinogenic process, resulting in constant supply of growth and survival factors.

Role of ROS and Oxidative Stress in Hepatocarcinogenesis

Ikkβ$^{\Delta hep}$ mice exhibit elevated oxidative stress and ROS accumulation after DEN administration. Consumption

(D) Frequencies of proliferating cells in livers of indicated mice were determined by BrdU pulse labeling before and after DEN injection. Results are averages ±S.E. Asterisks, $p < 0.05$ by Student's t test.

(E) Mice of indicated genotypes were subjected to partial hepatectomy. Before each time point (2 hr) mice were injected with BrdU. When indicated, liver sections were prepared and BrdU-positive cells were identified. Results are averages ±S.E. (n = 4 per each time point). Asterisks, $p < 0.05$ by Student's t test.

(F) A model explaining our results. The death of DEN-exposed hepatocytes, which is enhanced by an IKKβ deficiency, causes release of cellular constituents that activate IKKβ and NF-κB in Kupffer cells to induce production of growth factors (IL-6, TNFα, HGF) that stimulate proliferation of surviving hepatocytes harboring DEN-induced mutations.

of an antioxidant for a short duration around the time of DEN exposure reversed the elevation in HCC load caused by loss of IKKβ. BHA also blocks prolonged JNK activation and DEN-induced compensatory proliferation but has no effect on JNK activation and compensatory proliferation after partial hepatectomy (Kamata et al., 2005), suggesting a considerable degree of biological specificity. However, BHA can also induce drug metabolizing enzymes through the Nrf2-dependent antioxidant response (Kobayashi et al., 2004). Yet *Nrf2*$^{-/-}$ mice, which fail to induce phase 2 enzymes, do not show increased susceptibility to DEN-induced hepatocarcinogenesis (Satoh et al., 2002). Furthermore, DEN, like other nitrosamines, is not detoxified by phase 2 conjugating enzymes (Williams, 1993). Indeed, BHA consumption had no effect on p53 and IKK activation or the initial phase of DEN-induced JNK activation. Given that JNK activity is required for liver regeneration (Figure 3G), it is plausible that the major anticarcinogenic effect of BHA in *Ikkβ*$^{\Delta hep}$ mice is due to inhibition of prolonged JNK activation, which is ROS dependent (Kamata et al., 2005). Increased ROS accumulation was also observed in mice with hepatocyte-specific c-Myc and TGFα transgenes (Thorgeirsson et al., 2000). Feeding these mice with the antioxidant vitamin E decreased ROS production, hepatocyte proliferation, and tumor incidence (Factor et al., 2000). Although the mechanism by which vitamin E exerted its anticarcinogenic effect was not explored, it was suggested that ROS generated by TGFα signaling are the primary carcinogenic agents in this model. While ROS can cause DNA damage, their primary tumor-promoting effect could be mediated through JNK activation, the level and duration of which determine whether DEN-exposed hepatocytes will die or proliferate, rather than direct induction of oncogenic mutations. Nonetheless, we cannot exclude the contribution of oxidative DNA lesions to HCC development in *Ikkβ*$^{\Delta hep}$ mice.

Role of Inflammation and Similarity to Other Models of Liver Cancer

Although inflammation was not expected to be involved in chemical hepatocarcinogenesis, our results reveal that inflammatory crosstalk between dying hepatocytes and myeloid cells is central to HCC development. One likely mechanism accounting for this crosstalk entails activation of Kupffer cells by products released by dying hepatocytes, a well-established proinflammatory mechanism (Scaffidi et al., 2002).

Recently, inflammation-driven NF-κB activation was shown to be instrumental in another model of liver carcinogenesis in which chronic hepatitis due to *Mdr2* deficiency plays a central role (Pikarsky et al., 2004). In that study, it was suggested that inhibition of NF-κB in hepatocytes, via Tet-induced IκB superrepressor transgene, decreases rather than increases tumorigenesis. Yet it should be noted that the C/EBPβ promoter used to express the Tet transactivator is not specific to hepatocytes and is active in myeloid and other cell types (Akira et al., 1990; Talbot et al., 1994). Given the broader specificity of this promoter, the results obtained by inhibiting NF-κB in *Mdr2*$^{-/-}$ mice (Pikarsky et al., 2004) are similar to the results we obtained with the *Mx1-Cre*-driven deletion of IKKβ in the DEN model. Thus, HCC formation in *Mdr2*$^{-/-}$ mice is also likely to depend on NF-κB activation in myeloid cells. Indeed, the survival and progression of HCCs in that model require TNFα (Pikarsky et al., 2004), a cytokine produced by Kupffer cells and not hepatocytes. Similarly, adenovirus-mediated delivery of catalytically inactive IKKβ to transgenic mice expressing TGFα and c-Myc in hepatocytes led to killing of already-developed HCCs (Cavin et al., 2004). Although it was assumed that the adenovirus delivered the IKKβ mutant to hepatocytes, it should be noted that adenovirus more readily infects Kupffer cells (Lieber et al., 1997). It is also noteworthy that inhibition of NF-κB during the first 7 months of life in *Mdr2*$^{-/-}$ mice did not reduce HCC formation (Pikarsky et al., 2004), most likely because it exerts opposing effects on tumor development in hepatocytes and myeloid cells. Only after 7 months, when these mice exhibit full-blown chronic hepatitis due to activation of myeloid cells by dying hepatocytes, does NF-κB inhibition decrease tumor development. Thus, the results obtained by Pikarsky et al. (2004) may be consistent with our model (Figure 7F).

Another HCC model in which tumor development is highly dependent on inflammation is the HBsAg model, in which the inflammatory response is T cell mediated (Chisari, 1995). Although NF-κB may be activated in livers of patients suffering from chronic viral hepatitis or HCC (Tai et al., 2000), this activation is probably secondary to the inflammatory response induced by the virus, which drives expression of hepatomitogens. In light of our results, it is important to examine whether an early effect of viral infection is inhibition of NF-κB in hepatocytes, which facilitates both hepatocyte death and inflammation-driven expression of hepatomitogens in Kupffer cells.

Inhibition of NF-κB activation in keratinocytes also stimulates cell proliferation and promotes squamous cell carcinoma (SCC) when combined with oncogenic *Ha-ras* (Dajee et al., 2003). Interestingly, hyperproliferation of NF-κB-deficient keratinocytes depends on TNFα as well as persistent JNK activation (Zhang et al., 2004), just as observed in DEN-treated *Ikkβ*$^{\Delta hep}$ mice. Such findings raise the specter that IKKβ inhibitors, currently being developed (Karin et al., 2004), may increase the risk of HCC and SCC. However, the growth and survival of both cancers requires production of NF-κB-dependent cytokines by other cells. As pharmacological NF-κB inhibitors are unlikely to be cell type specific, their use may not result in increased cancer incidence after all.

Experimental Procedures

Animals

Jnk1$^{-/-}$, *Ikkβ*$^{F/F}$, *Ikkβ*$^{F/F}$*:Alb-Cre* (referred to as *Ikkβ*$^{\Delta hep}$), and *Ikkβ*$^{F/F}$*:Mx1-Cre* (referred to as *Ikkβ*$^{L+H}$ after poly[IC] injection), *Ikkβ*$^{F/F}$*:LysM-Cre* (referred to as *Ikkβ*$^{\Delta mye}$) mice were described (Hsu et al., 2004; Maeda et al., 2003). *Tnfr1*$^{-/-}$ and *IL-6*$^{-/-}$ (C57BL/6) mice were from The Jackson Laboratory. All mice were maintained in filter topped cages on autoclaved food and water at UCSD according to NIH guidelines.

Tumor Induction and Analysis

Fifteen-day-old mice and littermates on a mixed C57BL/6/129 background (*Ikkβ*$^{\Delta hep}$ and *Ikkβ*$^{F/F}$ mice were intercrossed at least

six times) were injected intraperitoneally (i.p.) with 5 or 25 mg/kg DEN (Sigma), as indicated. For the initiation-promotion model, 4-week-old mice were injected i.p. with 100 mg/kg DEN and after 4 weeks received 0.07% phenobarbital (Sigma) in drinking water for 6 months. When indicated, 16-day-old mice were given BHA-containing (0.7%) or normal chow for 4 days. These mice were injected with DEN (25 mg/kg) at day 18. After 8 months on normal chow, all mice were sacrificed and their livers removed and separated into individual lobes. Externally visible tumors ($\geq$0.5 mm) were counted and measured by stereomicroscopy. Large lobes were fixed in 4% paraformaldehyde overnight and paraffin embedded. Sections (5 μm) were H&E stained, and tumor-occupied areas were measured using Scion Image for Windows. Remaining lobes were microdissected into tumor and nontumor tissue and stored at −80°C until analyzed. Apoptosis was determined by the ApoAlert TUNEL Assay Kit (Clontech). To examine cell proliferation, mice were injected i.p. with 100 mg/kg BrdU (Sigma) 2 hr prior to sacrifice, and paraffin sections were stained using the BrdU in situ detection kit (Pharmingen). Liver injury was examined by measuring circulating transaminases (Maeda et al., 2003).

Kupffer Cell Isolation and $GdCl_3$ Treatment

Kupffer cells were isolated by collagenase digestion and differential centrifugation using Percoll (Pharmacia). The liver was perfused in situ as described (Maeda et al., 2003). The cell suspension was filtered through nylon gauze and the filtrate centrifuged twice at 50 g for 1 min to remove hepatocytes. The nonparenchymal cell fraction was washed with buffer and centrifuged through a Percoll cushion at 1000 g for 15 min to obtain the Kupffer cell fraction, followed by washing with buffer. Cells were plated in plastic culture dishes and cultured for 1 hr, and nonadherent cells were removed. Macrophages were cultured as described (Hsu et al., 2004).

To deplete Kupffer cells, mice were administered i.v. 10 mg $GdCl_3$-$6H_2O$/kg (Sigma) or saline 24 hr before DEN administration (Hardonk et al., 1992).

Biochemical and Immunohistochemical Analyses

IKK and JNK immunecomplex kinase assays, electrophoretic mobility shift assays, immunoblotting, and immunohistochemistry were described (Maeda et al., 2003). The following antibodies were used: anti-IKKα (Imgenex); anti-IKKβ (Upstate); anti-IκBα, anti-c-Myc, anti-cyclin D1, anti-p53, anti-CDK1, anti-CDK2, anti-RelA, and c-Rel (Santa Cruz Biotechnology); anti-BrdU and anti-PCNA (Pharmingen); anti-8-OHdG (Japan Institute for the Control of Aging); and anti-IL-6 (R&D). Analysis of mRNA expression by real-time PCR or Northern blot analysis was described (Hsu et al., 2004; Maeda et al., 2003).

GSH levels were measured as described (Kamata et al., 2005). Lipid peroxidation was measured using an assay kit (Calbiochem) that detects malondialdehyde (MDA) and 4-hydroxyalkenals (HAE), which are generated by peroxidation of polyunsaturated fatty acids and related esters. To assess levels of superoxide anions, freshly prepared frozen sections were incubated with 2 μM dihydroethidine hydrochloride (Molecular Probes) for 30 min at 37°C, after which they were observed by fluorescent microscopy and photographed.

Statistical Analysis

Data are expressed as means ±SEM. Differences were analyzed by Student's t test. P values $\leq$0.05 were considered significant. For overall survival analysis, Kaplan-Meier curves were analyzed by log rank test. In all cases, group size was chosen to produce results that are statistically unambiguous.

Supplemental Data

Supplemental Data include ten figures and can be found with this article online at http://www.cell.com/cgi/content/full/121/7/977/DC1/.

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References

Akira, S., Isshiki, H., Sugita, T., Tanabe, O., Kinoshita, S., Nishio, Y., Nakajima, T., Hirano, T., and Kishimoto, T. (1990). A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. EMBO J. *9*, 1897–1906.

Berenblum, I. (1954). Carcinogenesis and tumor pathogenesis. Adv. Cancer Res. *2*, 129–175.

Bosch, F.X., Ribes, J., Diaz, M., and Cleries, R. (2004). Primary liver cancer: worldwide incidence and trends. Gastroenterology *127*, S5–S16.

Cavin, L.G., Venkatraman, M., Factor, V.M., Kaur, S., Schroeder, I., Mercurio, F., Beg, A.A., Thorgeirsson, S.S., and Arsura, M. (2004). Regulation of α-fetoprotein by NF-κB protects hepatocytes from TNFα cytotoxicity during fetal liver development and hepatic oncogenesis. Cancer Res. *64*, 7030–7038.

Chisari, F.V. (1995). Hepatitis B virus transgenic mice: insights into the virus and the disease. Hepatology *22*, 1316–1325.

Coussens, L.M., and Werb, Z. (2002). Inflammation and cancer. Nature *420*, 860–867.

Dajee, M., Lazarov, M., Zhang, J.Y., Cai, T., Green, C.L., Russell, A.J., Marinkovich, M.P., Tao, S., Lin, Q., Kubo, Y., and Khavari, P.A. (2003). NF-κB blockade and oncogenic Ras trigger invasive human epidermal neoplasia. Nature *421*, 639–643.

Dyroff, M.C., Richardson, F.C., Popp, J.A., Bedell, M.A., and Swenberg, J.A. (1986). Correlation of O4-ethyldeoxythymidine accumulation, hepatic initiation and hepatocellular carcinoma induction in rats continuously administered diethylnitrosamine. Carcinogenesis *7*, 241–246.

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.

Esterbauer, H., and Cheeseman, K.H. (1990). Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. Methods Enzymol. *186*, 407–421.

Factor, V.M., Laskowska, D., Jensen, M.R., Woitach, J.T., Popescu, N.C., and Thorgeirsson, S.S. (2000). Vitamin E reduces chromosomal damage and inhibits hepatic tumor formation in a transgenic mouse model. Proc. Natl. Acad. Sci. USA *97*, 2196–2201.

Farber, J.L., and Gerson, R.J. (1984). Mechanisms of cell injury with hepatotoxic chemicals. Pharmacol. Rev. *36*, 71S–75S.

Fausto, N. (1999). Mouse liver tumorigenesis: models, mechanisms, and relevance to human disease. Semin. Liver Dis. *19*, 243–252.

Fausto, N. (2000). Liver regeneration. J. Hepatol. Suppl. 1 *32*, 19–31.

Ghosh, S., and Karin, M. (2002). Missing pieces in the NF-κB puzzle. Cell Suppl. *109*, S81–S96.

Greten, F.R., Eckmann, L., Greten, T.F., Park, J.M., Li, Z.W., Egan, L.J., Kagnoff, M.F., and Karin, M. (2004). IKKβ links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. Cell *118*, 285–296.

Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. Cell *100*, 57–70.

Hara, A., Yoshimi, N., Yamada, Y., Matsunaga, K., Kawabata, K., Sugie, S., and Mori, H. (2000). Effects of Fas-mediated liver cell apoptosis on diethylnitrosamine-induced hepatocarcinogenesis in mice. Br. J. Cancer *82*, 467–471.

Hardonk, M.J., Dijkhuis, F.W., Hulstaert, C.E., and Koudstaal, J.

(1992). Heterogeneity of rat liver and spleen macrophages in gadolinium chloride-induced elimination and repopulation. J. Leukoc. Biol. *52*, 296–302.

Horiguchi, N., Takayama, H., Toyoda, M., Otsuka, T., Fukusato, T., Merlino, G., Takagi, H., and Mori, M. (2002). Hepatocyte growth factor promotes hepatocarcinogenesis through c-Met autocrine activation and enhanced angiogenesis in transgenic mice treated with diethylnitrosamine. Oncogene *21*, 1791–1799.

Hsu, L.C., Park, J.M., Zhang, K., Luo, J.L., Maeda, S., Kaufman, R.J., Eckmann, L., Guiney, D.G., and Karin, M. (2004). The protein kinase PKR is required for macrophage apoptosis after activation of Toll-like receptor 4. Nature *428*, 341–345.

Im, Y.H., Kim, H.T., Kim, I.Y., Factor, V.M., Hahm, K.B., Anzano, M., Jang, J.J., Flanders, K., Haines, D.C., Thorgeirsson, S.S., et al. (2001). Heterozygous mice for the transforming growth factor-β type II receptor gene have increased susceptibility to hepatocellular carcinogenesis. Cancer Res. *61*, 6665–6668.

Kalinichenko, V.V., Major, M.L., Wang, X., Petrovic, V., Kuechle, J., Yoder, H.M., Dennewitz, M.B., Shin, B., Datta, A., Raychaudhuri, P., and Costa, R.H. (2004). Foxm1b transcription factor is essential for development of hepatocellular carcinomas and is negatively regulated by the p19ARF tumor suppressor. Genes Dev. *18*, 830–850.

Kamata, H., Honda, S., Maeda, S., Chang, L., Hirata, H., and Karin, M. (2005). Reactive oxygen species promote TNF-α–induced cell death and sustained JNK activation by oxidixing MAP kinase phosphatases. Cell *120*, 649–661.

Karin, M., Cao, Y., Greten, F.R., and Li, Z.W. (2002). NF-κB in cancer: from innocent bystander to major culprit. Nat. Rev. Cancer *2*, 301–310.

Karin, M., Yamamoto, Y., and Wang, Q.M. (2004). The IKK NF-κB system: a treasure trove for drug development. Nat. Rev. Drug Discov. *3*, 17–26.

Klein, G., and Klein, E. (1985). Evolution of tumours and the impact of molecular oncology. Nature *315*, 190–195.

Kobayashi, A., Ohta, T., and Yamamoto, M. (2004). Unique function of the Nrf2-Keap1 pathway in the inducible expression of antioxidant and detoxifying enzymes. Methods Enzymol. *378*, 273–286.

Korsmeyer, S.J. (1992). Bcl-2 initiates a new category of oncogenes: regulators of cell death. Blood *80*, 879–886.

Li, Q., Van Antwerp, D., Mercurio, F., Lee, K.F., and Verma, I.M. (1999). Severe liver degeneration in mice lacking the IκB kinase 2 gene. Science *284*, 321–325.

Liao, W.S., Conn, A.R., and Taylor, J.M. (1980). Changes in rat α 1-fetoprotein and albumin mRNA levels during fetal and neonatal development. J. Biol. Chem. *255*, 10036–10039.

Lieber, A., He, C.Y., Meuse, L., Schowalter, D., Kirillova, I., Winther, B., and Kay, M.A. (1997). The role of Kupffer cell activation and viral gene expression in early liver toxicity after infusion of recombinant adenovirus vectors. J. Virol. *71*, 8798–8807.

Maeda, S., Chang, L., Li, Z.-W., Luo, J.-L., Leffert, H.L., and Karin, M. (2003). IKKβ is required for prevention of apoptosis mediated by cell-bound but not by circulating TNFα. Immunity *19*, 725–737.

Mauad, T.H., van Nieuwkerk, C.M., Dingemans, K.P., Smit, J.J., Schinkel, A.H., Notenboom, R.G., van den Bergh Weerman, M.A., Verkruisen, R.P., Groen, A.K., and Oude Elferink, R.P. (1994). Mice with homozygous disruption of the mdr2 P-glycoprotein gene. A novel animal model for studies of nonsuppurative inflammatory cholangitis and hepatocarcinogenesis. Am. J. Pathol. *145*, 1237–1245.

Philip, M., Rowley, D.A., and Schreiber, H. (2004). Inflammation as a tumor promoter in cancer induction. Semin. Cancer Biol. *14*, 433–439.

Pierce, R.H., Vail, M.E., Ralph, L., Campbell, J.S., and Fausto, N. (2002). Bcl-2 expression inhibits liver carcinogenesis and delays the development of proliferating foci. Am. J. Pathol. *160*, 1555–1560.

Pikarsky, E., Porat, R.M., Stein, I., Abramovitch, R., Amit, S., Kasem, S., Gutkovich-Pyest, E., Urieli-Shoval, S., Galun, E., and Ben-Neriah, Y. (2004). NF-κB functions as a tumour promoter in inflammation-associated cancer. Nature *431*, 461–466.

Sakon, S., Xue, X., Takekawa, M., Sasazuki, T., Okazaki, T., Kojima, Y., Piao, J.H., Yagita, H., Okumura, K., Doi, T., and Nakano, H. (2003). NF-κB inhibits TNF-induced accumulation of ROS that mediate prolonged MAPK activation and necrotic cell death. EMBO J. *22*, 3898–3909.

Sarma, D.S., Rao, P.M., and Rajalakshmi, S. (1986). Liver tumour promotion by chemicals: models and mechanisms. Cancer Surv. *5*, 781–798.

Satoh, K., Itoh, K., Yamamoto, M., Tanaka, M., Hayakari, M., Ookawa, K., Yamazaki, T., Sato, T., Tsuchida, S., and Hatayama, I. (2002). Nrf2 transactivator-independent GSTP1-1 expression in "GSTP1-1 positive" single cells inducible in female mouse liver by DEN: a preneoplastic character of possible initiated cells. Carcinogenesis *23*, 457–462.

Scaffidi, P., Misteli, T., and Bianchi, M.E. (2002). Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature *418*, 191–195.

Schirmacher, P., Geerts, A., Jung, W., Pietrangelo, A., Rogler, C.E., and Dienes, H.P. (1993). The role of Ito cells in the biosynthesis of HGF-SF in the liver. EXS *65*, 285–299.

Sell, S., and Leffert, H.L. (1982). An evaluation of cellular lineages in the pathogenesis of experimental hepatocellular carcinoma. Hepatology *2*, 77–86.

Tai, D.I., Tsai, S.L., Chang, Y.H., Huang, S.N., Chen, T.C., Chang, K.S., and Liaw, Y.F. (2000). Constitutive activation of NF-κB in hepatocellular carcinoma. Cancer *89*, 2274–2281.

Talbot, D., Descombes, P., and Schibler, U. (1994). The 5′ flanking region of the rat LAP (C/EBP beta) gene can direct high-level, position-independent, copy number-dependent expression in multiple tissues in transgenic mice. Nucleic Acids Res. *22*, 756–766.

Tang, G., Minemoto, Y., Dibling, B., Purcell, N.H., Li, Z., Karin, M., and Lin, A. (2001). Inhibition of JNK activation through NF-κB target genes. Nature *414*, 313–317.

Thorgeirsson, S.S., and Grisham, J.W. (2002). Molecular pathogenesis of human hepatocellular carcinoma. Nat. Genet. *31*, 339–346.

Thorgeirsson, S.S., Factor, V.M., and Snyderwine, E.G. (2000). Transgenic mouse models in carcinogenesis research and testing. Toxicol. Lett. *112–113*, 553–555.

Verna, L., Whysner, J., and Williams, G.M. (1996). N-nitrosodiethylamine mechanistic data and risk assessment: bioactivation, DNA-adduct formation, mutagenicity, and tumor initiation. Pharmacol. Ther. *71*, 57–81.

Williams, G.M. (1993). Inhibition of chemical-induced experimental cancer by synthetic phenolic antioxidants. Toxicol. Ind. Health *9*, 303–308.

Wong, G.H., and Goeddel, D.V. (1988). Induction of manganous superoxide dismutase by tumor necrosis factor: possible protective mechanism. Science *242*, 941–944.

Yamada, Y., Kirillova, I., Peschon, J.J., and Fausto, N. (1997). Initiation of liver growth by tumor necrosis factor: deficient liver regeneration in mice lacking type I tumor necrosis factor receptor. Proc. Natl. Acad. Sci. USA *94*, 1441–1446.

Zhang, J.Y., Green, C.L., Tao, S., and Khavari, P.A. (2004). NF-κB RelA opposes epidermal proliferation driven by TNFR1 and JNK. Genes Dev. *18*, 17–22.

Zhou, Z., Wang, L., Song, Z., Saari, J.T., McClain, C.J., and Kang, Y.J. (2004). Abrogation of NF-κB ac tivation is involved in zinc inhibition of lipopolysaccharide-induced TNFα production and liver injury. Am. J. Pathol. *164*, 1547–1556.