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The transcription factor c-JUN/AP-1 promotes HBV-related liver tumorigenesis in mice

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Hepatocellular carcinoma (HCC) develops as a consequence of chronic inflammatory liver diseases such as chronic hepatitis B virus (HBV) infection. The transcription factor c-Jun/activator protein 1 (AP-1) is strongly expressed in response to inflammatory stimuli, promotes hepatocyte survival during acute hepatitis and acts as an oncogene during chemically induced liver carcinogenesis in mice. Here, we therefore aimed to characterize the functions of c-Jun during HBV-related liver tumorigenesis. To this end, transgenic mice expressing all HBV envelope proteins (*HBV*$^+$), an established model of HBV-related HCC, were crossed with knockout mice lacking *c-Jun* specifically in hepatocytes and tumorigenesis was analyzed. Hepatic expression of c-Jun was strongly induced at several time points during tumorigenesis in *HBV*$^+$ mice, whereas expression of other AP-1 components remained unchanged. Importantly, formation of premalignant foci and tumors was strongly reduced in *HBV*$^+$ mice lacking *c-Jun*. This phenotype correlated with impaired hepatocyte proliferation and increased expression of the cell cycle inhibitor p21, whereas hepatocyte survival was not affected. Progression and prognosis of HBV-related HCC correlates with the expression of the cytokine osteopontin (Opn), an established AP-1 target gene. Opn expression was strongly reduced in *HBV*$^+$ livers and primary mouse hepatocytes lacking *c-Jun*, demonstrating that c-Jun regulates hepatic Opn expression in a cell-autonomous manner. These findings indicate that c-Jun has important functions during HBV-associated tumorigenesis by promoting hepatocyte proliferation as well as progression of dysplasia. Therefore, targeting c-Jun may be a useful strategy to prevent hepatitis-associated tumorigenesis.

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Hepatocellular carcinoma (HCC) frequently develops as a consequence of chronic inflammatory liver diseases such as chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, chronic inflammation, liver injury, fibrosis and subsequent cirrhosis. HCC is the seventh and fifth most common cancer in women and men, respectively, and its incidence is particularly high in developing countries, in which HBV and HCV infections are endemic.[1] HBV is considered to be non-cytopathic in most cases and the majority of HBV-related liver damage has been attributed to hepatic immune responses.[2] However, HBV may directly promote tumorigenesis by genomic integration of viral DNA or by the action of viral transactivators such as HBx or mutant preS2 surface proteins.[3] In keeping with this notion, overexpression of hepatoviral proteins in transgenic mice is sufficient to cause HCC as shown in Tg(*Alb-1HBV*)Bri44 mice (hereafter referred to as *HBV*$^+$ mice), which express the large, middle and small isoforms of HBV surface proteins (LHBs, MHBs and HBs antigens) under the control of the albumin promoter as well as HBx from its endogenous promoter.[4] Although liver disease in this model does not include viral replication, cccDNA formation and HBV-specific immune cell responses, liver disease in these animals is characterized by accumulation of hepatoviral proteins in the endoplasmic reticulum (ER) and appearance of 'ground glass hepatocytes', liver damage with compensatory proliferation, aneuploidy, dysplasia and eventually HCC, thereby mimicking several aspects of disease pathogenesis in HBV-infected patients.[5–7] *HBV*$^+$ mice therefore represent a useful tool to genetically dissect molecular pathways related to hepatitis-associated hepatocarcinogenesis. HBV replication and expression of hepatoviral proteins such as HBx strongly activate c-Jun.[3,8–10] c-Jun is a member of the dimeric activator protein 1 (AP-1) transcription factor family, which mainly consists of Jun (c-Jun, JunB and JunD) and Fos proteins (c-Fos, FosB, Fra-1 and Fra-2).[11] c-Jun is expressed as an immediate early gene in response to a variety of stress stimuli, growth factors and subsequent signaling through MAP kinase pathways. It is a major determinant of cell fate in the liver and regulates hepatocyte survival during acute hepatitis and ER stress[8,12] as well as hepatocyte proliferation following 2/3 partial hepatectomy.[13] Moreover, c-Jun acts as an oncogene in the liver and strongly promotes liver tumorigenesis in models of chemically induced HCC.[14–16] However, the functions of c-Jun during HBV-related liver disease are only poorly defined, although the above-mentioned findings strongly suggest that c-Jun may be a central regulator of HBV-mediated hepatocarcinogenesis. To address this issue experimentally, we generated *HBV*$^+$ transgenic mice

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Abbreviations: HCC, hepatocellular carcinoma; HBV, hepatitis B virus; ER, endoplasmic reticulum; ConA, concanavalin A; ALT, alanine aminotransferase; PMH, primary mouse hepatocytes; AP-1, activator protein 1

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specifically lacking *c-Jun* in hepatocytes. Our findings indeed indicate that c-Jun has important functions during HBV-related tumorigenesis by regulating hepatocyte proliferation as well as progression of dysplasia.

Results

Increased expression of c-Jun in *HBV*$^+$ mice. To analyze the expression of c-Jun at various stages of HBV-related tumorigenesis, hepatic expression of c-Jun was determined by quantitative PCR (qPCR) in *HBV*$^+$ mice. In comparison to *HBV*$^-$ controls, *c-Jun* expression was increased at all time points studied and peaked at around 6 months of age (Figure 1a, Supplementary Figure S1A). Moreover, hepatic c-Jun expression was also increased in a separate infectious mouse model of hepatitis B (Supplementary Figure S1B). In contrast, expression of *JunB* was not induced in *HBV*$^+$ livers at 6 months of age, whereas JunD expression was slightly increased in *HBV*$^+$ livers irrespective of c-Jun expression (Figure 1b). Expression of *Fos* genes could not be detected (Figure 1b). Immunohistochemistry of *HBV*$^+$ livers revealed that c-Jun was expressed in hepatocytes and inflammatory cells (Figure 1c). Hepatic *c-Jun* expression was substantially reduced in *HBV*$^-$ *c-Jun*$^{\Delta li}$ and *HBV*$^+$ *c-Jun*$^{\Delta li}$ livers (Figure 1b), in which residual c-Jun expression occurred in non-parenchymal cells (Figure 1c). These findings indicate that c-Jun expression is induced following HBV infection and in HBV transgenic mice. Moreover, transgene expression of small and large HBsAg at 6 months of age was analyzed by ELISA and immunoblotting, respectively, and revealed that transgene expression was not altered in the absence of *c-Jun* (Supplementary Figure S1C), indicating that this mouse model was indeed suitable to further study the functions of c-Jun in HBV-related tumorigenesis.

***c-Jun* strongly promotes HBV-related tumorigenesis.** To determine the impact of c-Jun expression on HBV-related tumorigenesis, *HBV*$^+$ *c-Jun*$^{f/f}$ and *HBV*$^+$ *c-Jun*$^{\Delta li}$ mice were analyzed at 12 months of age. No obvious macroscopical liver abnormalities were observed in *HBV*$^-$ *c-Jun*$^{f/f}$ and *c-Jun*$^{\Delta li}$ mice (Figure 2a). In contrast, liver tumors were apparent macroscopically in 15/22 *HBV*$^+$ *c-Jun*$^{f/f}$ mice, whereas livers appeared normal and tumor-free in all 16 *HBV*$^+$ *c-Jun*$^{\Delta li}$ mice (Figure 2a). These findings correlated with a significant increase in liver weight in *HBV*$^+$ *c-Jun*$^{f/fi}$ as compared with *HBV*$^+$ *c-Jun*$^{\Delta li}$ mice (Figure 2b). Moreover, tumorigenesis was also quantified microscopically and confirmed that tumorigenesis was strikingly reduced at this time point in *HBV*$^+$ mice lacking *c-Jun* (Figure 2c). Histopathological hallmarks of *HBV*$^+$ livers include the appearance of ground glass hepatocytes due to accumulation of surface proteins in the ER, inflammation, cellular atypia and the appearance of atypical foci, which progress to hyperplastic nodules, adenoma, and eventually HCC.[5] Infiltration of CD3$^+$ T cells and Ly6G$^+$ neutrophils was not altered in *HBV*$^+$ mice lacking *c-Jun* (Supplementary Figure S2A). The appearance of ground glass hepatocytes, inflammation and cellular atypia were comparable in *HBV*$^+$ *c-Jun*$^{f/f}$ and *HBV*$^+$ *c-Jun*$^{\Delta li}$ livers as determined by histopathological analysis, whereas numbers of atypical foci and subsequent pro-tumorigenic alterations were profoundly reduced in the absence of *c-Jun* (Figures 3a and b). c-Jun expression was observed in most HBV-related pathologies including ground glass hepatocytes, adenoma and HCC (Supplementary Figure S2B). It should be noted that most of the tumor lesions observed in *HBV*$^+$ livers here were hyperplastic nodules or adenomas and that the prevalence of HCC in *HBV*$^+$ *c-Jun*$^{f/f}$ mice was low at the age of 12 months. However, it has been shown that the precursor lesions observed here can be used as tumor surrogate and

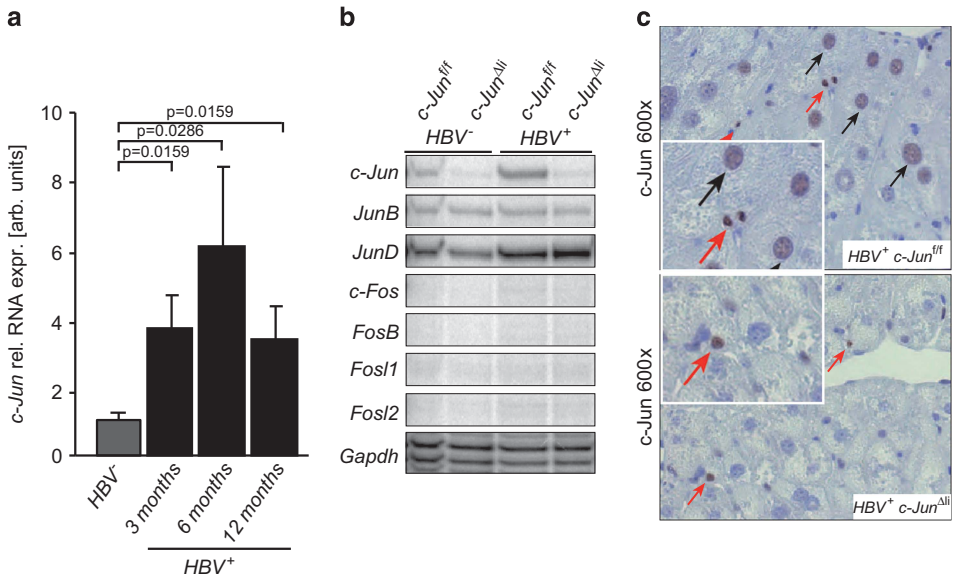

Figure 1 c-Jun expression is induced in *HBV*$^+$ mice. (**a**) Hepatic *c-Jun* mRNA expression at the indicated time points was determined by qPCR and normalized to the expression in *HBV*$^-$ livers ($n \geq 4$ per genotype and time point, significance was tested by Mann–Whitney test). (**b**) Hepatic expression of AP-1 family members in mice with the indicated genotypes at 6 months of age was analyzed by RNase protection assay. *Gapdh* expression was used as loading control. (**c**) c-Jun expression in livers of 6-month-old mice with the indicated genotypes as determined by immunohistochemistry. Note the expression in hepatocytes (black arrows) and inflammatory cells (red arrows)

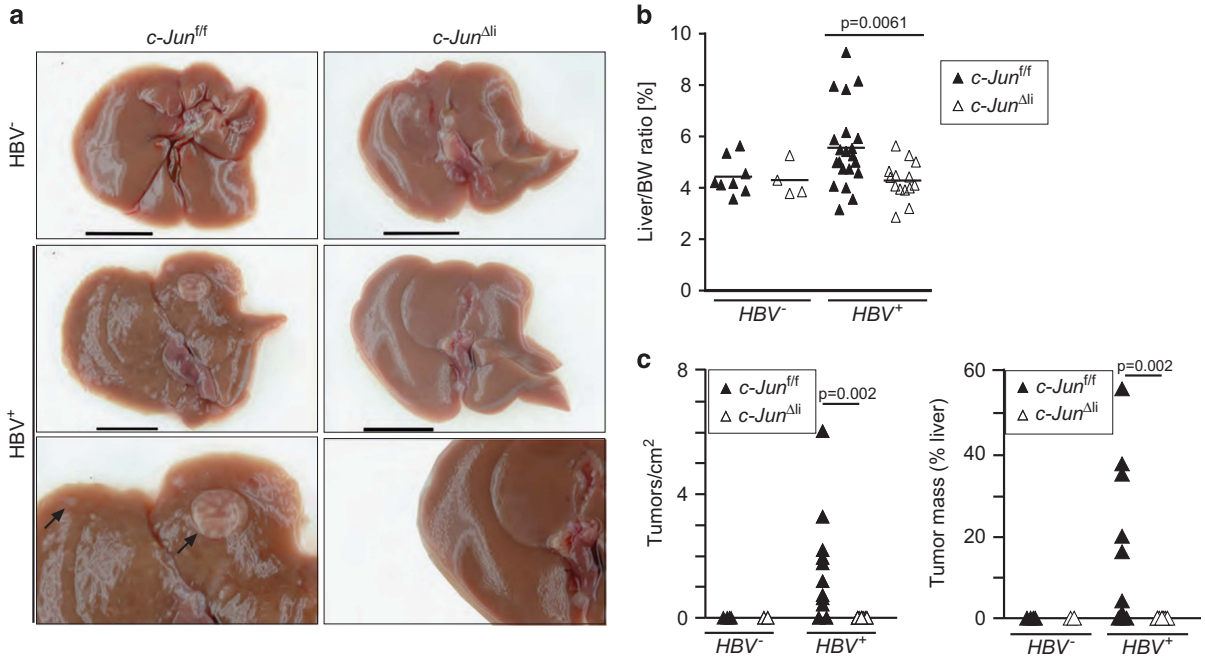

Figure 2 Liver tumorigenesis is strongly reduced in 12-month-old *HBV*+ mice lacking *c-Jun*. (**a**) Macroscopical images of representative livers of mice with the indicated genotypes. Black bar = 1 cm. (**b**) Liver weight was determined as liver/body weight ratio, ($n = 4$–21 per genotype, significance was tested by Mann–Whitney test (MWT)). (**c**) Liver tumorigenesis was quantified histologically and is given as tumor numbers per cm^2 liver tissue. Tumor load was determined as a ratio of tumorous tissue and non-tumorous tissue and is given in (%). Significance was tested by MWT

predict hepatocarcinogenesis at later time points.[7] Moreover, expression of HBsAg is gradually downregulated during neoplastic transformation and areas lacking transgene expression can therefore be used as a predictor of tumorigenesis.[7] Although transgene expression was comparable at 6 months of age, immunohistochemistry revealed that HBsAg expression was lost in many liver areas of *HBV*+ *c-Jun*f/f mice at 12 months of age consistent with the formation of preneoplastic foci, but rarely in *HBV*+ *c-Jun*Δli mice (Supplementary Figure S1D). Tumor analysis was therefore not extended beyond 12 months. In conclusion, our findings indicate that c-Jun is an important regulator of tumorigenesis in *HBV*+ mice.

c-Jun promotes hepatocyte proliferation in *HBV*+ livers. c-Jun is an important regulator of hepatocyte proliferation, survival and related stress responses. We have previously shown that c-Jun expression determines hepatocyte survival during T-cell-mediated hepatitis as well as during chemically induced ER stress,[8,12] raising the question whether similar functions may be relevant in *HBV*+ mice. However, expression of the established ER stress marker genes *BiP* and *Gadd153* as well as *XBP-1* splicing were not significantly induced in *HBV*+ as compared with *HBV*- livers and not altered in the absence of *c-Jun* (Supplementary Figure S3A). To further analyze the impact of c-Jun expression on hepatocyte damage, serum transaminase concentrations were measured. Serum alanine aminotransferase (ALT) concentrations in *HBV*+ mice peaked at the age of 6 months and decreased thereafter, but were not altered in the absence of *c-Jun* (Figure 4a), suggesting that c-Jun is not involved in regulating hepatocyte damage or survival in this model. In keeping with this notion, no difference in cell death could be observed by TUNEL staining, whereas the numbers of TUNEL+ cells were very low overall (Supplementary Figure S4). Previous findings suggest that c-Jun promotes DEN-mediated and HCV-related hepatocarcinogenesis by regulating the expression of *Birc5* or activation of Stat3.[15,16] In *HBV*+ mice, hepatic *Birc5* expression was not altered in the absence of *c-Jun* (Supplementary Figure S3A). Although Stat3 phosphorylation was evident in *HBV*+ livers, its phosphorylation as well as expression of Socs3 was not changed in *c-Jun*-deficient livers (Supplementary Figure S3B), indicating that the Stat3 pathway does not contribute to the differences observed in *HBV*+ *c-Jun*Δli mice.

Hepatocarcinogenesis in *HBV*+ mice is preceded by sustained hepatocyte proliferation.[17] Hepatocyte proliferation was significantly reduced in *HBV*+ livers lacking *c-Jun* at the age of 6 and 12 months (Figures 4b and c), whereas hepatic expression of the cell cycle inhibitor and tumor suppressor p21 was increased (Figure 4c; Supplementary Figure S5A). Moreover, *HBV*+ *c-Jun*f/f primary mouse hepatocytes (PMH) were isolated and infected with adenoviral vectors expressing *Cre* recombinase, which resulted in profound inhibition of c-Jun expression, whereas p21 expression was significantly induced (Supplementary Figure S5B). These findings strongly suggest that c-Jun interferes with hepatic p21 expression in a cell-autonomous manner.

c-Jun regulates hepatic Opn expression. Our finding that progression of dysplasia was reduced in *c-Jun* mutant livers raises the question whether *c-Jun* also regulates the

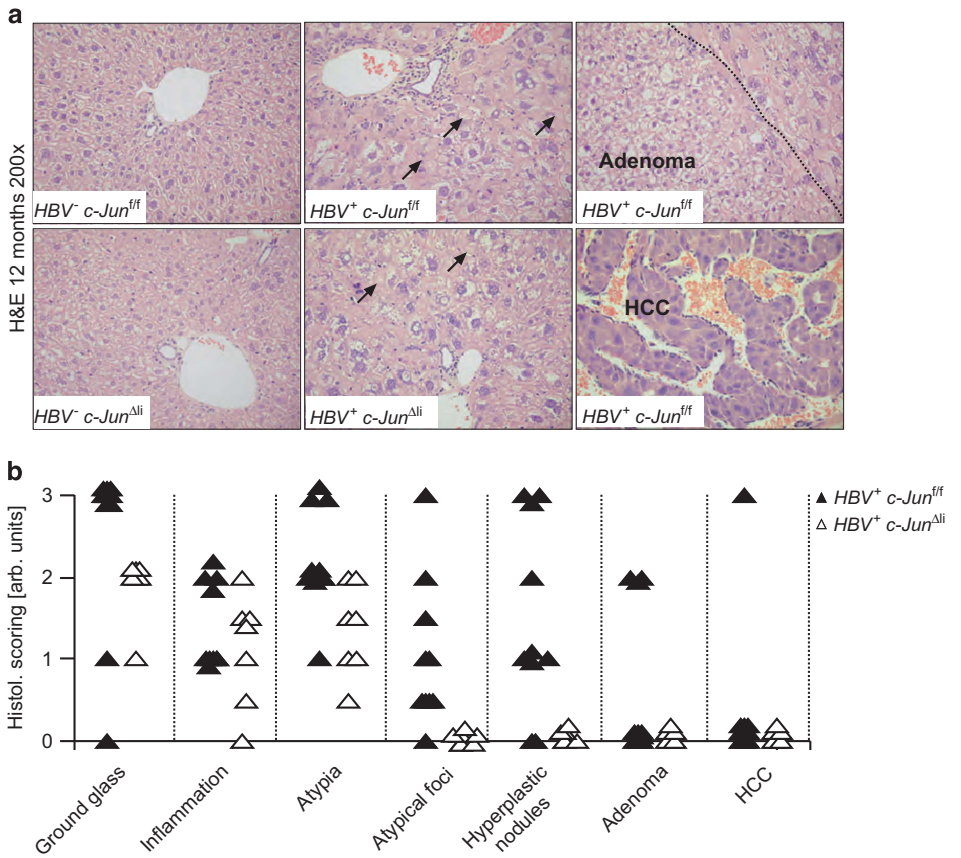

Figure 3 Absence of atypical foci and dysplastic progression in *HBV*+ mice lacking *c-Jun*. (**a**) Representative histological stainings of mice with the indicated genotypes are shown (12 months of age). Note the appearance of ground glass hepatocytes in *HBV*+ mice, which occurred irrespective of c-Jun expression. (**b**) Histological alterations observed in mice with the indicated genotypes were scored using an arbitrary scale from 0 to 3

expression of genes related to the progression of dysplastic lesions such as osteopontin (Opn). *Opn* is highly expressed in HCC and correlates with histological tumor grading, tumor stage as well as early recurrence following surgical resection, in particular in HBV-infected patients.[18] Importantly, hepatic *Opn* expression was induced in *HBV*+ mice at the age of 12 months as determined by qPCR, ELISA and immunoblotting, but reduced in the absence of *c-Jun* (Figures 5a and b; Supplementary Figure S3B). To address the question whether c-Jun promotes hepatocellular Opn expression in a cell-autonomous manner, PMH were isolated. Opn expression was indeed reduced in *HBV*+ *c-Jun*$^{\Delta li}$ PMH as determined by qPCR and ELISA (Figure 5c). Opn expression was also impaired in *HBV*+ *c-Jun*$^{f/f}$ PMH upon adenoviral transfer of Cre recombinase (Supplementary Figure S5B), indicating that this phenotype was independent of the AlfpCre transgene used here. Immunohistochemistry further revealed that Opn expression was restricted to bile duct epithelia in *HBV*+ *c-Jun*$^{\Delta li}$ livers. In *HBV*+ *c-Jun*$^{f/f}$ livers, it occurred in some dysplastic foci and scattered hepatocytes as well as in bile duct epithelia and proliferative ductular cells. These cells also expressed Sox9 and cytokeratin 19 (CK19) (Figure 5d), which also identify hepatic and liver cancer progenitor cells.[19] Moreover, hepatic Opn expression was also reduced in *c-Jun*$^{\Delta li}$ mice during acute concanavalin A (ConA)-mediated hepatitis (Supplementary Figure S5C), indicating that c-Jun regulates Opn expression also in other models of inflammatory liver disease. In conclusion, these findings indicate that c-Jun regulates hepatic Opn expression in a cell-autonomous manner, which in turn promotes HBV-related tumorigenesis.

Discussion

Expression of the AP-1 transcription factor c-Jun has been observed in many molecular subtypes of human HCC [20] and is particularly increased in HBV-related HCC.[21] c-Jun strongly promotes liver tumorigenesis in mouse models of chemically induced HCC[14–16] and also determines hepatocyte fate in a variety of inflammatory stress conditions including T-cell-mediated hepatitis and ER stress,[8,12] suggesting that c-Jun may also contribute to the pathogenesis of hepatitis-associated HCC. Here, we demonstrate that c-Jun strongly promotes liver tumorigenesis in *HBV*+ mice, a mouse model that mimics many aspects of the series of pathophysiological events leading to HCC in patients with chronic HBV infection.[5,17] c-Jun regulates hepatocyte survival following DEN-mediated tumor initiation by interfering with c-Fos and Birc5[15] or with Nos2 and Stat3 signaling in DEN-treated HCV transgenic mice.[16] Interestingly, no differences in liver damage

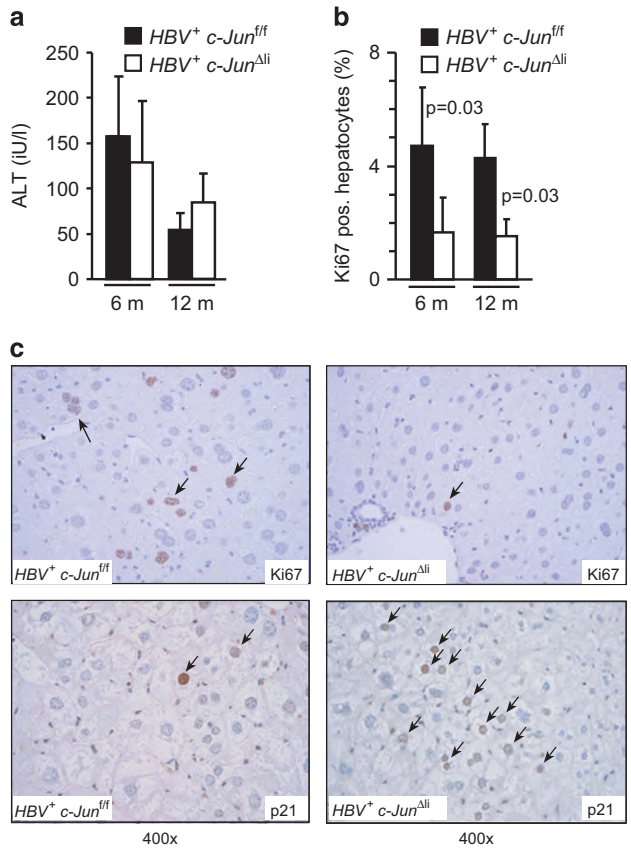

Figure 4 c-Jun promotes hepatocyte proliferation by interfering with p21. (**a**) Liver damage in mice with the indicated genotypes was determined by analysis of serum ALT concentrations at 6 and 12 months, respectively. ($n = 12$–16 per genotype and time point). (**b** and **c**) Hepatocyte proliferation was assessed by immunohistochemistry for Ki67 at 6 and 12 months of age, respectively. For quantification, three representative high-power fields were analyzed per mouse. ($n \geq 4$ per genotype and time point; significance was tested by Mann–Whitney test). p21 expression was analyzed by immunohistochemistry

could be detected in *HBV*$^+$ mice lacking *c-Jun*, in which Birc5 and Stat3 expression were also not altered. In contrast, hepatocyte proliferation was reduced in *HBV*$^+$ mice lacking c-Jun, indicating that c-Jun rather regulates hepatocyte proliferation in these mice. Increased hepatocyte proliferation may occur in response to HBV-mediated liver damage or through the hepatoviral transactivator HBx or truncated preS2 proteins, all of which are expressed in *HBV*$^+$ mice and have been shown to promote c-Jun expression.[22,23] Impaired hepatocyte proliferation in the absence of *c-Jun* correlated with increased expression of the cell cycle inhibitor p21, which is consistent with previous findings in *c-Jun*$^{-/-}$ mouse embryonic fibroblasts and in *c-Jun*$^{\Delta li}$ mice following 2/3 partial hepatectomy.[13,24]

Expression of the cytokine Opn is an early predictor of HCC formation and predicts its recurrence upon surgical resection, in particular in HBV-infected patients.[18,25] Interestingly, hepatic Opn expression was reduced in the absence of *c-Jun* in a cell-autonomous manner. Opn is an established AP-1 target gene and its expression is likely regulated by c-Jun and the Fos proteins Fra-1 or Fra-2.[26–28] In *HBV*$^+$ mice, Opn expression occurred predominantly in bile duct epithelia, proliferating ductular cells and hepatocytes. Recent findings indicate that Opn promotes proliferative ductular reactions following liver damage.[29] Interestingly, this ductular reaction and cell numbers expressing the progenitor markers Opn, Sox9 and CK19 were reduced in *HBV*$^+$ mice lacking *c-Jun*. These findings suggest that c-Jun may be involved in regulating progenitor cell responses during HBV-related liver damage and hepatocarcinogenesis, which is consistent with the clinical observation that AP-1 expression identifies a subgroup of liver progenitor cell-derived tumors with poor prognosis.[30]

In conclusion, our findings indicate that c-Jun has important functions during HBV-related hepatocarcinogenesis. Interactions between c-Jun and p21 very likely promote compensatory hepatocyte proliferation, which is a prerequisite for HBV-related hepatocarcinogenesis. Moreover, our findings suggest that c-Jun regulates hepatic Opn expression, thereby contributing to the progression of dysplasia, possibly by interfering with progenitor cell responses. Targeting of c-Jun may therefore be a reasonable approach to interfere with the formation of hepatitis-associated HCC.

Materials and Methods

Mice. Mice with conditional alleles of *c-Jun* (*c-Jun*$^{f/f}$) were crossed with transgenic *AlfpCre* mice to obtain animals with hepatocyte-specific knockout of *c-Jun* (*c-Jun*$^{\Delta li}$).[31] These mice were crossed with *HBV*$^+$ mice (Tg(*Alb-1HBV*)Bri44).[4] Animals were bred on a mixed genetic background (C57BL/6 × 129/Sv × FVB/N) and housed under specific pathogen-free conditions. Either *c-Jun*$^{f/f}$ or *AlfpCre c-Jun*$^{f/f}$ littermates lacking the HBV transgene (*HBV*$^-$) were used as controls. All animals received humane care and experiments were performed in accordance with local and institutional regulations. In some animals, acute T-cell-mediated hepatitis was induced by injection of ConA (10 mg/kg body weight, Sigma, Taufkirchen, Germany) as previously described.[12] Isolation of PMH was performed as described.[8] In some experiments, PMH were infected by adenoviral vectors expressing GFP or Cre recombinase (Gene Transfer Vector Core Facility, University of Iowa, Iowa, IA, USA). Briefly, PMH were incubated with adenovirus (10 MOI) for 3 h and collected after 48 h. To study c-Jun expression in a mouse model of HBV infection, C57BL/6 mice were infected with adenovirus (10^9 U per mouse) encoding an 1.3 HBV overlength genome (AdenoHBV) or empty control virus as described.[32] Mice were analyzed 7 days after adenoviral infection.

Cytotoxicity assays and ELISA. Liver damage was determined by analysis of serum ALT using semi-automated clinical routine methods. Opn or LHB concentrations in liver lysates, serum and cell culture supernatants were determined by ELISA according to the manufacturer's protocol (DY441, R&D Systems, Wiesbaden, Germany and EL10018, Abazyme, Needham, MA, USA).

Histology and Immunohistochemistry. For histology, livers were fixed in 3.7% neutral buffered formaldehyde at 4 °C and embedded in paraffin. Immunohistochemistry was performed using the Envision kit (Dako, Hamburg, Germany) and antibodies for c-Jun (#9165; Cell Signaling, NEB, Frankfurt, Germany), Ki67 (#301119; Novocastra, Newcastle, UK), p21 (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), Opn (#AF808; R&D Systems), HBsAg (HB100; BioLogo, Kronshagen, Germany), Sox9 (AB5535; Millipore, Schwalbach, Germany), CK19 (TROMA, kindly provided by R. Kemler), CD3 (clone SP7; Thermo Scientific, Fremont, CA, USA) or Ly6G (#551459; BD, Heidelberg, Germany) were used.

qPCR and RNA protection analysis. For isolation of RNA, macrodissected liver tissue was snap-frozen in liquid nitrogen and total RNA was isolated using Qiazol (Qiagen, Hilden, Germany). For isolation of total RNA from PMH, cells were lysed on their culture plate and RNA was extracted using the RNeasy kit (Qiagen). Complementary DNA synthesis was performed using the First Strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany). qPCR was performed with SYBR Green (Invitrogen, Karlsruhe, Germany) and 10% dimethylsulfoxide

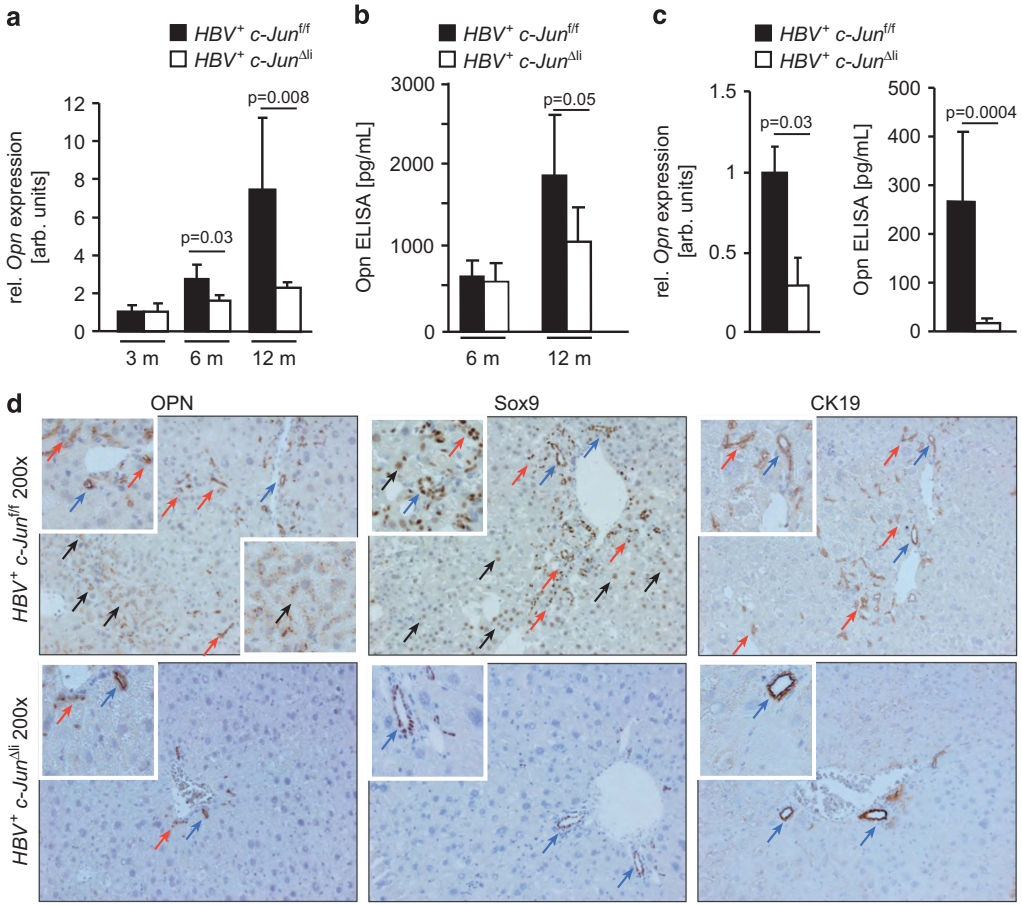

Figure 5 c-Jun regulates hepatic Opn expression. (**a** and **b**) Hepatic Opn expression was determined at the indicated time points by qPCR (**a**) and ELISA (**b**), respectively. *Opn* RNA expression was normalized to expression in *HBV* controls. ($n \geq 4$ per genotype and time point, significance was tested by Mann–Whitney test (MWT)). (**c**) Opn expression in primary hepatocytes was determined by qPCR and ELISA from the supernatant, respectively. ($n \geq 4$ per genotype, significance was tested by MWT). (**d**) Expression analysis of Opn, Sox9 and CK19 by immunohistochemistry. Hepatocytes, bile duct epithelia and proliferative ductular cells are indicated by black, blue and red arrows, respectively

(Sigma) on a 480 Lightcycler (Roche, Mannheim, Germany; 40 cycles: 30″ 95 °C; 30″ 60 °C; 40″ 72 °C). Loading was normalized to *Hprt* and *Actin* messenger RNA and 18 S ribosomal RNA. All primers were designed using Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast). Primers were synthesized by Microsynth, Balgach, Switzerland, and the specificity of the PCR products was analyzed by melting curve analysis.

Primer sequences were as follows: *Actin*: 5′-AGCCATGTACGTAGCCATCC-3′; 5′-CTCTCAGCTGTGGTGGTGAA-3′; *18 S*: 5′-CGCGGTTCTATTTTGTTGGT-3′; 5′-AGTCGGCATCGTTTATGGTC-3′; *Hprt:* 5′-GCTTGCTGGTGAAAAGGACCTC-3′; 5′-CAAATCAAAAGTCTGGGGACGC-3′ *c-Jun:* 5′-AAAACCTTGAAAGCGCAAAA-3′; 5′-CGCAACCAGTCAAGTTCTCA'; *Opn*: 5′-TGCACCCAGATCCTATAGCC-3′; 5′-CTCC ATCGTCATCATCATCG-3′ *BiP*: 5′-AGTGGTGGCCACTAATGGAG-3′; 5′-CAATCC TTGCTTGATGCTGA-3′ *Gadd153*: 5′-GCATGAAGGAGAAGGAGCAG-3′; 5′-CTTCC GGAGAGACAGACAGG-3′ spliced Xbp-1: 5′-GAGTCCGCAGCAGGTG-3′; 5′-GTGT CAGAGTCCATGGGA-3′ *Birc5:* 5′-CTCTCCCGGCATGCTCTGCG-3′; 5′-CCGCC ATTCGCTCTGGGGTG-3′ *p21:* 5′-AACTACCAGCTGTGGGGTGA-3′; 5′-GACAAC GGCACACTTTGCTC-3′; *Sesn2*: 5′-TAGCCTGCAGCCTCACCTAT-3′; 5′-GATTTTGA GGTTCCGTTCCA-3′; *Ho-1*: 5′-CACGCATATACCCGCTACCT-3′; 5′-CCAGAGTGT TCATTCGAGCA-3′; *Atf-3*: 5′-CTAGAATCCCAGCAGCCAAG-3′; 5′-GGCCAGCTAG GTCATCTGAG-3′; RNase protection analyses were performed using the Riboquant multiprobe RPA system mFos/Jun (BD).

Western blot analysis. Hepatocyte and total liver lysates were analyzed by immunoblot using antibodies for c-Jun (#9165; Cell Signaling), phospho-c-Jun (Ser63, #9261; Cell Signaling), β-Actin (#A2066; Sigma), phospho-Stat3 (Tyr705, #9131; Cell Signaling), Stat3 (#06-596; Millipore), Socs3 (#2932; Cell Signaling), p21 (Santa Cruz), Opn (#AF808; R&D Systems) or LHBs (MA18/07; kindly provided by M Nassal).

Statistics. Data in bar graphs represent mean $\pm$ S.D. Normal distribution of the data was tested using Graphpad PRISM software (La Jolla, CA, USA). Statistical analysis was performed using the nonparametric Mann–Whitney test or nondirectional two-tailed Student's *t*-test as indicated in the figure legends.

Conflict of Interest

The authors declare no conflict of interest.

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Author contributions

EFW, HEB and PH designed the study; CT, BH and PH performed experiments; CT, KZ, RT and PH analyzed the data and PH wrote the paper.

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