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HBV-related HCC development in mice is STAT3 dependent and indicates an oncogenic effect of HBx

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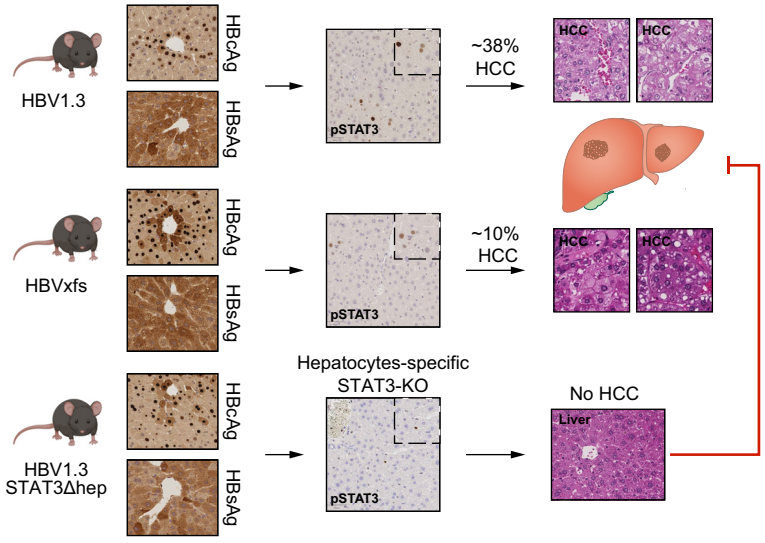
Graphical abstract



Highlights

- HBV1.3 transgenic mice developed HCC in the absence of inflammation.
- HBV-RNAs, HBx-protein, and pregenomic RNA were detected in HCC tissue.
- HBVxfs mice, expressing all HBV proteins but a truncated HBx, display significantly reduced HCC incidence.
- Hepatocyte-specific STAT3-knockout abrogated HCC development.

Impact and implications

Although most HCC cases in patients with chronic HBV infection occur after a sequence of liver damage and fibrosis, a subset of patients develops HCC without any signs of advanced liver damage. We demonstrate that the expression of all viral transcripts in HBV-transgenic mice suffices to induce HCC development independent of inflammation and fibrosis. These data indicate the direct oncogenic effects of HBV and emphasize the idea of early antiviral treatment in the 'immune-tolerant' phase (HBeAg-positive chronic HBV infection).

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HBV-related HCC development in mice is STAT3 dependent and indicates an oncogenic effect of HBx

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Background & Aims: Although most hepatocellular carcinoma (HCC) cases are driven by hepatitis and cirrhosis, a subset of patients with chronic hepatitis B develop HCC in the absence of advanced liver disease, indicating the oncogenic potential of hepatitis B virus (HBV). We investigated the role of HBV transcripts and proteins on HCC development in the absence of inflammation in HBV-transgenic mice.

Methods: HBV-transgenic mice replicating HBV and expressing all HBV proteins from a single integrated 1.3-fold HBV genome in the presence or absence of wild-type HBx (HBV1.3/HBVxfs) were analyzed. Flow cytometry, molecular, histological and *in vitro* analyses using human cell lines were performed. Hepatocyte-specific Stat3- and Socs3-knockout was analyzed in HBV1.3 mice.

Results: Approximately 38% of HBV1.3 mice developed liver tumors. Protein expression patterns, histology, and mutational landscape analyses indicated that tumors resembled human HCC. HBV1.3 mice showed no signs of active hepatitis, except STAT3 activation, up to the time point of HCC development. HBV-RNAs covering HBx sequence, 3.5-kb HBV RNA and HBx-protein were detected in HCC tissue. Interestingly, HBVxfs mice expressing all HBV proteins except a C-terminally truncated HBx (without the ability to bind DNA damage binding protein 1) showed reduced signs of DNA damage response and had a significantly reduced HCC incidence. Importantly, intercrossing HBV1.3 mice with a hepatocyte-specific STAT3-knockout abrogated HCC development.

Conclusions: Expression of HBV-proteins is sufficient to cause HCC in the absence of detectable inflammation. This indicates the oncogenic potential of HBV and in particular HBx. In our model, HBV-driven HCC was STAT3 dependent. Our study highlights the immediate oncogenic potential of HBV, challenging the idea of a benign highly replicative phase of HBV infection and indicating the necessity for an HBV 'cure'.

Introduction

Hepatitis B virus (HBV) infection, with an estimated 296 million chronic carriers worldwide, is one of the most common chronic infections despite screening programs and the availability of a prophylactic vaccine.[1–4] Chronic HBV infection is often asymptomatic until symptoms of late-stage liver disease are evident.[2] Chronic hepatitis B (CHB) includes signs of active hepatitis and subsequent risk for liver cirrhosis and is the leading cause of hepatocellular carcinoma (HCC),[1,3–5] resulting in an estimated 820,000 deaths worldwide in 2019[1,3] and HBV-related mortality is increasing.[1–4] Potentially curative treatment options for HCC are only suitable for patients with limited disease and preserved liver function. Despite the approval of additional systemic therapies (*e.g.* atezolizumab plus bevacizumab, or durvalumab–tremelimumab), advanced HCC still has a poor prognosis, and overall survival remains limited.[6] Thus, new markers for early detection of HCC and unraveling oncogenic processes to abrogate HCC development are warranted.

HBV is a partially double-stranded DNA virus that replicates via reverse transcription and persists via an episomal

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transcription template within the nucleus of infected hepatocytes (covalently closed circular DNA [cccDNA]).[7] Viral genomes can integrate into the host cell genome, which is a dead end for replication. Viral integrations can express full-length and truncated HBV proteins and can induce both genomic instability and direct insertional mutagenesis of diverse cancer-related genes.[8] The HBV genome has four overlapping open reading frames encoding the structural core protein (HBc), which includes the pre-core protein (HBe), the envelope proteins (S/M/L HBs), the viral polymerase, and the regulatory X (HBx) protein.[9] As with other viral accessory proteins, HBx stimulates HBV replication by linking a host protein to the DNA damage binding protein 1 (DDB1) associated E3 ubiquitin ligase complex.[10] Thereby, HBx influences the degradation of the structural maintenance of chromosomes proteins 5 and 6 (SMC5/6), enabling extrachromosomal HBV-DNA transcription.[11]

The 'natural' history of CHB is often assessed by the HBeAg status to decipher viral replication and risk for disease progression. However, studies indicate that HBV viral load is the best predictor to estimate the risk for disease progression and HCC development.[5,12] Nonetheless, only patients showing apparent signs of liver damage (HBeAg-positive or HBeAg-negative chronic hepatitis B or established cirrhosis) meet clear treatment criteria in current practice guidelines.[13,14] Nucleos(t)ide analogs such as tenofovir or entecavir inhibit reverse transcription and block HBV replication, reducing liver inflammation and disease progression. Interferon treatment can lead to a 'functional cure' (HBs loss/anti-HBs seroconversion) at a low percentage (<20%) but has side effects.[13,14] Nevertheless, even after years of antiviral therapy, HCC risk is present[15,16] and available treatments rarely achieve a 'cure' of HBV infection and eradication of viral cccDNA.[7]

Although antiviral therapy may reduce the HCC risk to that of a minimally active CHB,[17] there are debates on when to start and stop treatment. Current guidelines do not recommend treatment of HBeAg-negative chronic infection ('inactive carriers').[13,14] However, definitions of inactive carriers with a low risk for detrimental effects are not uniform, and these patients are still at risk to develop HCC.[18] Furthermore, HBV-DNA integrations and clonal expansions of hepatocytes bearing these integrations are already present in young patients with HBeAg-positive chronic infection and high viral loads but without an 'active' hepatitis.[19] Some studies showed an HCC risk of 0–1.4% in 5–10 years in these (formerly: 'immune tolerant') patients.[20,21] However, other studies especially from Asia reported a 10-year HCC incidence of 12.7% in the highly replicative phase of HBV infection accompanied by a higher risk of clinical events. Treatment of these patients strongly reduced disease-risk, emphasizing the advantage of early treatment.[22,23]

In CHB, and in other etiologies causing HCC, the main driver is a sequence of chronic inflammation, cell death, compensatory hepatocyte proliferation, and development of liver fibrosis/cirrhosis, referred to as necro-inflammation.[6,24,25] On a molecular level, many aspects of hepatocarcinogenesis, including driver mutations or methylation status of genes, show a wide diversity, and the involvement of cytokine network activation, including nuclear factor kappa B- and signal transducer and activator of transcription 3 (STAT3)-signaling, have been described.[26,27]

In patients with CHB however, HCC development can also occur in the absence of liver cirrhosis, suggesting direct oncogenic effects of HBV.[8,28] As such, the integration of HBV DNA into the host genome is well studied, but no direct activation of oncogenes has been found.[19,29,30] Studies describe a direct oncogenic potential of HBV proteins or mutants, including spliced HBV protein,[31] Pre-S2 mutants[32] and overexpression of large HBs.[33] Furthermore, HBx protein seems to play a direct role in HCC development and progression by interfering with multiple pathways.[34–38] Nonetheless, the impact of physiological levels of HBV protein expression on HCC development remains unclear.

In this study, we used 1.3-overlength HBV transgenic mice (HBV1.3), expressing all viral proteins driven by the endogenous HBV promoters enabling high-level HBV replication[39,40] and compared them with mice replicating HBV but lacking full-length HBx expression. These mice neither show viral spread nor cccDNA formation as a result of restriction factors or a host cell dependency factor.[39,41] As HBV1.3 mice show high viral replication and lack evidence of hepatitis,[39,40] we aimed to dissect inflammation and integration independent direct oncogenic effects of HBV in this model.

Materials and methods

Mice

Mice were maintained under specific pathogen free conditions. Experiments were conducted so that they conformed to ARRIVE guidelines and in accordance with the guidelines of the German Animal Protection Law and were approved by local committees of government of Bavaria (license number: 55.1-1-54-2531.3-27-08, 55.2-1-54-2532-120-12, 55.2-1-54-2532-144-2014) and by local committees of government of Baden-Württemberg (license number: 35-9185.81/G-50/20). Mice transgenic for a 1.3 × overlength HBV genotype D ayw genome (HBV1.3) have been provided by Prof. F. Chisari and have been described.[39,40] Mice transgenic for a 1.3 × overlength HBV genotype D ayw genome replicating HBV but lacking full-length HBx expression (HBVxfs) were described previously.[42] C57BL/6 mice were bred in-house, used for backcrossing to transgenic animals and co-housed with HBV1.3 mice as control (wild-type [WT]) mice on the same background. For hepatocyte-specific STAT3 or suppressor of cytokine signaling 3 (SOCS3) inactivation, mice expressing hepatocyte-specific Cre-recombinase under the albumin promoter (AlbCre)[43] were crossed with $STAT3^{f/f}$ animals,[44] which harbor loxP-sites in exon 21 of STAT3, or with $SOCS3^{f/f}$ animals.[45] These mice were further crossed with HBV1.3 animals resulting in HBV1.3_*AlbCreSTAT3*$^{f/f}$ or HBV1.3_*AlbCreSOCS3*$^{f/f}$ mice. *AlbCre*-negative mice (HBV1.3_*STAT3*$^{f/f}$ or HBV1.3_*SOCS3*$^{f/f}$) were used as HBV1.3 littermate controls. *AlbCre*, *STAT3*$^{f/f}$, and *SOCS3*$^{f/f}$ mice were provided by Prof. Algül. Both male and female mice were used in mouse experiments in an even proportion between different strains. We analyzed between four and 96 mice per time point in each experiment as indicated in the figure legends.

Cell lines

HepaRG cells were cultured as previously described[46] by growing the cells for 2 weeks in supplemented William's E medium (10% FBS Fetalclone II, 100 U/ml penicillin/streptomycin, 2 mM glutamine, 0.023 U/ml human insulin, 0.0047 mg/

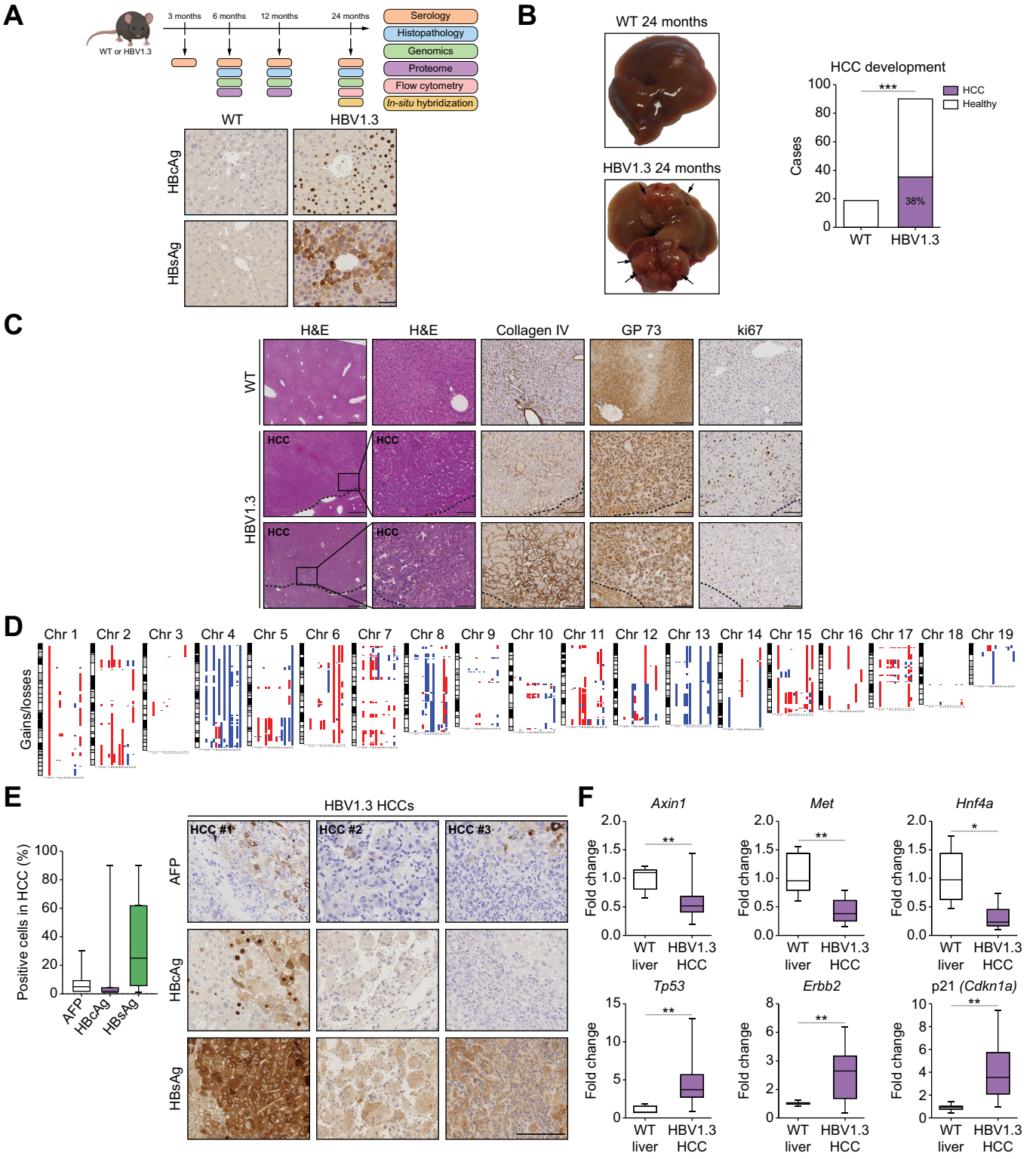

Fig. 1. HBV1.3 mice spontaneously develop tumors resembling human HCC. (A) Top: Graphical summary of experimental design (created with BioRender.com). Below: IHC staining for HBcAg and HBsAg in representative liver sections of control (WT) and HBV1.3 mice at 24 months of age (scale bar: 100 μm). (B) Macroscopic pictures of unaffected WT mouse liver and HBV1.3 liver with tumor nodules (arrows indicate tumors) and bar graph of total number of mice analyzed at 20–24 months of age (cases without tumor in white and livers with HCC in purple) of WT and HBV1.3 mice (Fisher's exact test, ***p <0.001). (C) Representative H&E and IHC staining of collagen IV, GP73, and ki67 of WT and HCC-bearing HBV1.3 mice (scale bar: 100 μm). (D) aCGH displaying chromosomal aberrations in micro-dissected HCC samples (gains in red and losses in blue). Each row resembles an HCC sample. (E) Quantification and IHC staining for AFP, HBcAg, and HBsAg in HCC of HBV1.3 mice (n = 25; three different staining patterns of HCC are shown, scale bar: 100 μm). (F) Quantitative RT-qPCR for up/downregulated genes in HCC of HBV1.3 mice (n = 12) compared with and normalized to 24-month-old WT liver tissue (n = 6). Box plots indicate interquartile range and median of relative expression. The whiskers extend

ml hydrocortisone, 0.08 mg/ml gentamicin) and differentiation for 2 weeks by adding 1.8% DMSO (Sigma-Aldrich, Steinheim, Germany) to the growth medium.

HuH7 and HepG2-based cells were cultured in supplemented DMEM (10% FCS, 100 U/ml penicillin/streptomycin, 2 mM glutamine, 1 × non-essential amino acids, 1 mM sodium pyruvate). Primary human hepatocytes (PHHs) were obtained from the Department of General Surgery, MRI, TU-Munich (Munich, Germany) and cultured as previously described,[47] after informed patient's consent and authorization by the Technical University Munich ethics committee (ref: 5846/13).

Statistical analysis

Statistical analyses were performed with Prism software (Graphpad Prism version 9.5.1, GraphPad Software, San Diego, CA, USA). The standard error of the mean was calculated from the average of at least four independent samples per condition as indicated in the figure legends. To evaluate statistical significance, data were subjected to Student's *t* test (unpaired, two-tailed test), or with Welch's correction, ordinary one-way ANOVA with Tukey's multiple comparisons test, Brown-Forsythe and Welsh ANOVA, or Fisher's exact test. A value of $p < 0.05$ was considered significant.

For further details regarding the materials and methods used, please refer to the supplementary information.

Results

HBV1.3 mice spontaneously develop tumors resembling human HCC

HBV1.3 mice express all viral transcripts and proteins, driven exclusively by the endogenous HBV promoters enabling high-level HBV replication.[39,40] Expression of viral surface proteins (HBsAg) and the HBV core protein (HBcAg) were readily detected in HBV1.3 mice until the age of 24 months (Fig. 1A). Expression patterns were comparable to that in patients with HBV infection with HBsAg diffuse/focal cytoplasmic and membranous staining, and HBcAg predominantly nuclear staining.[48]

We observed that 35 of the 91 HBV1.3 mice studied (38%) spontaneously developed macroscopically visible liver tumors when aged between 20 and 24 months (Fig. 1B). No liver tumors were detected in co-housed WT control mice at that age (Fig. 1B). H&E staining and immunohistochemistry (IHC) of these tumors showed broadening of liver cords, loss of collagen IV structure, Golgi protein-73 (GP73) positivity, and variable positivity for antigen KI-67/MKI67 (ki67) as typically found in HCC (Fig. 1C). Besides the most common trabecular growth pattern some HCC or sub-nodules showed either pseudoglandular pattern, acinar pattern, clear cell changes, and solid type growth patterns (Fig. S1A). HCC development showed a sex disparity, with twice as many tumors in male mice (Fig. S1B). Typical for HCC, most tumors were negative for biliary or stemness markers,[49] but some had morphologic intrahepatic cholangiocellular carcinoma patterns. These stained positive for CK19, CD44, and a few HCC-nodules showed single A6/CD44-positive cells (Fig. S1C). Array-based comparative genomic hybridization (aCGH) of micro-dissected HCC samples showed typical chromosomal aberrations, also found in human HBV-induced HCC such as losses of 4q and 13q, even though mice have their genetic material contained in 20 pairs of chromosomes (Fig. 1D).[50] Analyses of multiple micro-dissected HCC-nodules within one liver by aCGH revealed that these multiple intrahepatic nodules resemble intrahepatic metastasis as chromosomal aberrations showed clonality (Fig. S1D).

Interestingly, only a few cells in HCC nodules of HBV1.3 mice stained positive for alpha-fetoprotein (AFP). Most HCC cells showed a loss of HBcAg expression, whereas up to one quarter still stained positive for HBsAg as shown by IHC (Fig. 1E). Quantitative RT-qPCR of typical oncogenes and tumor-suppressors showed mRNA downregulation of *Axin1, Hnf4α* and *Met* expression as well as upregulation of *Erbb2*, *Tp53* (Tumor Protein P53) and *Cdkn1a* expression (Fig. 1F). Furthermore, HCC in HBV1.3 mice displayed reduced *Egfr1* and *Ctnnb1* gene expression, but upregulation of *Cdh1* and *Cdkn2a* by RT-qPCR (Fig. S1E). We conclude that HBV1.3 mice spontaneously develop HCC, with typical features of human CHB-related HCC.[6]

HBV1.3 mice develop HCC in the absence of inflammation

We questioned whether chronic inflammation as a well-established driver of human CHB-induced HCC may account for HCC development in HBV1.3 mice. We therefore searched for signs of liver damage and innate or adaptive immune responses. We observed normal alanine transaminase (ALT) serum levels and no histological signs of hepatitis in HBV1.3 mice before HCC development. ALT increased in mice with HCC at 20–24 months (Fig. 2A). Also, some animals that did not develop HCC displayed increased ALT associated with other diseases commonly found in older mice (*e.g.* lymphoma).

We next analyzed the HBV integration and gene expression. Sequencing of target locus amplification using two primer sets showed only one 1.3 × overlength HBV genotype D ayw genome (transgene [TG]) integrated at chr5:114480565-114480884 in a non-coding region of HBV1.3 mice (Fig. S2A). Thus, the integration site of the TG is unlikely to cause aberrant cellular processes. Furthermore, no TG–TG fusions were found. Three single nucleotide polymorphisms (TG:331 T→C, TG:3513 T→C; TG:5625 C→T) but no INDELs were detected compared with the reference sequence.

Serum HBsAg levels declined in HBV1.3 mice between 3 and 12 months of age and further on remained stable up to 24 months. However, single animals showed measurable anti-HBs without loss of HBsAg. Except for one HBs/anti-HBs double-positive animal, no anti-HBs seroconversion was detected (Fig. 2B and Fig. S2B). HBeAg expression showed a similar pattern with detectable HBeAg until 24 months of age and no significant increase or decrease in mice with HCC (Fig. 2B). No anti-HBe antibodies were detected (Fig. S2B). HBsAg staining of

above and below min. to max., *t* test, two-tailed with Welch's correction: **p* <0.05, ***p* <0.01. aCGH, array-based comparative genomic hybridization; AFP, alpha-fetoprotein; GP73, Golgi protein-73; HBcAg, hepatitis B core antigen; HBsAg, hepatits B surface antigen; HCC, hepatocellular carcinoma; H&E, hematoxylin and eosin; IHC, immunohistochemistry; ki67, antigen KI-67/MKI67; RT-qPCR, quantitative reverse transcription polymerase chain reaction; WT, wild-type.

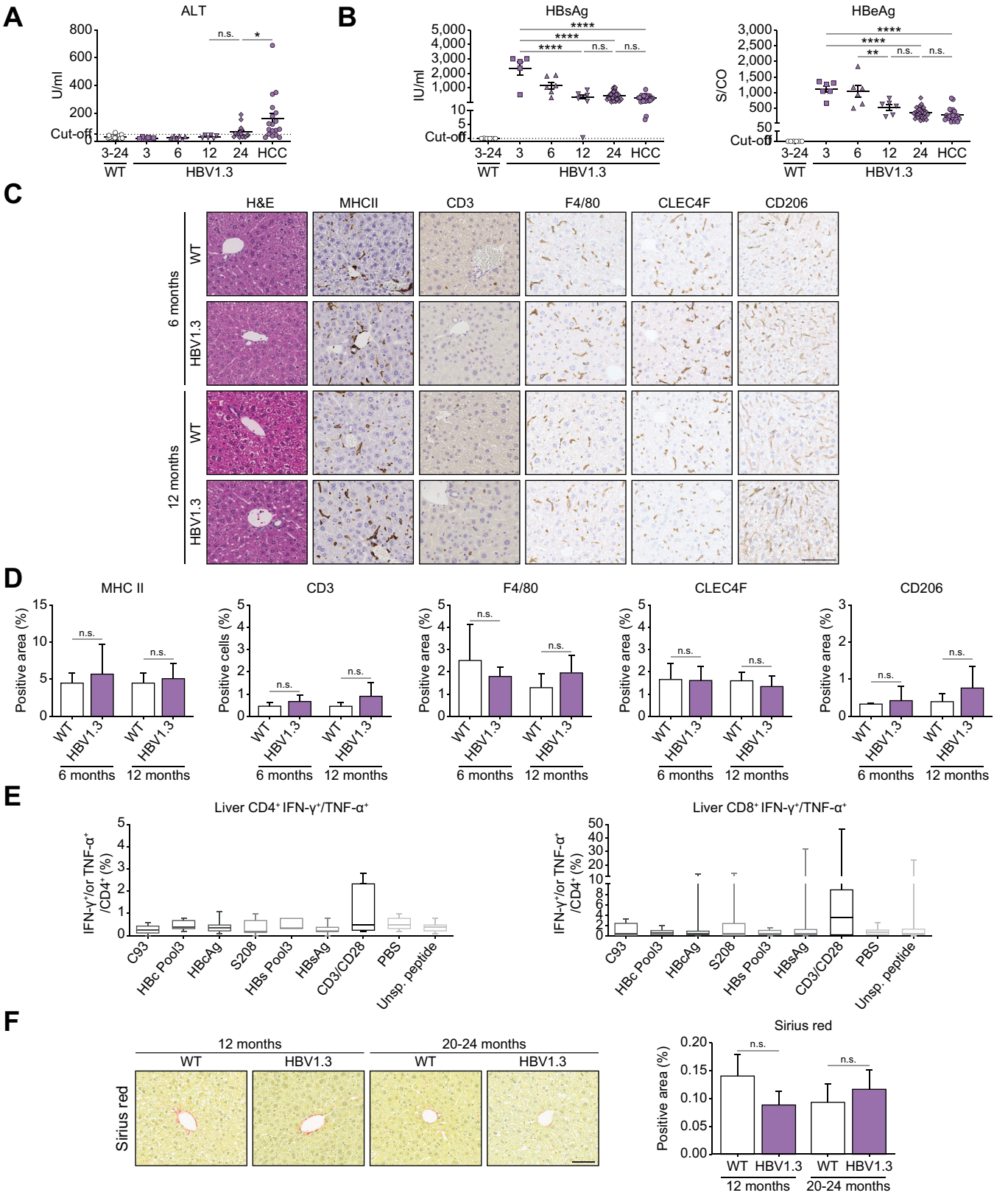

Fig. 2. HBV1.3 mice develop HCC in the absence of inflammation. (A) Serum ALT levels and (B) HBsAg and HBeAg levels in serum of HBV1.3 and WT mice at the indicated months of age and in HBV1.3 mice with HCC (ns = not significant, **p* <0.05, ***p* <0.01, *****p* <0.0001 in ordinary one-way ANOVA). (C) Representative H&E and IHC staining for MHCII, CD3, F4/80, CLEC4F, CD206 at indicated time points (scale bar: 100 μm) and (D) quantification of positive cells/positive area at 6 and 12 months of age (data represent mean with SD; WT n = 3–5; HBV1.3 n = 4–6 for each time point, ordinary one-way ANOVA). (E) Flow cytometry analysis of CD4+ and CD8+ T cells isolated from livers of 24-month-old HBV1.3 mice after *ex vivo* stimulation with indicated antigens/controls and intracellular staining for IFN-γ and TNF-α.

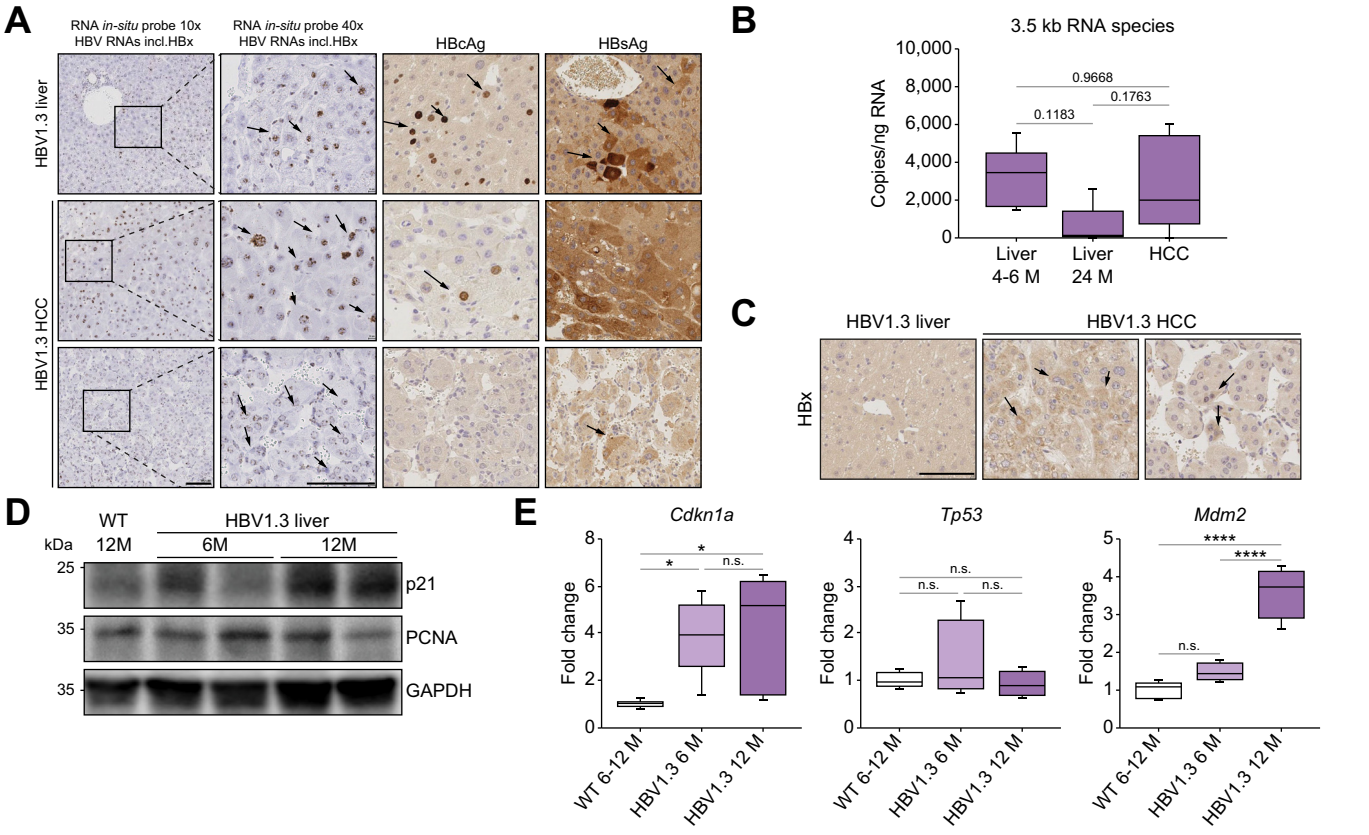

Fig. 3. Expression of 3.5-kb RNA species and HBx in HCC. (A) Representative pictures of RNA *in situ* hybridization on FFPE slides using a labeling probe covering all HBV transcripts including HBx and IHC for HBcAg and HBsAg on consecutive FFPE cuts of HBV1.3 liver and HCC tissue (scale bar: 100 μm). (B) Amount of HBV 3.5-kb RNA species by ddPCR using HBV primers for 3.5-kb RNA in liver and HCC tissue at indicated time points (*p* values indicated, ordinary one-way ANOVA, n = 5 per group). (C) IHC staining for HBx protein in liver and HCC tissue of HBV1.3 mice (40 × magnification). (D) Western blot analysis for p21, PCNA, and GAPDH as loading control in liver tissue of WT and HBV1.3 mice at 6 and 12 months of age. (E) RT-qPCR for expression of *Cdkn1a*, *Tp53*, and *Mdm2* in liver tissue of control (WT; n = 3) and HBV1.3 (n = 4) mice at 6 and 12 months of age. Box plots (indicating interquartile range and median) show relative expression. The whiskers extend above and below minimum to maximum, ns = not significant, **p* <0.05, *****p* <0.0001, ordinary one-way ANOVA. Cnkn1a, Cyclin Dependent Kinase Inhibitor 1A; ddPCR, droplet digital polymerase chain reaction; FFPE, formalin-fixed, paraffin-embedded; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HBcAg, hepatitis B core antigen; HBsAg, hepatits B surface antigen; HCC, hepatocellular carcinoma; IHC, immunohistochemistry; p21, CDK-inhibitor 1; Mdm2, mouse double minute 2 homolog; PCNA, proliferating cell nuclear antigen; RT-qPCR, quantitative reverse transcription polymerase chain reaction; Tp53, Tumor Protein P53; WT, wild-type.

liver tissue showed diffuse/focal cytoplasmic and membranous staining and HBcAg predominantly nuclear staining of hepatocytes with predominant positivity near central veins (Fig. S2C) as reported for human samples.[48] Importantly, HBsAg and HBeAg levels were comparable between 24-month-old HBV1.3 mice that did and did not develop HCC (Fig. 2B and Fig. S2B).

Mirroring ALT levels, no histological signs of hepatitis were found in HBV1.3 mice. IHC staining for major histocompatibility complex class II (MHCII), cluster of differentiation 3 (CD3), as well as for EGF-like module-containing mucin-like hormone receptor-like 1 (F4/80), C-type lectin domain family 4 member F (CLEC4F), and cluster of differentiation 206 (CD206) did not show differences between WT and HBV1.3 mice at 6 and 12 months (Fig. 2C and D).

To detect HBV-specific T cells, cells isolated from the spleen and liver of 20- to 24-month-old HBV1.3 animals were stimulated by overlapping peptide pools covering the complete core- and S-protein. Analysis of liver-associated lymphocytes by fluorescence-activated cell sorting for TNF-α- or IFN-γ-positive CD4+ or CD8+ T cells detected no T cell population that could react with HBV antigens in HBV1.3 mice (Fig. 2E). The same pattern was found for lymphocytes isolated from spleen (Fig. S2D and E). In line with the absence of liver inflammation, HBV1.3 mice did not develop liver fibrosis as confirmed by Sirius Red staining (Fig. 2F). Thus, these data indicate that HBV-induced HCC development occurred in HBV1.3 mice carrying a single 1.3 × TG in the absence of inflammation or fibrosis and any detectable adaptive or innate immune response.

Box plots (indicating interquartile range and median) represent pooled data for TNF-α and/or INF-γ positive cells. The whiskers extend above and below min. to max. (for single stains and quantification see Fig. S2). (F) Sirius Red staining of livers from WT and HBV1.3 mice at indicated time points and quantification of positive area (data represent mean with SD; n = 4–5, ordinary one-way ANOVA; ns: not significant. ALT, alanine aminotransferase; CD206, cluster of differentiation 206; CD3, cluster of differentiation 3; CD4, cluster of differentiation 4; CD8, cluster of differentiation 8; CLEC4F, C-type lectin domain family 4 member F; F4/80, EGF-like module-containing mucin-like hormone receptor-like 1; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e-antigen; HBsAg, hepatits B surface antigen; HCC, hepatocellular carcinoma; H&E, hematoxylin and eosin; IFN-γ Interferon gamma; IHC, immunohistochemistry; MHCII, major histocompatibility complex class II; SD, standard deviation; TNF-α, tumor necrosis factor alpha; WT, wild-type.

Expression of 3.5-kb RNA species and HBx in HCC

The non-structural HBx protein was previously implicated in HBV-related HCC development and progression by direct/epigenetic interference with tumor suppressors.[8,37,38] As direct detection and quantification of HBx protein may be difficult,[51] we first used RNA *in situ* hybridization (ISH) to visualize HBV RNA expression. As all HBV RNA transcripts overlap, gene-specific ISH is not possible, and staining was performed using a probe targeting the common co-terminus of all HBV RNAs including HBx. Using consecutive histological sections for IHC and ISH staining, we observed that before HCC development, ISH staining of HBV RNAs corresponded to HBs and HBc expression (Fig. 3A). In contrast, in HCC tissue, we detected a marked expression of HBV RNAs in areas without HBsAg/HBcAg protein expression (Fig. 3A). These data indicate that the expression profile from the integrated HBV genome is altered in HCC, potentially as well favoring HBx gene expression.

Given the positivity for HBeAg in serum throughout the lifespan of HBV1.3 mice (Fig. 2B) we tested for the presence of 3.5-kb RNA species (including precore RNA for HBeAg expression and pregenomic RNA for genome replication) by droplet digital PCR (ddPCR). Expression of 3.5-kb RNA species showed the same tendency to decrease from younger mice to 24-month-old animals (Fig. 3B) and to increase again in HCC tissues as compared with non-tumor tissue in age-matched animals (Fig. 3B). In line with the idea of altered expression of HBV transcripts in HCC of HBV1.3 mice, IHC analysis for HBx indicated that in normal liver tissue HBx protein was hardly detectable but stained positive in HCC areas (Fig. 3C and S3A). These HBx-positive areas were also positive in HBV RNA ISH but HBsAg/HBcAg-negative in IHC.

HBx has been reported to directly inhibit several forms of DNA repair.[52] HBx also blocks transcriptional activity and protein–protein interactions of p53, disrupting apoptotic pathways[53,54] and has been described to contribute to oncogenic transformation by upregulating *Cdkn1a* (p21) (Cyclin Dependent Kinase Inhibitor 1A).[55,56] We detected p21 protein in liver lysates of HBV1.3 animals before HCC development, whereas the proliferation marker, proliferating cell nuclear antigen (PCNA), was unchanged (Fig. 3D). Indeed, at 6 and 12 months HBV1.3 animals expressed significantly more p21 mRNA (Fig. 3E). The mRNA expression level of p53 was not affected. In contrast, mouse double minute 2 homolog (MDM2) was significantly overexpressed in HBV1.3 mice at 12 months of age (Fig. 3E). MDM2 is involved in long-term survival of hepatocytes and inhibition of p53 transcriptional activity, potentially playing a role in genetic instability and cellular stress. Thus, we next analyzed HCCs found in HBV1.3 mice by whole-exome sequencing (WES) to rule out a high, uniform number of somatic single nucleotide variants as reported in the chemical-genotoxic diethylnitrosamine (DEN) mouse model.[57] Copy number variation profiles recapitulated findings from aCGH (*e.g.* loss of chromosome 4), and WES showed a broad spectrum of mutations with somatic mutations in genes including *Keap1* and *Arid2* (Fig. S3B–E), which were previously reported in HCC.[6] However, no recurrence of specific somatic mutations or alterations associated with the presence of the transgene was detected (Fig. S3, Tables S1 and S2). Taken together, these data suggest that HBV transcripts, other than HBs and HBc protein, are predominantly found in HCC, and that HBx could be involved in HCC development in HBV1.3 mice with its proposed pro-tumorigenic functions.

Functional knockout of HBx in HBVxfs mice reduces signs of DNA damage response

We next asked whether precluding DDB1- and/or p53-binding by HBx would prevent HCC development. Therefore, we used a second mouse model, the X frame-shift mouse (HBVxfs), that also replicates HBV from a 1.3 ayw, genotype D overlength genome, but with a frame-shift mutation at amino acid 87 in both copies of the HBx open reading frame. This HBVxfs mouse line expresses all viral transcripts, but the frame-shifts result in the expression of a truncated HBx protein that lacks the well-defined DDB1- and p53-binding sites (XFS) (Fig. S4A). The TG is integrated in Chr10: 6523201-6523204 in HBVxfs mice (Fig. S4B). Loss of HBx does not prevent HBV replication from integrated DNA, and its functional knockout should not affect viral protein expression (Fig. 4A and B). The higher expression of some viral proteins in HBVxfs as compared with HBV1.3 mice is most likely an effect of the different integration site. HBVxfs mice also showed no significant signs of hepatitis using H&E staining (Fig. 4A) or aberrant ALT serum values up to the age of 12 months (Fig. S4C).

By inducing Structural Maintenance of Chromosomes 5/6 (SMC5/6) degradation, HBx drives expression from HBV cccDNA.[11] The XFS protein lacks the domain responsible for interaction with DDB1 and consequently does not induce SMC6 degradation when expressed *in vitro* (Fig. 4C). Consequently, XFS is not able to stimulate the activity of luciferase reporter constructs containing either an upstream HBx promoter sequence (pX-Luciferase) or an upstream HBc promoter sequence (pCore-Luciferase) (Fig. 4D) and does not rescue transcription of HBx-deficient HBV in PHHs (Fig. S4D) or HepRG cells (Fig. S4E).

We observed various markers of DNA damage response in HBV1.3 mice previously described to be related to HBx.[38,58,59] HBV1.3 mice showed increased phosphorylation of the H2A histone family member X (γH2AX) in hepatocytes as a sign of accumulating DNA damage response at 12 months compared with the control (Fig. 4E). Nevertheless, HBVxfs mice also showed positive γH2AX hepatocytes but reduced compared with HBV1.3 mice (Fig. 4E). Western blot analyses of liver lysates also showed abundance of γH2AX and phosphorylated protein kinase ataxia telangiectasia mutated (ATM) in HBV1.3 and HBVxfs mice at 12 months of age (Fig. S4F). ATM is a kinase which is upregulated and auto-phoshorylated in response to double-stranded DNA breaks, leading to a cascade of kinase reactions that regulate cell cycle, apoptosis and DNA damage repair, including H2AX phosphorylation. In line, HBV1.3 mice showed a small but significant increase in cleaved Caspase 3 positive hepatocytes as a sign of cell death by apoptosis (Fig. 4E). In contrast, HBVxfs mice showed no increase of cleaved Caspase 3 staining over control mice, and no significant difference for ki67 was observed (Fig. 4E). Furthermore, HBV1.3 and HBVxfs mice overexpressed *Cdkn1a* and *Mdm2* at 12 months. However, only HBV1.3 mice showed a significant increase in *Atm* expression by RT-qPCR compared with WT and HBVxfs mice (Fig. 4F). Taken together, HBV1.3 mice accumulated DNA damage response over time,

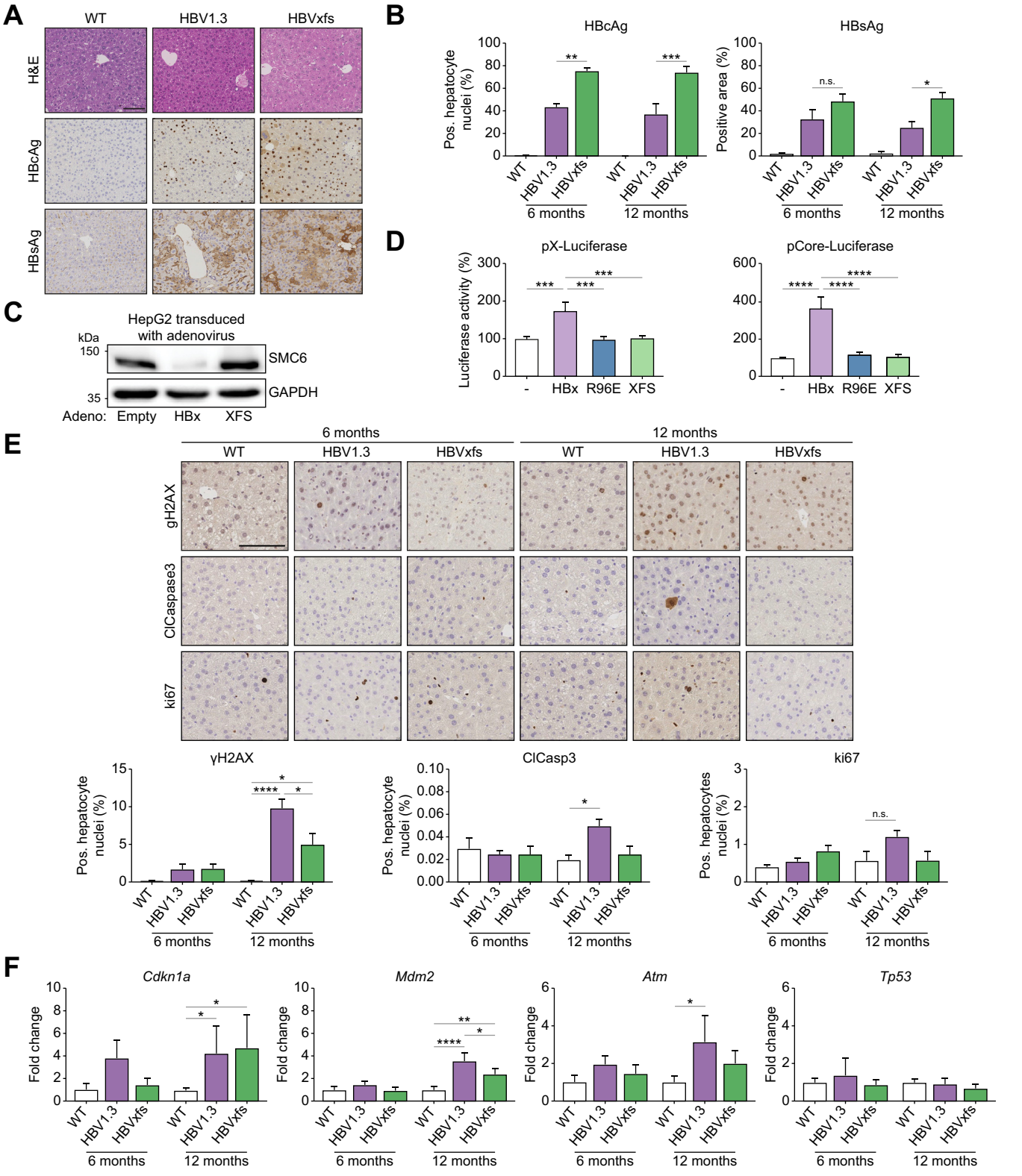

Fig. 4. Functional knockout of HBx in HBVxfs mice reduces signs of DNA damage. (A) Representative H&E and IHC staining for HBcAg and HBsAg at indicated time points in WT, HBV1.3, and HBVxfs livers (scale bar: 100 μm). (B) Quantification of HBcAg-positive hepatocytes and HBsAg-positive area (data represent mean with SD; n = 5–6; ns = not significant, **p* <0.05, ***p* <0.01, ****p* <0.001, ordinary one-way ANOVA). (C) Western blot analysis of SMC6 in cell lysates from HepG2 cells 48 h after transduction with empty, HBx, or XFS expressing adenovirus. (D) Relative activity of HBV X and HBV Core promoter driven luciferase reporter constructs after co-transfection with empty vector or constructs expressing HBx, HBx.R96E, or HBx-XFS as indicated (data represent mean with SD; ****p* <0.001, *****p* <0.0001, ordinary one-way ANOVA). (E) Representative IHC staining of livers from WT, HBV1.3, and HBVxfs mice for γH2AX, ClCaspase3, and ki67 at indicated time points (scale bar: 100 μm) and quantification of % positive hepatocytes at 6 and 12 months of age (data represent mean with SD; n = 4–5, ordinary one-way ANOVA). (F) RT-qPCR for *Cdkn1a*, *Mdm2, Atm*, and *Tp53* in liver tissue of 6- and 12-month-old control, HBV1.3, and HBVxfs mice (data represent mean with SD of relative gene expression; WT

which was reduced but not abolished in HBV transgenic mice lacking functional HBx expression.

HCC incidence is significantly higher in HBV1.3 mice expressing WT HBx

Both HBV1.3 (see Fig. 1) and HBVxfs mice developed HCC at the age of 20–24 months, with a significantly higher incidence in HBV1.3 mice (relative risk 3.750, 95% CI 2.067–6.803) (Fig. 5A and Fig. S5A). In addition, the average tumor nodule size and the number of intrahepatic HCC nodules were higher in HBV1.3 mice (Fig. 5B). Higher HCC incidence in HBV1.3 occurred despite similar HBsAg and HBeAg abundance in the serum of HBVxfs mice (Fig. 5C) compared with HBV1.3 (Fig. 2B). HBVxfs also showed abundance of HBV 3.5-kb RNA species in liver and HCC tissue (Fig. 5C), as transcription of those derive from the 1.3 × overlength TG.

Histological analysis of HCC in both mouse lines showed typical HCC patterns (Fig. 5D) and analysis of HCC tissue clustered typically for HCC in an mRNA-based 16-gene array (Fig. S5B).[60] However, no significant difference was found in chromosomal aberrations between HCC of the two mouse models using aCGH, as displayed by frequency blots (Fig. 5E) and principal component analysis (Fig. S5C).

HBVxfs HCC presented frequently with a histologically well-differentiated pattern (Fig. 5D and Fig. S5A), and had fewer infiltrating CD3-positive cells, as well as a significantly increased amount of AFP, glutamine synthetase, and HBsAg-positive hepatocytes (Fig. S5D and E). Less differentiated HCC of HBV1.3 mice could explain the latter, which is also reflected by the RNA expression pattern, including differentiation factors, oncogenes and tumor-suppressors analyzed by RT-qPCR (Fig. 5F). HCC of HBVxfs mice showed fewer intrahepatic metastasis but more frequent lung metastasis (Fig. S5F). These data indicate that WT HBx is a major driver of HCC development in HBV1.3 mice, but also that other HBx/HBV functions might be involved in HCC development and metastasis in this model.

HCC in HBV1.3 critically depends on STAT3 activation

We screened for altered pathways that could be involved in HCC development in our model. HBV infection has been shown to directly activate STAT3, which may support HBV replication by preventing apoptosis of infected hepatocytes *in vitro*.[61] In HCC development the significance of STAT3 activation has been convincingly demonstrated,[27,62] and STAT3 phosphorylation is increased in up to 60% of human HCC cases correlating with high tumor aggressiveness.[27,63]

As one potential pathway in HCC development driven by HBV, we identified single clusters of phosphorylated STAT3 (pSTAT3)-positive hepatocyte nuclei in livers of HBV1.3 mice before HCC development (Fig. 6A). Upon HCC development, tumor tissue of HBV1.3 mice showed a significant increase in pSTAT3-positive hepatocyte nuclei in IHC when compared with HBV1.3 non-tumor tissue (Fig. 6A and B). Searching for upstream regulators that could be responsible for the observed activation of STAT3, we found that the cytokine receptor subunit glycoprotein 130 (gp130) was significantly upregulated on mRNA level at 6 months of age in HBV1.3 compared with control and with HBVxfs mice. HBVxfs also showed increased expression but significantly less than HBV1.3 (Fig. 6C). Gp130 is known to be phosphorylated after complexing with other proteins and to activate STAT3 which indicates that gp130-mediated STAT3 activation could be involved in HCC development in the HBV1.3 and HBVxfs model. Using a multiplex cytokine assay, we screened for potential factors that could activate STAT3 signaling in liver tissue lysates. From the analyzed factors (*i.e.* IL-6, IL-12p70, IL-21, IL-27p28, IL-31, and oncostatin M), only IL-6 showed a significant increase in expression on protein level at 12 months of age in HBV1.3 mice compared with controls (Fig. S6A).

To demonstrate the critical involvement of STAT3 signaling in HBV-driven HCC at a functional level, we crossed HBV1.3 mice with mice carrying hepatocyte-specific conditional knockout of STAT3 (HBV1.3-*AlbCre*STAT3$^{f/f}$).[62,64] As HBV was reported to favor epigenetic silencing of Suppressor of cytokine signaling 3 (*SOCS3*) mRNA, which is a negative regulator of JAK/STAT signaling, leading to the sustained activation of IL-6/STAT3 pathway,[65] we investigated whether over-activation of STAT3 signaling would increase HCC incidence. Therefore, we crossed HBV1.3 mice with mice with hepatocyte-specific conditional knockout of SOCS3 (HBV1.3-*AlbCre*SOCS3$^{f/f}$).[45] Albumin-Cre-negative animals (HBV1.3-STAT3$^{f/f}$ or HBV1.3-SOCS3$^{f/f}$) served as HBV1.3 littermate controls (Fig. 6D). HBV1.3-*AlbCre*STAT3$^{f/f}$ mice showed abrogation of pSTAT3-positive hepatocyte nuclei, whereas HBV1.3-*AlbCre*SOCS3$^{f/f}$ mice harbored big foci of hepatocytes with nuclear pSTAT3 (Fig. 6E). Although the number of infiltrating CD3-positive cells and MHCII-positive area were not significantly changed (Fig. S6B and C), HBV1.3-*AlbCre*STAT3$^{f/f}$ mice showed reduced ki67-positive hepatocytes compared with HBV1.3 and HBV1.3-*AlbCre*SOCS3$^{f/f}$ animals, but no change in the frequency of cleaved Caspase3-positive hepatocytes (Fig. S6B and C). Nevertheless, despite extensive screening of IL-6/gp130/STAT3-related signatures/pathways in liver tissue by cytokine arrays and proteomics analysis before HCC development, the source of STAT3 activation in this mouse model could not be identified in 12-month-old animals (Fig. S6D).

Increased STAT3 activity in HBV1.3-*AlbCre*SOCS3$^{f/f}$ animals did not change HCC incidence as compared with HBV1.3 mice (Alb-Cre-negative HBV1.3-STAT3$^{f/f}$ or HBV1.3-SOCS3$^{f/f}$ animals at 20–24 months of age) (Fig. 6F).

In sharp contrast, the hepatocyte-specific knockout of STAT3 in HBV1.3-*AlbCre*STAT3$^{f/f}$ mice abrogated HCC development (Fig. 6F). Altogether, these data indicate a STAT3-dependent mechanism in directly HBV-driven HCC in our model.

n = 3–6; HBV1.3 n = 4–5, HBVxfs n = 4–5 each time point; **p* <0,05, ***p* <0,01, *****p* <0,0001, ordinary one-way ANOVA). γH2AX, phosphorylation of the H2A histone family member X; Atm, protein kinase ataxia telangiectasia mutated; ClCaspase3, Cleaved Caspase-3; H&E, hematoxylin and eosin; HBcAg, hepatitis B core antigen; HBsAg, hepatits B surface antigen; IHC, immunohistochemistry; ki67, antigen KI-67/MKI67; p21, CDK-inhibitor 1; Mdm2, mouse double minute 2 homolog; RT-qPCR, quantitative reverse transcription polymerase chain reaction; SMC6, Structural Maintenance of Chromosomes 6; Tp53, Tumor Protein P53; WT, wild-type; XFS, X frame-shift.

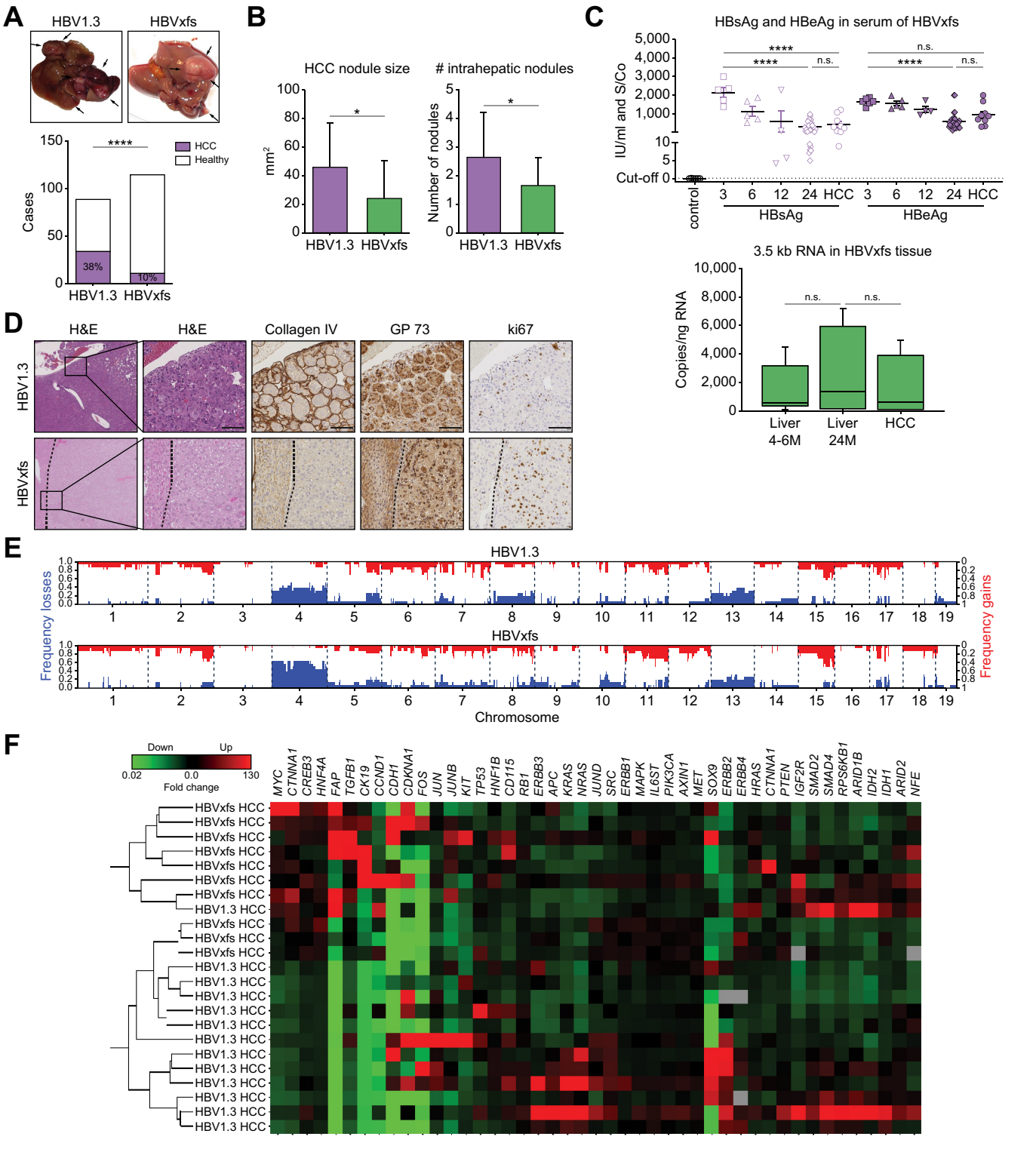

Fig. 5. HBV1.3 mice expressing WT HBx develop significantly more HCC compared with HBVxfs. (A) Macroscopic pictures of HBV1.3 and HBVxfs livers with tumor nodules (arrows indicate tumors) and bar graph indicating number of HCCs in total number of HBV1.3 (same group of mice from Fig. 1) and HBVxfs mice analyzed (unaffected livers in white and livers with tumors in purple, Fisher's exact test, ****p <0.0001). (B) Quantification of maximum tumor nodule size measured in H&E-stained FFPE sections and total number of tumor nodules found in one liver (data represent mean with SD, *p <0.05, t test). (C) HBsAg and HBeAg levels in serum of WT, HBVxfs mice and HBVxfs mice with HCC at the indicated time points (top graph). Below, quantification of HBV 3.5-kb RNA species analyzed by ddPCR in liver and HCC tissue of HBVxfs mice at indicated time points (ns = not significant, ****p <0.0001, ordinary one-way ANOVA). (D) H&E and IHC staining of Collagen IV, GP73, and ki67 (scale bar, 100 μm) of HCC from HBV1.3 and HBVxfs mice. (E) aCGH of micro-dissected tumor samples displaying chromosomal aberrations (gains red and losses blue) in HBV1.3 and HBVxfs mice. Each row resembles a tumor sample, frequency of chromosomal aberrations is visualized. (F) Heatmap visualizing expression pattern of indicated genes in tumor tissue of HBV1.3 and HBVxfs animals analyzed in triplicate by RT-qPCR. Color code indicates downregulation (green) and upregulation (red) (0.02–130 in fold change, normalized to WT liver tissue). aCGH, array-based comparative genomic hybridization; ddPCR, droplet digital polymerase

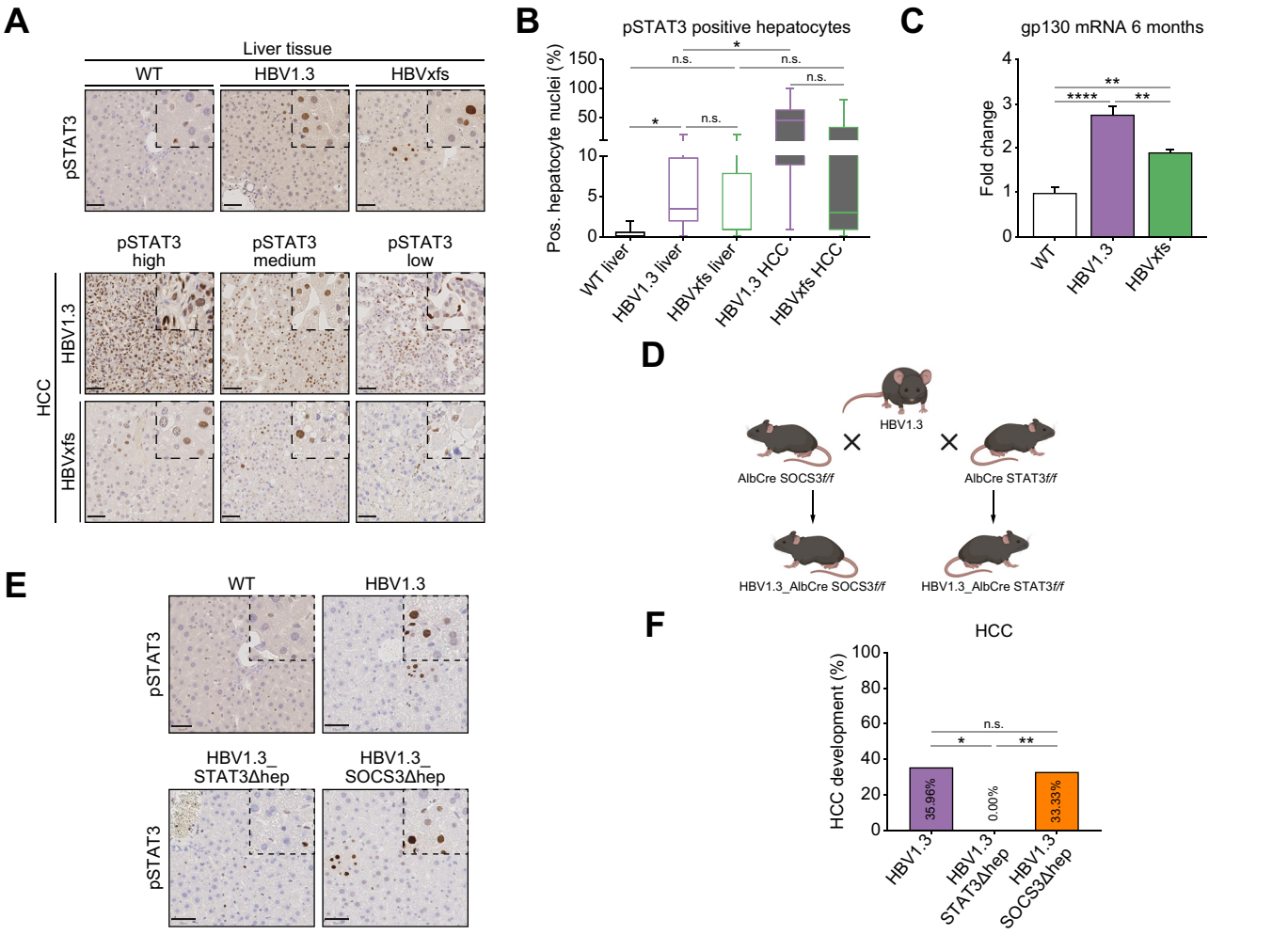

Fig. 6. HCC in HBV1.3 critically depends on STAT3 activation. (A) Representative IHC staining for pSTAT3 in WT, HBV1.3 and HBVxfs liver tissue at 24 months of age and three examples for low, medium and high pSTAT3-positive HCCs in HBV1.3 and HBVxfs mice (scale bar, 50 μm). (B) Quantification of pSTAT3-positive hepatocyte nuclei shown as box plots indicating interquartile range and median. The whiskers extend above and below minimum to maximum (ns = not significant, *p <0.05 in Brown-Forsythe and Welsh ANOVA; n = 6–14). (C) RT-qPCR for *gp130* in liver tissue of 6-month-old WT (n = 3), HBV1.3 (n = 5), and HBVxfs (n = 5) mice (data represent mean with SEM; **p <0.005, ****p <0.0001, ordinary one-way ANOVA). (D) Breeding scheme for hepatocyte-specific knockout of STAT3 (HBV1.3_*AlbCre*STAT3$^{f/f}$ = HBV1.3_STAT3Δhep) and SOCS3 (HBV1.3_*AlbCre*SOCS3$^{f/f}$ = HBV1.3_SOCSΔhep) in HBV1.3 mice (created with BioRender.com). (E) Representative IHC staining for pSTAT3 in liver tissue of 12 months old WT, HBV1.3, HBV1.3_STAT3Δhep and HBV1.3_SOCS3Δhep mice. (scale bar, 50 μm) (F) Incidence of HCC development in HBV1.3, HBV1.3_STAT3Δhep and HBV1.3_SOCS3Δhep mice. Compared were HBV1.3 (n=29: including HBV.1.3_STAT3$^{f/f}$ and HBV1.3_SOCS3$^{f/f}$) *vs.* HBV1.3_STAT3Δhep (n=21) mice using Fisher's exact test (*p <0.05) and HBV1.3_STAT3Δhep (n = 21) *vs*. HBV1.3_SOCS3Δhep (n = 32) mice using Fisher's exact test (**p <0.005). gp130, Glycoprotein 130; HCC, hepatocellular carcinoma; IHC, immunohistochemistry; pSTAT3, phosphorylated Signal transducer and activator of transcription 3; RT-qPCR, quantitative reverse transcription polymerase chain reaction; SOCS3, Suppressor of cytokine signaling 3; WT, wild-type.

Discussion

Here, we show that the expression of HBV transcripts and proteins from an integrated TG in HBV1.3 mice is sufficient to drive HCC development in 35 out of 91 mice analyzed at 20–24 months of age. Previously, mouse models expressing mutated HBs or overexpressing HBs or HBx were generated to assess the effect of HBV proteins on HCC development, which partially required additional challenge by an oncogenic chemical toxin.[31–33,35,36] Unlike these models, HBV1.3 mice in this study express all viral transcripts at physiological levels and replicate HBV from a single 1.3 × overlength, HBV genotype D (ayw) transgene. Thorough characterization of liver tumors in HBV1.3 mice showed typical histological patterns and genetic aberrations, including a wide variety of HCC subtypes resembling the multitude of patterns of human HCC.[30,50] Of note, well-established changes in expression levels of oncogenes and

chain reaction; FFPE, formalin-fixed, paraffin-embedded; GP73, Golgi protein-73; H&E, hematoxylin and eosin; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e-antigen; HBsAg, hepatits B surface antigen; HCC, hepatocellular carcinoma; IHC, immunohistochemistry; ki67, antigen KI-67/MKI67; RT-qPCR, quantitative reverse transcription polymerase chain reaction; SD, standard deviation; WT, wild-type.

tumor-suppressors (*e.g. AXIN1*, *MYC*, *MET*, *CDKN2a*, and *CDKN1a*)[6,30,60] were detected in HBV1.3 mice. HCC in HBV1.3 mice show no uniform patterns or overexpression of stemness markers. In contrast to other studies, we show that expression of physiological levels of HBV transcripts from the original promoters is sufficient to cause liver tumors resembling human HCC.

A typical main oncogenic driver for HCC is necro-inflammation, a sequence of chronic inflammation, cell death and compensatory hepatocyte proliferation, and includes the development of liver cirrhosis.[24,25] We found no significant signs of active hepatitis or significant HBV-specific immunity, including innate immune responses in HBV1.3 mice up to the age of 24 months, which is in line with previous reports.[40] In different HBs-transgenic mice, incompetent anti-HBV CD8+ and CD4+ T cells were described to majorly contribute to immune tolerance in HBs-transgenic mice,[66] possibly also explaining the lack of significant HBV-specific immune reactions in HBV1.3 mice. However, a therapeutic vaccine approach in HBV1.3 animals was able to induce HBV-specific antibodies and CD8+ T cell immunity.[67]

Notably, in CHB the risk for HCC is known to be already present in patients without cirrhosis, illustrating the direct oncogenic effect of HBV.[4,15,16,29] Mutagenesis or genetic instability by integration of HBV-DNA in host hepatocyte genomes is a well-accepted oncogenic factor of HBV.[8,29,68] However, by sequencing of target locus amplifications we found only a single HBV TG in a non-coding region in the HBV1.3 strain and no evidence of HBV DNA integration in mice. Missing HBV DNA ('re')-integration in host DNA of murine hepatocytes can be explained by species specificity of HBV (no human NTCP)[69] and secondly by a second restriction very likely at the step of capsids entry into the nucleus or rcDNA repair and cccDNA formation, or as proposed previously, a different host cell dependency factor.[41] In conclusion, necro-inflammation and insertional mutagenesis can be excluded as oncogenic drivers in HBV1.3 mice.

The expression of HBeAg in HBV1.3 and HBVxfs up to the age of 24 months strengthens the notion of a HBeAg-positive HBV infection model. The abundance of 3.5-kb RNA species, which comprise precore RNA for HBeAg expression and the pregenomic (pg)RNA for genome replication in liver and HCC of HBVxfs and HBV1.3 is a possibly important finding. In HCC of HBsAg-positive patients treated with active antiviral drugs expression of episomal forms of HBV DNA in 40% and of pgRNA in 66% of cases was reported.[70] The presence of 3.5-kb RNA in HBxfs animals can be explained by expression from the linear 1.3 × HBV TG independent of cccDNA and thus independent of WT HBx. An oncogenic potential of HBV pgRNA was previously reported.[71] Furthermore, we found a tendency towards higher 3.5-kb RNA species expression in HCC of HBV1.3 mice as compared with liver tissue of 24-month-old HBV1.3 mice.

Accumulated evidence has shown that the non-structural HBx protein can interfere with or alter the expression of tumor suppressor genes and oncogenes, as well as cause epigenetic aberrations, and that plays a role in HCC development and progression.[8,37,38] We found only scarce signals of HBx protein in HBV1.3 mice. However, our observation of HBV RNA expression in areas without HBs/HBc protein as well as finding HBx protein in HCC is indicative of altered expressions from the HBV TG in HCC, favoring HBx gene expression, and is in line with previous reports.[34,38] Thus, we postulated an effect of HBx in HCC development in HBV1.3 mice. Maintenance of genomic integrity through the recognition and repair of damaged or altered DNA is essential for cellular integrity. It has been reported that HBx inhibits several forms of DNA repair and blocks transcriptional activity and protein–protein interactions of p53 disrupting apoptotic pathways.[53,54] In HBV1.3 mice, we found overexpression of *Cdkn1a* and *Cdkn2a* and chromosomal aberrations in HCC and increased *Cdkn1a* (p21) and *Mdm2* expression before HCC development. Upregulation of p21 by HBx has previously been reported,[55,56] and MDM2 is involved in the long-term survival of hepatocytes resulting in genetic instability, cellular stress and abnormalities in antitumor genes in HCC development.[72] Interestingly, WES of HBV1.3 HCC showed a diverse spectrum of mutations but no recurrence of specific somatic mutations and no high numbers of single nucleotide variants, as usually seen in for example the genotoxic DEN-mouse model.[57] Nevertheless, stochastic mutation events leading to gene abnormalities are likely to be involved as HCC promoters in this model.

Firstly, DDB1-dependent degradation of SMC5/6 by HBx is a crucial host restriction factor of HBV gene expression from cccDNA and maintains virus replication.[11] Secondly, the SMC5/6 complex has an essential role in DNA replication through natural pausing sites and in endogenous DNA damage tolerance.[73] Thus, we used a second mouse model, transgenic for the same HBV1.3-overlength genome (HBVxfs mice). They also express all HBV gene products from a TG integrated at a different site and produce HBV particles but express a truncated HBx lacking the previously described DDB1 and p53 binding sites.

HBVxfs mice showed decreased signs of accumulating DNA damage response compared with HBV1.3 mice expressing WT HBx. Besides changes in DNA damage response caused by c-terminal truncated HBx, HBV1.3 mice developed significantly more HCC as compared with HBVxfs mice (38% *vs.* 10% HCC incidence, relative risk 3,750, 95% CI 2.067–6.803). In contrast, the generally well-differentiated HCC in HBVxfs mice fostered a higher frequency of pulmonary metastases. This could for example be explained by an epithelial–mesenchymal transition independent mechanism such as vessels that encapsulated tumor clusters that predicted higher metastasis and recurrence rates in humans.[74] In our model a distinct pattern with higher expression of differentiation markers but a lower number of infiltrating T cells was present. Because of usage of co-housed C57Bl/6 mice from backcrossings as controls and based on the different origin of HBV1.3 and HBVxfs mice, minor differences in the genetic background and an effect on the observed tumor incidence cannot be excluded. However, the thorough backcrossings of the lines to the C57Bl/6 background and co-housing in the same animal facility should minimize confounding factors. Moreover, observed molecular patterns in HBV1.3 mice that are known to be driven by HBx support the notion of HBx being one oncogenic factor in this model. We concluded that the c-terminal action of HBx has potent oncogenic potential by increasing HCC incidence and leading to less differentiated HCC subtypes, however, metastatic disease seems to rely on different factors. Nonetheless, our data

suggest additional oncogenic factors in these models, independent of mechanisms related to functions of C-terminal HBx as HBVxfs animals still developed HCC.

Thus, we screened for a common factor altered in HBV transgenic models. We found clusters of pSTAT3-positive hepatocytes in young HBV1.3 mice without active signs of hepatitis and a significant increase of nuclear pSTAT3 in HCC. The critical link between continuous hepatocyte death and compensatory liver regeneration with STAT3 signaling as intrinsic signaling pathways driving or mediating these processes in HCC development has been identified previously[27,62] and STAT3 is frequently found phosphorylated in human HCC.[27,63] Nevertheless, the functional consequence of STAT3 activation is highly context- and tissue-dependent.[75] Previously, HBV infection was shown to activate STAT3 signaling by induction of Y705 phosphorylation, supporting virus replication and preventing apoptosis,[61,76] as well as favoring epigenetic silencing of *SOCS3* mRNA which leads to the sustained activation of the IL-6/STAT3 pathway.[65] Nevertheless, in our model a protein screen performed in 12-month-old animals did not detect a significant candidate (Fig. S6D), and we did not find the source of STAT3 activation in HBV1.3 mice.

Given the STAT3 activation in hepatocytes of HBV1.3 mice and the transient upregulation of gp130, and as STAT3 is known to be essential for efficient repair of damaged DNA,[77] we asked whether over-activation of STAT3 signaling by hepatocyte-specific conditional knockout of SOCS3 would increase HCC incidence. However, HCC incidence in HBV1.3-*AlbCre*SOCS3$^{f/f}$ animals was unchanged to HBV1.3 mice. This could be partially explained as IL-6 signaling is selectively inhibited by SOCS3 binding to gp130. However, SOCS3 does not interfere with, for example, IL-10R-mediated STAT3 activation.[75]

In contrast, hepatocyte-specific conditional knockout of STAT3 in HBV1.3-*AlbCre*STAT3$^{f/f}$ mice abrogated HCC development. This finding reinforces the idea for STAT3 signaling from previously described pro-viral effects by supporting virus replication and preventing apoptosis[61,76] to being a host factor in directly HBV-driven HCC development in HBV1.3 mice.

Whether STAT3 is indispensable for HCC development in our model because of cellular effects, such as prevention of apoptosis, or by supporting virus replication, remains unclear. Moreover, how HBV increases nuclear pSTAT3 in HBV transgenic mice and whether this is mediated by an increase in STAT3 phosphorylation or changes in nuclear translocation remains elusive and further research is warranted.

Our data provide *in vivo* proof of direct oncogenic effects of HBV, independent of inflammation and insertional mutagenesis. Therapeutic targeting of, for example, HBx or a hepatocyte-specific STAT3 inhibition – without detrimental effects – is warranted but not reasonable in every patient with chronic HBV infection. Furthermore, the direct oncogenic effects of HBV found in this model further challenge the idea of a benign 'immune tolerant'-phase not requiring antiviral treatment.[12,19] Given that antiviral therapy can reduce HCC risk by 70% among patients with indeterminate phase CHB,[78] indications for treatment should be adapted. Our data once again emphasize the necessity for HBV 'cure' in patients chronically infected with HBV.

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Abbreviations

aCGH, array-based comparative genomic hybridization; AFP, alpha-fetoprotein; ALT, alanine aminotransferase; ATM, ataxia telangiectasia mutated; cccDNA, covalently closed circular DNA; CD206: cluster of differentiation 206; CD3, cluster of differentiation 3; CHB, chronic hepatitis B; ClCaspase3, Cleaved Caspase-3; CLEC4F, C-type lectin domain family 4 member F; DDB1, DNA damage binding protein 1; ddPCR, droplet digital PCR; DEN, diethylnitrosamine; F4/80, EGF-like module-containing mucin-like hormone receptor-like 1; FFPE, formalin-fixed, paraffin-embedded; GAPDH, glycerinaldehyde-3-phosphate dehydrogenase; gp130, glycoprotein 130; GP73, Golgi protein-73; γH2AX, phosphorylation of the H2A histone family member X; HBcAg, hepatits B core antigen; HBeAg, hepatitis B e-antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; IHC, immunohistochemistry; ISH, *in situ* hybridization; ki67, antigen KI-67/MKI67; Mdm2, mouse double minute 2 homolog; MHCII, major histocompatibility complex class II; p21/Cdkn1a, Cyclin Dependent Kinase Inhibitor 1A; PCNA, proliferating cell nuclear antigen; pgRNA, pregenomic RNA; PHHs, primary human hepatocytes; pSTAT3, phosphorylated Signal transducer and activator of transcription 3; RT-qPCR, quantitative reverse transcription polymerase chain reaction; SMC6, Structural Maintenance of Chromosomes 6; SOCS3, Suppressor of cytokine signaling 3; STAT3, Signal transducer and activator of transcription 3; TG, transgene; TNF-α: tumor necrosis factor alpha; Tp53, Tumor Protein P53; WES, whole-exome sequencing; WT, wild-type.

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Conflicts of interest

The authors declare no conflicts of interest that pertain to this work. Please refer to accompanying ICMJE disclosure forms for further details.

Authors' contributions

Study design: MR, UP, MH. Experiments: MR, SS, MvdK, EK, SFD, MLP, TR, KW, FT, CCC, DY, VL, RS, JH. Data curation: SL, KU. Interpretation of data: MR, SS, MvdK, SFD, MLP, TR, SL, KW, FG, KU, ML, UP, MH. Writing the draft manuscript: MR. Critical revision of the manuscript: SS, MvdK, EK, SFD, TR, FG, HA, RMS, RR, HW, ML, UP, MH.

Data availability statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Supplementary data

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Author names in bold designate shared co-first authorship

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