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Hepatitis B surface antigen expression impairs endoplasmic reticulum stress-related autophagic flux by decreasing LAMP2

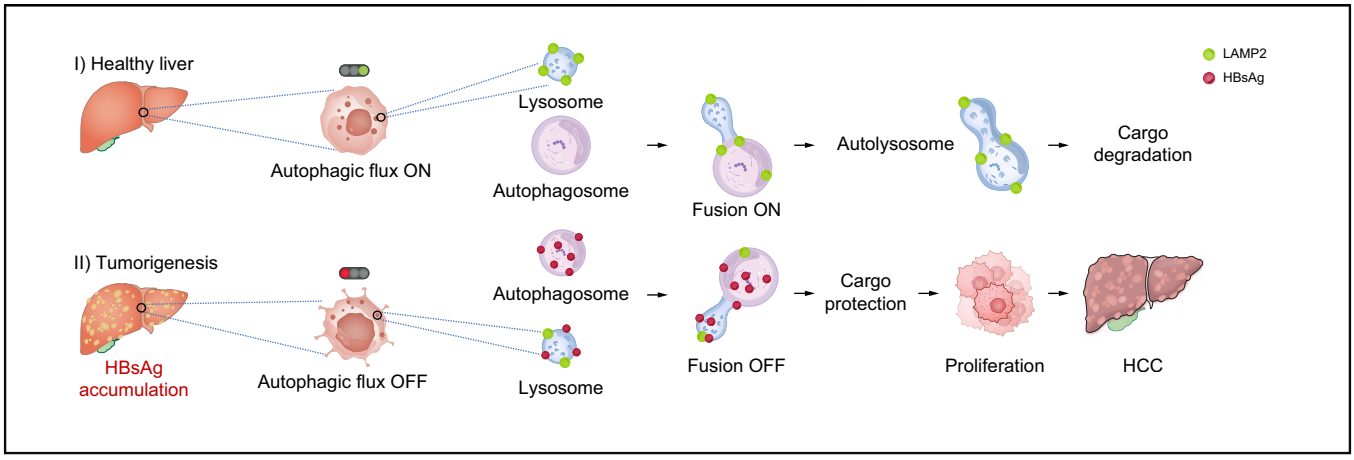
Authors

Yaojie Liang, Xufeng Luo, Stefan Schefczyk, Lorraine T. Muungani, Hui Deng, Baoju Wang, Hideo A. Baba, Mengji Lu, Heiner Wedemeyer, Hartmut H. Schmidt, Ruth Broering

Correspondence

ruth.broering@uni-due.de (R. Broering).

Graphical abstract



Highlights

- HBsAg accumulation induces ER stress and hepatocarcinogenesis *in vivo*.
- After HBsAg challenge, ATF4 and ATF6 downregulate the expression of LAMP2.
- Reduced LAMP2 worsens ER stress-related autophagic flux and promotes proliferation.
- ER stress inhibitors reverse HBsAg-reduced LAMP2 and suppress proliferation.

Impact and implications

Factors and mechanisms involved in hepatocarcinogenesis driven by hepatitis B surface antigen (HBsAg) are poorly defined, hindering the development of effective therapeutic strategies. This study showed that HBsAg-induced endoplasmic reticulum stress suppressed LAMP2, thereby mediating autophagic injury. The present data suggest that restoring LAMP2 function in chronic HBV infection may have both antiviral and anti-cancer effects. This study has provided insights into the role of HBsAg-mediated intracellular events in carcinogenesis and thereby has relevance for future drug development.

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Hepatitis B surface antigen expression impairs endoplasmic reticulum stress-related autophagic flux by decreasing LAMP2

Yaojie Liang,[1] Xufeng Luo,[1,2] Stefan Schefczyk,[1] Lorraine T. Muungani,[1] Hui Deng,[3] Baoju Wang,[3] Hideo A. Baba,[4] Mengji Lu,[5] Heiner Wedemeyer,[1,6] Hartmut H. Schmidt,[1] Ruth Broering[1,*]

[1]*Department of Gastroenterology, Hepatology and Transplant Medicine, Medical Faculty, University of Duisburg-Essen, Essen, Germany;* [2]*Institute for Lymphoma Research, Henan Cancer Hospital, The Affiliated Cancer Hospital of Zhengzhou University, Zhengzhou University, Zhengzhou, China;* [3]*Department of Infectious Diseases, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China;* [4]*Institute of Pathology, Medical Faculty, University of Duisburg-Essen, Essen, Germany;* [5]*Institute for Virology, Medical Faculty, University of Duisburg-Essen, Essen, Germany;* [6]*Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Hannover, Germany*

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Background & Aims: Hepatitis B surface antigen (HBsAg) drives hepatocarcinogenesis. Factors and mechanisms involved in this progression remain poorly defined, hindering the development of effective therapeutic strategies. Therefore, the mechanisms involved in the HBsAg-induced transformation of normal liver into hepatocellular carcinoma (HCC) were investigated.

Methods: Hemizygous Tg(Alb1HBV)44Bri/J mice were examined for HBsAg-induced carcinogenic events. Gene set-enrichment analysis identified significant signatures in HBsAg-transgenic mice that correlated with endoplasmic reticulum (ER) stress, unfolded protein response, autophagy and proliferation. These events were investigated by western blotting, immunohistochemical and immunocytochemical staining in 2-, 8- and 12-month-old HBsAg-transgenic mice. The results were verified in HBsAg-overexpressing Hepa1-6 cells and validated in human HBV-related HCC samples.

Results: Increased BiP expression in HBsAg-transgenic mice indicated induction of the unfolded protein response. In addition, early-phase autophagy was enhanced (increased BECN1 and LC3B) and late-phase autophagy blocked (increased p62) in HBsAg-transgenic mice. Finally, HBsAg altered lysosomal acidification via ATF4- and ATF6-mediated downregulation of lysosome-associated membrane protein 2 (LAMP2) expression. In patients, HBV-related HCC and adjacent tissues showed increased BiP, p62 and downregulated LAMP2 compared to uninfected controls. *In vitro,* the use of ER stress inhibitors reversed the HBsAg-related suppression of LAMP2. Furthermore, HBsAg promoted hepatocellular proliferation as indicated by Ki67, cleaved caspase-3 and AFP staining in paraffin-embedded liver sections from HBsAg-transgenic mice. These results were further verified by colony formation assays in HBsAg-expressing Hepa1-6 cells. Interestingly, inhibition of ER stress in HBsAg-overexpressing Hepa1-6 cells suppressed HBsAg-mediated cell proliferation.

Conclusions: These data showed that HBsAg directly induces ER stress, impairs autophagy and promotes proliferation, thereby driving hepatocarcinogenesis. In addition, this study expanded the understanding of HBsAg-mediated intracellular events in carcinogenesis.

Impact and implications: Factors and mechanisms involved in hepatocarcinogenesis driven by hepatitis B surface antigen (HBsAg) are poorly defined, hindering the development of effective therapeutic strategies. This study showed that HBsAg-induced endoplasmic reticulum stress suppressed LAMP2, thereby mediating autophagic injury. The present data suggest that restoring LAMP2 function in chronic HBV infection may have both antiviral and anti-cancer effects. This study has provided insights into the role of HBsAg-mediated intracellular events in carcinogenesis and thereby has relevance for future drug development.

Introduction

Liver cancer is the sixth most common cancer worldwide and is characterised by a high degree of malignancy, aggressive metastasis and insidious presentation.[1] HBV infection is the leading risk factor for HCC, accounting for approximately 60% of HCC cases in Asia and Africa and 20% in the West.[1] Hepatitis B surface antigen (HBsAg) continues to be generated and accumulates in hepatocytes during long-term chronic HBV infection, culminating in the classic histological finding of “ground glass” hepatocytes.[2] Studies have shown a positive correlation between HBsAg levels and the incidence of HCC in patients.[3] The risk of HCC is 2.46-fold higher in patients with residual HBsAg titres greater than 1,000 IU/ml.[4] However, the mechanisms driving

Keywords: HBV surface protein; lysosome; incomplete autophagy; liver cancer; cellular homeostasis; tumorigenesis.

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* Corresponding author. Address: University of Duisburg-Essen, Medical Faculty, Department of Gastroenterology, Hepatology and Transplant Medicine, Hufelandstr. 55, 45147 Essen, Germany; Tel.: +49-201-723-6015, Fax: +49-201-723-6915.

E-mail address: ruth.broering@uni-due.de (R. Broering).

HBsAg-induced hepatocarcinogenesis remain unclear, thus hindering the development of new drugs for the treatment of HCC.

HBV encodes three surface proteins, the large, medium and small HBsAg (PreS1, PreS2 and S, respectively). All HBsAg proteins are synthesised at the ER, the largest membranous organelle, which plays a major role in the processing and secretion of at least one-third of all proteins.[5,6] Disruption of ER homeostasis leads to an abnormal accumulation of unfolded or misfolded proteins, causing ER stress, which in turn activates an adaptive mechanism called the unfolded protein response (UPR) to repair and restore the ER to a steady state.[7] BiP is the key chaperone and regulator of ER homeostasis and binds to the UPR sensors IRE1, PERK and ATF6 to keep them inactive.[7,8] However, because it has a higher affinity for proteins that are misfolded or unfolded, BiP dissociates from the ER stress sensors, releasing them to activate their downstream signalling.[9]

Autophagy plays a critical protective role in the clearance of misfolded proteins and protein aggregates during ER stress.[10] Early autophagy is characterised by phagocytosis of cytoplasmic components and the formation of autophagosomes. Late autophagy is characterised by the formation of autolysosomes by fusion of autophagosomes with lysosomes and subsequent degradation of cargo by lysosomal hydrolases.[11] Both up- and down-regulation of autophagy have been found in human malignancies, suggesting that autophagy plays a complex role in tumour initiation and progression.[12] Studies have shown that, in the healthy liver, autophagy primarily has a tumour suppressive function, but the detailed role of autophagy in hepatocarcinogenesis is unclear.[13] In this study, the role of HBsAg in inducing ER stress and autophagic injury in hepatocytes was investigated, providing insights into the underlying mechanisms of HBsAg-induced carcinogenesis.

Materials and methods

See supplementary materials and supplementary CTAT table for details regarding mouse experiments, primary mouse hepatocyte isolation, human HCC tissue samples, Hepa1-6 cell culture,

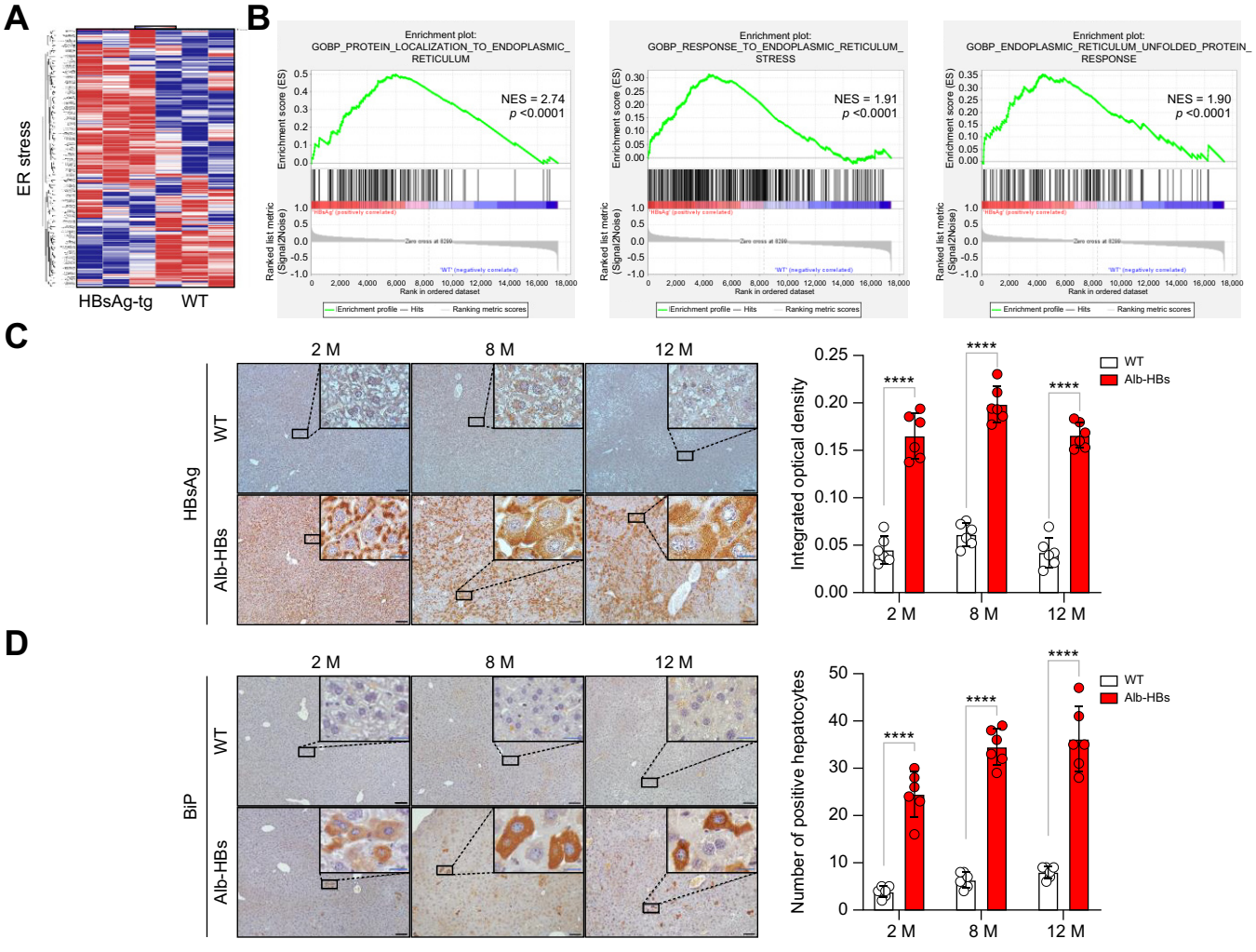

Fig. 1. ER stress is induced in HBsAg-tg mice. (A) Clustering heat map of GSE84429 was acquired for hepatic ER stress signature comparing HBsAg-tg and WT mice. (B) Gene set enrichment analysis of GSE84429 for ER localization, ER stress and UPR distinguished HBsAg-tg and WT livers. Immunohistochemical staining show (C) HBsAg and (D) BiP expression in livers of WT and Alb-HBs mice at different ages (2-, 8-, 12-month-old, group sizes n = 3). Quantitative analysis based on integrated optical density of two randomly selected areas of each mouse (mean±SD). Scale bars: blue bar = 20 μm, black bar = 100 μm; *p* values (unpaired t test, unequal variances t-test) ****p <0.0001. ER, endoplasmic reticulum; HBsAg, hepatitis B surface antigen; HBsAg-tg, HBsAg-transgenic; NES, normalised enrichment score; UPR, unfolded protein response; WT, wild-type.

HBsAg overexpression, western blot, autophagosome staining, lysosomal tracking, acridine orange staining, dual-luciferase reporter assay, immunofluorescent and immunohistochemical staining, cell proliferation assay, colony formation assay and data mining.

Statistical analysis

The GraphPad Prism version 9 (GraphPad Software, La Jolla, CA) was used to create graphs and perform statistical analysis. Representative data expressed as mean ± SD. Two groups were compared using unpaired t-test, F-test and unequal variances t-test (Welsh's t-test). Statistical significance was determined by *p* values less than 0.05.

Results

HBsAg induces the UPR in HBsAg-transgenic mice

To determine whether HBsAg affects ER stress and thereby alters a number of HCC-related intracellular events, public microarray data (GSE84429),[14] generated from liver samples of wild type

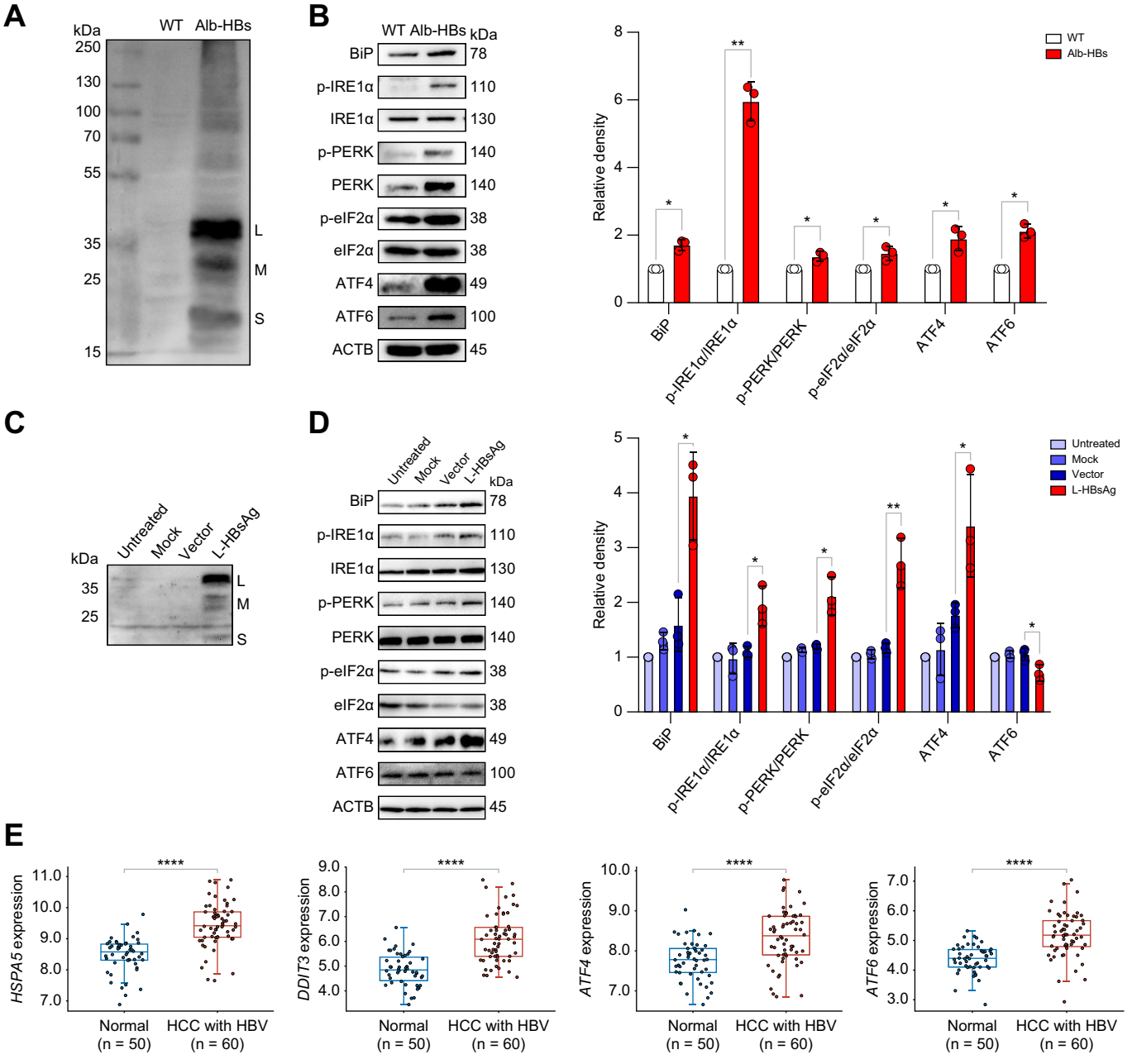

Fig. 2. UPR is induced in HBsAg-transgenic mice. Western blot was performed to detect (A) HBsAg and (B) UPR components in primary murine hepatocytes isolated from WT and Alb-HBs mice (4-6-months-old). Western blot visualised (C) HBsAg and (D) UPR components in HBsAg-overexpressing Hepa1-6 cells (2 μg/well, 6 well plate, 24 h). Relative density is given as mean±SD of three independent experiments. (E) Gene expression of *HSPA5*, *DDIT3*, *ATF4* and *ATF6* was compared between HBV-associated HCC tissues and normal tissues (The Cancer Genome Atlas). *p* values (unpaired t-test, unequal variances t-test) **p* <0.05, ***p* <0.01, *****p* <0.0001. HBsAg, hepatitis B surface antigen; HCC, hepatocellular carcinoma; L-HBsAg, large HBsAg; UPR, unfolded protein response; WT, wild-type.

and HBsAg-transgenic mice, were reanalysed by gene set-enrichment analysis (GSEA). This analysis revealed the potential candidate signatures of intracellular events driven by HBsAg accumulation. ER stress and UPR signatures were significantly enriched in HBsAg-transgenic mice (Fig. 1A and B). These events were investigated in 2-, 8- and 12-month-old HBsAg-transgenic male mice (Tg(Alb1HBV)44Bri/J, Alb-HBs), representing non-HCC, early HCC and HCC stages, respectively.[15,16] Immunohistochemical staining showed that large amounts of HBsAg already accumulated in hepatocytes of at least 2-month-old HBsAg-transgenic mice (Fig. 1C). To determine whether HBsAg triggers the UPR in these transgenic mice, BiP was visualised as a master regulator of the UPR. Immunohistochemical (IHC) staining of paraffin sections showed that BiP expression was significantly upregulated in 2-, 8- and 12-month-old transgenic mice (Fig. 1D). Western blot was performed to examine UPR markers in primary murine hepatocytes (PMHs) of wild-type and HBsAg-transgenic mice. HBsAg (Fig. 2A) significantly upregulated the abundance of BiP, PERK, p-PERK, p-eIF2α, ATF4 and p-IRE1α in PMHs (Fig. 2B), indicating that the UPR is activated. Notably, increased ATF6 (Fig. 2B) but no cleaved ATF6 was observed in HBsAg-transgenic mice, suggesting that ATF6 may not be involved in the HBsAg-mediated UPR.

To mimic the HBsAg-mediated activation of the UPR *in vitro*, HBsAg was overexpressed in Hepa1-6 cells using the large HBsAg-coding plasmid[17] (Fig. 2C). Western blot showed increased levels of BiP, p-PERK, p-eIF2α, ATF4 and p-IRE1α in the HBsAg-transfected cells (Fig. 2D). In contrast to the animal model, a slight decrease in total ATF6 protein expression was observed. To confirm HBsAg-mediated effects on ER conditions, HBV-associated HCC samples were examined by re-analysis of TCGA (The Cancer Genome Atlas) data, including 50 normal

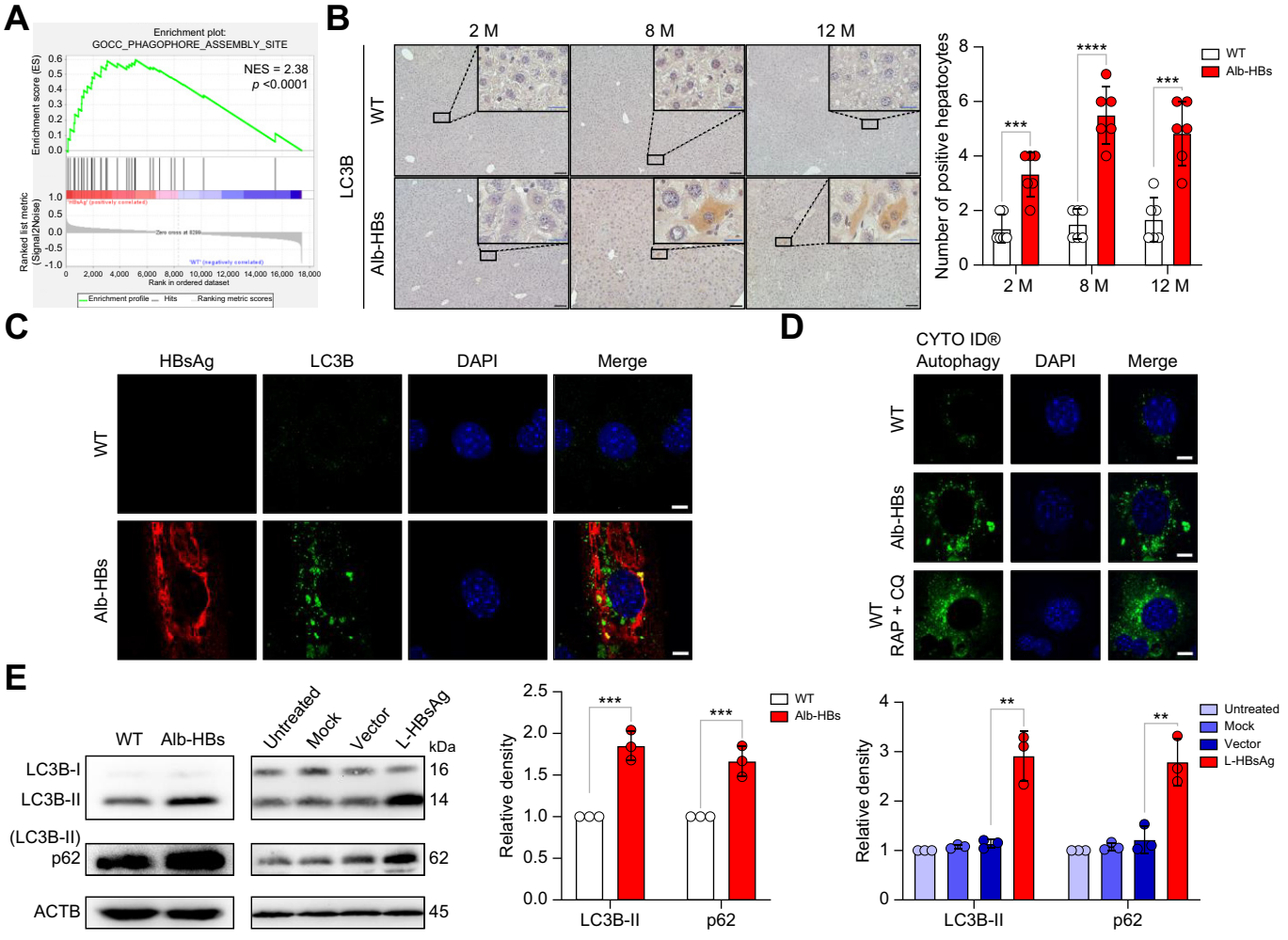

Fig. 3. Autophagosomes are enriched in HBsAg-transgenic mice. (A) Gene set-enrichment analysis of GSE84429 for autophagosomes discriminates HBsAg-transgenic and WT mouse livers. (B) Immunohistochemistry detects LC3B in WT and Alb-HBs livers at different ages (2-, 8-, 12-month-old, group sizes n = 3). LC3B was quantified (mean±SD) by counting the positive hepatocytes from two randomly selected areas for each mouse. (C) Immunofluorescence staining shows HBsAg (red) and LC3B (green) in primary murine hepatocytes isolated from WT and Alb-HBs mice (4-6-months-old, representative of three independent experiments. (D) Immunofluorescence staining visualises autophagosomes in primary murine hepatocytes isolated from WT and Alb-HBs (4-6-months-old) mice. Hepatocytes were labelled with CYTO-ID® green detection reagent to detect autophagosomes. Hepatocytes treated with RAP (1 μM) in the presence of CQ (10 μM) for 24 h were used as a positive control; representative of three independent experiments. (E) Western blot detects LC3B-I, LCRB-II, p62 and ACTB in primary murine hepatocytes isolated from WT and Alb-HBs mice (4-6-months-old, left panel) and Hepa1-6 transfected with HBsAg-encoding plasmid (2 μg/well, 6 well plate, 24 h; right panel). Relative density is given as mean±SD of three independent experiments. Scale bars: blue bar = 20 μm, black bar = 100 μm; white bar, 10 μm; *p* values (unpaired t-test, unequal variances t-test); ***p* <0.01, ****p* <0.001, *****p* <0.0001. CQ, chloroquine; HBsAg, hepatitis B surface antigen; L-HBsAg, large HBsAg; NES, normalized enrichment score; RAP, rapamycin; WT, wild-type.

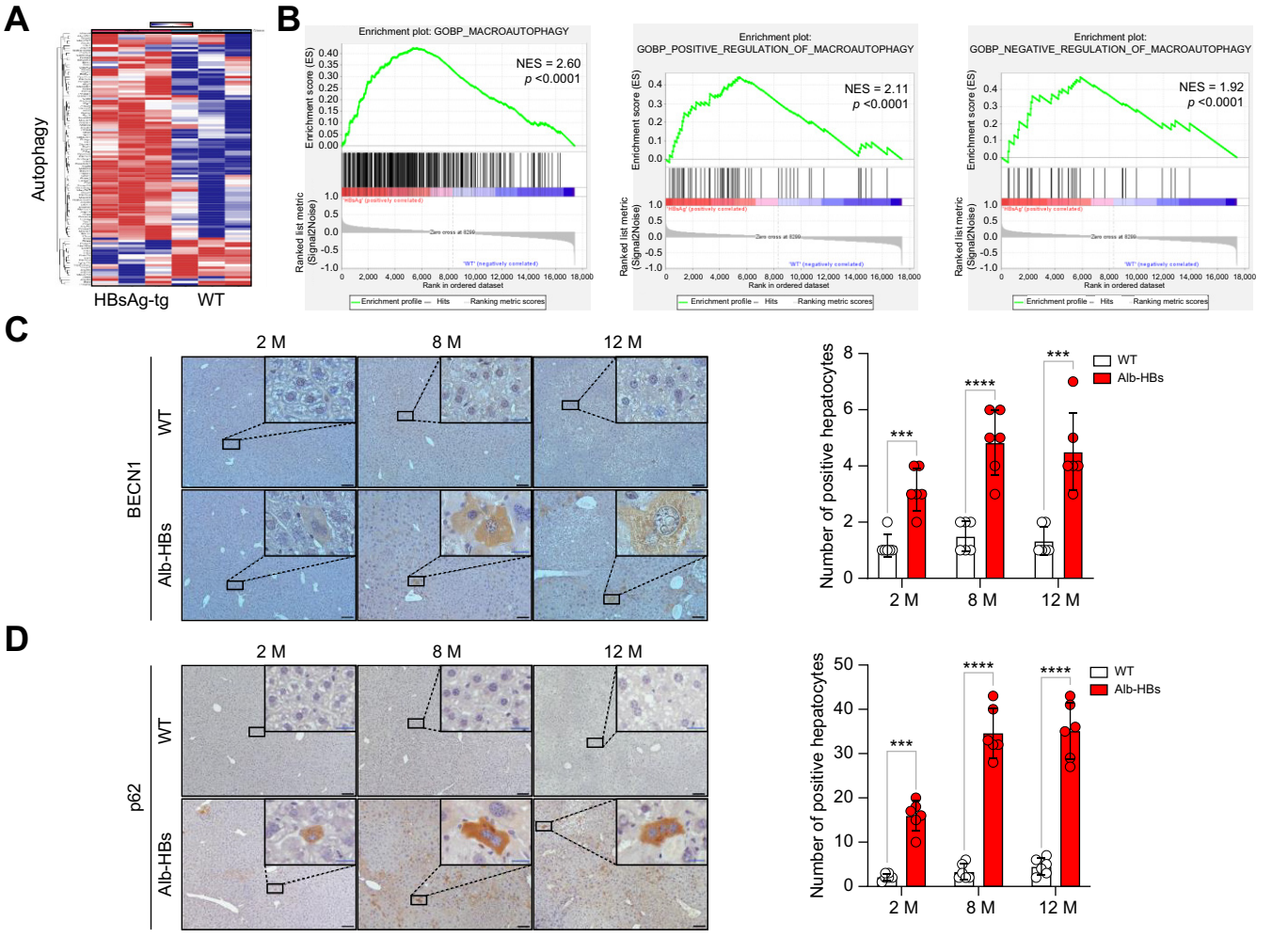

Fig. 4. Autophagy signalling is abnormal in HBsAg-tg mice. (A) Clustering heat map of GSE84429 was acquired for hepatic autophagy signature comparing HBsAg-tg and WT mice. (B) Gene set-enrichment analysis of GSE84429 for autophagy, autophagy positive regulation and autophagy negative regulation distinguished HBsAg-tg and WT livers. Immunohistochemical staining shows (C) BECN1 and (D) p62 expression in Alb-HBs and WT mouse livers at different ages (2-, 8-, 12-months-old, group sizes n = 3). BECN1 and p62 were quantified (mean±SD) by counting the positive hepatocytes from two randomly selected areas for each mouse (n = 3). Scale bars: blue bar = 20 μm, black bar = 100 μm; white bar = 10 μm; *p* values (unpaired t-test, unequal variances t-test), ***p <0.001, ****p <0.0001. HBsAg, hepatitis B surface antigen; HBsAg-tg, HBsAg-transgenic; NES, normalized enrichment score; WT, wild-type.

tissues and 60 HCC samples from HBV-positive patients (excluding hepatitis C virus coinfection). In tumour samples, expression of *HSPA5* (encoding BiP protein), *DDIT3* (encoding CHOP protein), *ATF4* and *ATF6* was significantly upregulated compared to non-tumour samples (Fig. 2E). These data showed that UPR-related genes were induced in HBV-associated HCC tissue, demonstrating the relevance of our previous findings.

Accumulation of autophagosomes is observed in HBsAg-transgenic mice

Next, whether HBsAg promotes autophagosomes in hepatocytes was investigated. The microarray dataset (GSE84429) was re-analysed by GSEA. Genes associated with autophagosomes (phagophore assembly) were significantly enriched in HBsAg-transgenic mice (Fig. 3A). LC3B is often used to monitor autophagy, as LC3B-II levels are closely correlated with the number of autophagosomes.[18] Increased expression of LC3B was demonstrated by IHC staining in 8- and 12-month-old transgenic mice (Fig. 3B), whereas immunofluorescence (IF) staining also showed increased LC3B in PMHs of 4- to 6-month-old transgenic mice (Fig. 3C). Western blot analysis further showed increased expression of LC3B-II in HBsAg-transgenic mouse livers and HBsAg-expressing Hepa1-6 cells (Fig. 3E). Phagophore assembly was confirmed by the CYTO-ID® Autophagy Detection Kit 2.0, which visualised an increased number of autophagosomes in PMHs from HBsAg-transgenic mice (Fig. 3D), similar to that observed in the positive control, represented by wild-type PMHs stimulated with rapamycin and chloroquine. These data showed a clear accumulation of autophagosomes in HBsAg-transgenic mice.

HBsAg impairs autophagic flux in HBsAg-transgenic mice

Whether HBsAg promotes autophagy in hepatocytes was further investigated. First, the microarray dataset (GSE84429) was reanalysed. Autophagy signalling, including positive and negative regulation, were significantly enriched in HBsAg-transgenic mice (Fig. 4A and B). BECN1[19] and p62[20] are known as markers of early and late autophagy activation, repectively. To investigate the effect of HBsAg on autophagy in transgenic mice, IHC was

performed and indicated a clear upregulation of both BECN1 and p62 in HBsAg-transgenic mice (Fig. 4C and D), suggesting that autophagy might be enhanced in the early stage and impaired in the late stage in HBsAg-transgenic mice.

To confirm the enhanced early and impaired late autophagy, western blot was performed to examine autophagy markers in PMHs (wild-type and Alb-HBs) and HBsAg-expressing Hepa1-6 cells. Western blot analysis showed that HBsAg significantly increased the expression of LC3B-II and p62 (Fig. 3E), suggesting that autophagic degradation may be impaired in hepatocytes. IF staining was performed and the results clearly confirmed increased p62 in PMHs from HBsAg-transgenic mice (Fig. 5A).

The autophagic flux was investigated by assessing lysosomal activity, using LysoTracker™ Red DND-99. Chloroquine is known to inhibit acidification of the lysosomal compartment, thereby preventing the degradation of cargo in lysosomes.[21] As a positive control, wild-type PMHs were treated with 10 μM of chloroquine for 24 h *in vitro*. The results showed that the fluorescence intensity of LysoTracker™ staining was clearly reduced in PMHs isolated from HBsAg-transgenic mice, compared to PMHs from

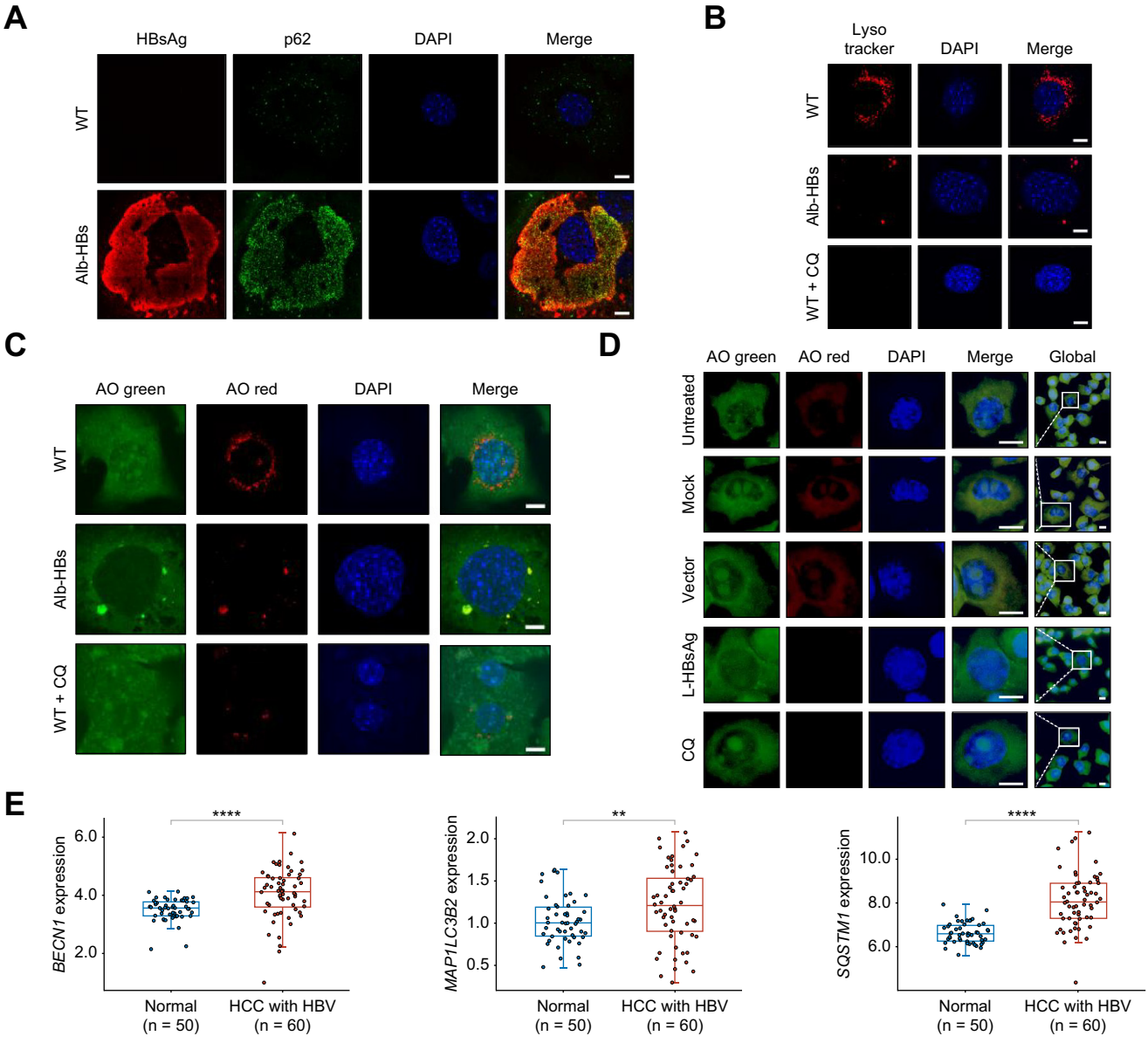

Fig. 5. Autophagic flux is impaired in HBsAg-transgenic mice. (A) Immunofluorescence staining shows HBsAg (red) and p62 (green) in primary murine hepatocytes isolated from WT and Alb-HBs (4-6-months-old), representative of three independent experiments. (B) Immunofluorescence staining shows lysosomal activity in primary murine hepatocytes isolated from WT and Alb-HBs mice (4-6-months-old). Primary murine hepatocytes were stained with 50 nM LysoTracker Red for 30 min. WT hepatocytes treated with CQ (10 mM) for 24 h were used as a positive control; representative of three independent experiments. Immunofluorescence staining shows lysosomal acidification in (C) primary murine hepatocytes isolated from WT and Alb-HBs mice (4-6-months-old) and (D) HBsAg-overexpressing Hepa1-6 cells. Cells were stained with AO (5 μM) for 15 min. Controls were treated with CQ (10 mM) for 24 h; representative of three independent experiments. The fluorescence intensity of AO-Red was analysed using an Olympus BX 51, upright epifluorescence microscope. (E) *BECN1*, *MAP1LC3B2* and *SQSTM1* gene expression was compared between HBV-associated HCC tissues and normal tissues (The Cancer Genome Atlas). Scale bars: white bar = 10 μm; *p* values (unpaired t-test, unequal variances t-test) $^{**}p$ <0.01, $^{****}p$ <0.0001. AO, acridine orange; CQ, chloroquine; HBsAg, hepatitis B surface antigen; HCC, hepatocellular carcinoma; WT, wild-type.

wild-type mice (Fig. 5B), suggesting compromised autophagy and reduced cargo degradation. Fusion between the autophagosome and lysosome depends on normal lysosomal activity, which is related to pH. Acridine orange is a dye that can stain acidic bodies such as lysosomes. By emitting red light after excitation and blue light under low pH conditions, it can be used to assess lysosomal pH.[22] Acridine orange staining of PMHs (wild-type and Alb-HBs) showed that the red fluorescence was clearly reduced in the HBsAg-transgenic PMHs, similar to the chloroquine-treated controls, indicating abnormal lysosomal acidification due to HBsAg expression (Fig. 5C). These results were confirmed in HBsAg-expressing Hepa1-6 cells (Fig. 5D). Thus, the autophagic flux seemed to be impaired in the presence of HBsAg accumulation.

Again, analysis of TCGA expression data from HBV-associated HCC (n = 60) and control tissues (n = 50) indicated increased gene expression of *BECN1*, *MAP1LC3B2* (encoding LC3B protein) and *SQSTM1* (encoding p62 protein) in tumour samples, compared to non-tumour controls (Fig. 5E). Thus genes related to incomplete autophagy were induced in HBV-associated HCC tissue.

LAMP2 expression is reduced in HBsAg-transgenic mice due to HBsAg accumulation

Re-analysis of the microarray dataset (GSE84429) indicated that the lysosome gene signature was significantly enriched in HBsAg-transgenic mice compared to wild-type mice (Fig. 6A and B). The role of lysosome-associated membrane proteins 1 and 2 (LAMP1 and LAMP2) in abnormal lysosomal acidification was analysed in HBsAg-transgenic mice by western blot. In particular, LAMP2 is an important regulator of the successful maturation of autophagosomes and phagosomes.[23] As shown in Fig. 6C, LAMP2 but not LAMP1 expression was significantly decreased in PMHs isolated from HBsAg-transgenic mice. IF staining confirmed the reduced LAMP2 expression in HBsAg-transgenic mice (Fig. 6D). Suppression of LAMP2 was also observed in Hepa1-6 cells transiently expressing HBsAg (Fig. 6E), suggesting that HBsAg appears to directly inhibit LAMP2 expression.

To determine whether the HBsAg-induced UPR contributes to the reduction of LAMP2, western blotting was performed in PMHs (wild-type and Alb-HBs) treated with the ER stress inhibitor tauroursodeoxycholic acid (TUDCA) *in vitro*. Interestingly, the results showed that LAMP2 expression was partially restored in PMHs treated with 1 mM TUDCA for 48 h (Fig. 6F). In addition, the decrease in p62 level in TUDCA-treated PMHs indirectly indicated that the corresponding autophagic flux was restored and improved to some extent with the normalisation of LAMP2 (Fig. 6F). As shown in Fig. 2B and D, the phosphorylation of eIF2α was induced by HBsAg expression in transgenic mice and transfected cells. To determine whether HBsAg-induced activation of PERK-eIF2α signalling was involved in decreased LAMP2 expression, the selective PERK inhibitor GSK2606414 was applied. GSK2606414 partially restored LAMP2 expression levels in HBsAg-transgenic mice (Fig. 6G). These results suggest that HBsAg-mediated activation of PERK-eIF2α signalling is involved in the reduction of LAMP2 expression in HBsAg-transgenic mice. Since ATF4 and ATF6 were upregulated in Alb-HBs mice (Fig. 2B) and ATF4 was induced in HBsAg-expressing Hepa1-6 cells (Fig. 2E), the role of ATF4 and ATF6 in LAMP2 expression was analysed. Dual-luciferase reporter assays determined the effect of ATF4 and ATF6 overexpression on *LAMP2* promoter activity. Transfection with higher doses of ATF4- or ATF6-encoding plasmids (80 ng/reaction) significantly suppressed luciferase activity. Functionality of the assay was confirmed by overexpression of PafA. These data highlight a potential role for ATF4 and ATF6 in HBsAg-mediated suppression of LAMP2.

HBsAg contributes to proliferation and drives hepatocarcinogenesis

Intracellular homeostasis, cell fate and tumourigenesis are often associated with ER stress and impaired autophagy.[7] Herein, it was found that gene signatures related to proliferation and liver cancer were significantly enriched in HBsAg-transgenic mice (Fig. 7A). In contrast, no significant enrichment of signatures related to apoptosis was observed. IHC staining for Ki67, a well-accepted marker of proliferation and a potential prognostic or predictive marker in malignant diseases, was performed to further investigate whether HBsAg induces proliferation in transgenic mice (2-, 8- and 12-month-old). Ki67-positive hepatocytes were observed in 8- and 12-month-old HBsAg-transgenic mice (Fig. 7B), suggesting increased hepatocyte proliferation due to HBsAg accumulation. IHC staining detected cleaved caspase-3, a critical executioner of apoptosis,[24] in HBsAg-transgenic mice (Fig. 7C), indirectly indicating apoptotic events due to HBsAg expression. Uncontrolled aggressive proliferation usually leads to tumourigenesis; alpha-fetoprotein (AFP), a well-known HCC marker, was investigated by IHC, indicating that AFP expression was massively increased in the older groups of HBsAg-transgenic mice (Fig. 7D). These results suggest that ER stress and impaired autophagic flux occurred in HBsAg-transgenic mice, coinciding with cell proliferation and subsequent tumourigenesis.

Colony formation was studied in Hepa1-6 cells overexpressing HBsAg, demonstrating the ability of individual cells to proliferate and form colonies (Fig. 7E). HBsAg-expressing Hepa1-6 cells showed a significant increase in cell colony formation. The relationship between ER stress and proliferation in HBsAg-transfected Hepa1-6 cells was analysed following TUDCA and GSK2606414 treatment for 24 h. ER stress inhibition significantly reduced HBsAg-induced cell proliferation, determined by a CCK8 assay (Fig. 7F). Thus, HBsAg appears to promote cell proliferation by inducing ER stress.

LAMP2 levels are suppressed in HBV-associated HCC and adjacent tissue

ER stress and autophagy impairment was analysed by IHC staining of HCC and adjacent tissue from HBV-infected (n = 5) and non-infected (n = 5) patients. The expression of HBsAg, BiP, p62, and LAMP2 (Fig. 8A) as well as BECN1 and LC3B (Fig. S1) was evaluated. The observed HBsAg signals were lower in HCC tissues compared to adjacent tissue controls, in agreement with other studies.[25,26] Consistent with our previous findings, the expression of BiP, LC3B, p62 was increased, whereas the expression of LAMP2 was decreased in HBV-related HCC and adjacent tissues compared to uninfected controls. In contrast, staining for BECN1 showed overall high expression in HCC and adjacent tissues (Fig. S1). These differences, except for BECN1, appeared to be significant when the integrated optical density was compared in five randomly selected areas from each patient. Finally, the integrated optical density of HBsAg negatively correlated with that of LAMP2 in HCC ($r = -0.5095$, $p = 0.0093$) and adjacent tissue ($r = -0.5651$, $p = 0.0001$) from HBV-infected patients (Fig. 8B). Thus, there appears to be a close relationship between HBsAg accumulation, hyperactivation of ER stress and LAMP2 decline-driven impaired autophagic flux in HBV-related HCC samples.

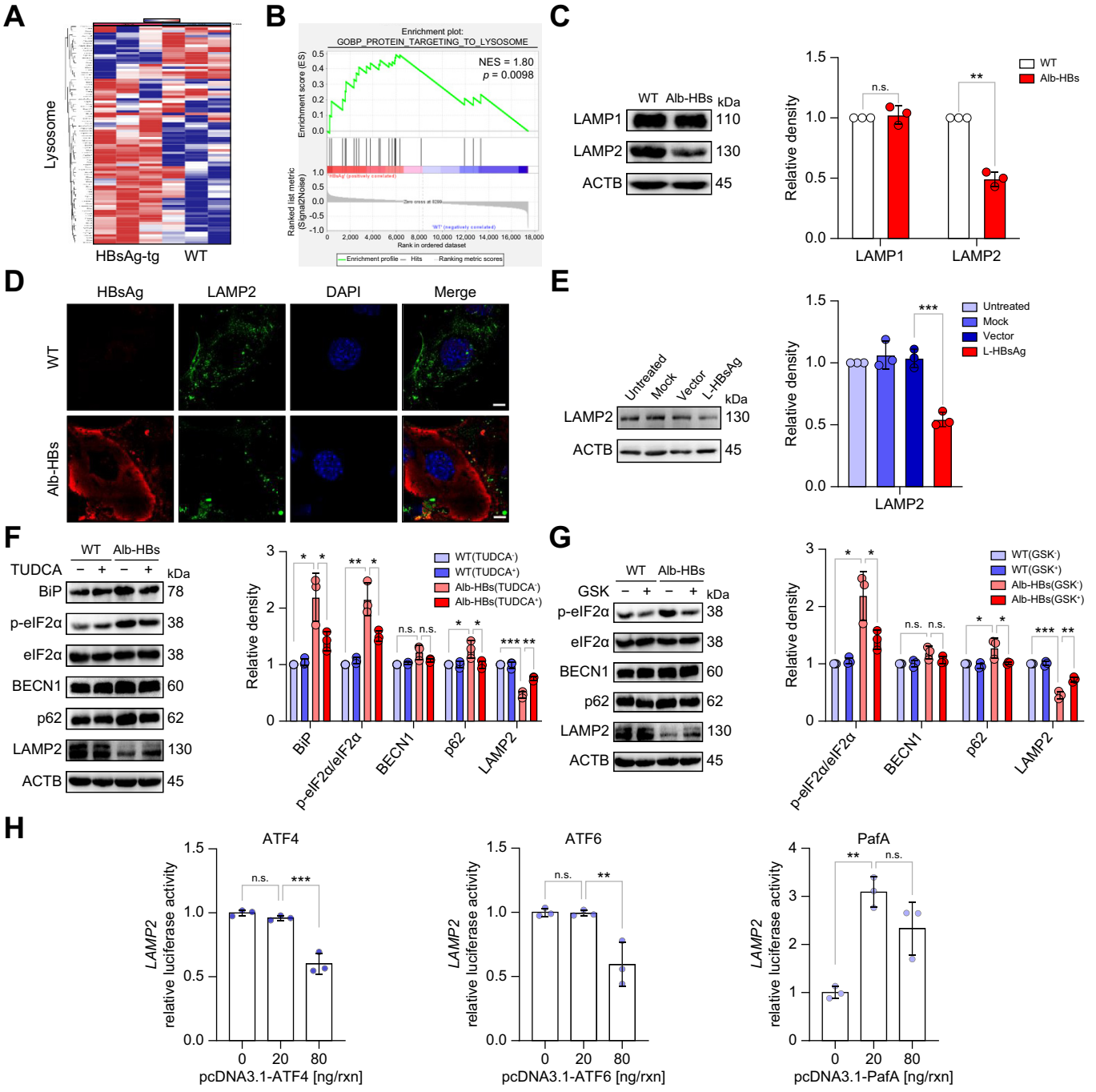

Fig. 6. HBsAg suppresses LAMP2 expression. (A) Clustering heat map of GSE84429 for lysosome signature was acquired comparing HBsAg-tg and WT mouse livers. (B) Gene set-enrichment analysis of GSE84429 for lysosome signature distinguished HBsAg-tg and WT mouse livers. (C) Western blot shows LAMP1/2 proteins in primary murine hepatocytes isolated from WT and Alb-HBs mice (4-6-months-old). (D) Immunofluorescence staining shows HBsAg (red) and LAMP2 (green) expression in primary murine hepatocytes isolated from WT and Alb-HBs mice (4-6-months-old), representative of three independent experiments. (E) Western blot shows LAMP2 expression in Hepa1-6 cells transfected with HBsAg-encoding plasmid and controls (2 μg/well, 6 well plate, 24 h). Western blot shows ER stress and autophagy components in primary murine hepatocytes isolated from WT and Alb-HBs mice (4–6-months-old) treated with (F) TUDCA (1 mM, 48 h) and (G) GSK2606414 (GSK, 1 mM, 48 h). (H) Dual luciferase reporter assay for the LAMP2 promoter was performed 48 h after the transfection with 20 ng/rxn and 80 ng/rxn of pcDNA3.1-ATF4, pcDNA3.1-ATF6 and pcDNA3.1-PafA in HEK293T cells. For western blots, relative densities are given as mean±SD of three independent experiments. Scale bars: white bar = 10 μm; *p* values (unpaired t-test, unequal variances t-test) **p* <0.1, ***p* <0.01, ****p* <0.001; n.s., not significant. HBsAg, hepatitis B surface antigen; L-HBsAg, large HBsAg; NES, normalized enrichment score; rxn, reaction; TUDCA, tauroursodeoxycholic acid; WT, wild-type.

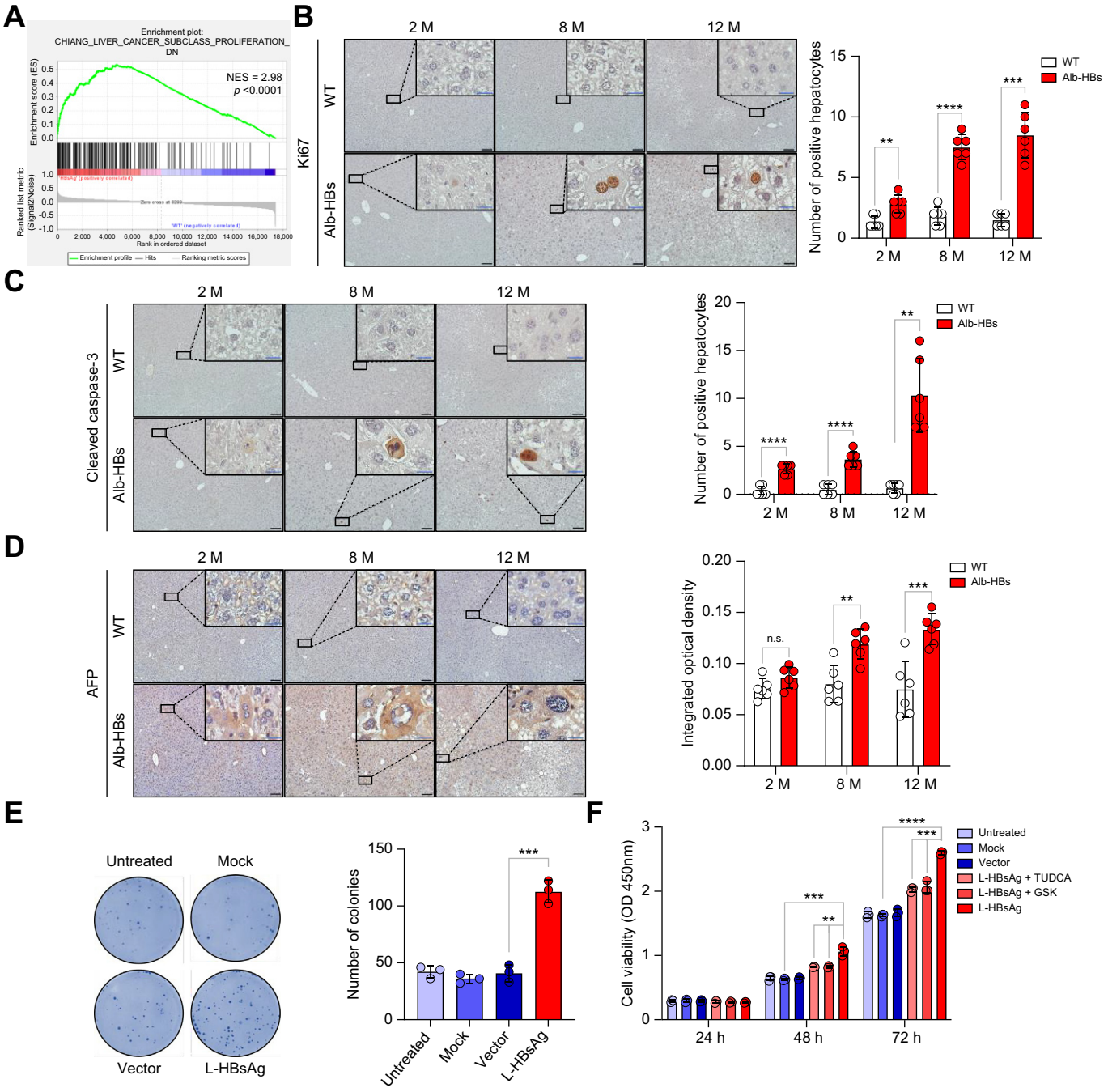

Fig. 7. HBsAg-induced ER stress drives proliferative activity. (A) Gene set-enrichment analysis of GSE84429 for proliferation distinguished HBsAg-tg and WT mouse livers. Immunohistochemical staining shows (B) Ki67, (C) cleaved caspase-3 and (D) AFP positive hepatocytes in WT and Alb-HBs mouse livers at different ages (2-, 8-, 12-months-old, group sizes n = 3), quantified in two randomly selected areas for each mouse. (E) Colony formation assays were performed with Hepa1-6 cells transfected with HBsAg plasmid for 24 h (2 μg/well, 6-well plate). The number of colonies was counted (mean±SD of three independent experiments). (F) CCK-8 viability analysis was performed. Hepa1-6 cells, transfected with HBsAg-encoding plasmid for 24 h, were trypsinized, seeded (5×10^5/well) and cultured in 6-well culture plates. TUDCA or GSK were added for 24 h. Hepa1-6 cells were trypsinized again, seeded (5×10^3/well) and cultured in 96-well culture plate. Cell viability was assessed at 24 h, 48 h and 72 h. Scale bars: blue bar = 20 μm, black bar = 100 μm; *p* values (unpaired t-test, unequal variances t-test) $^{**}p$ <0.01, $^{***}p$ <0.001, $^{****}p$ <0.0001; n.s., not significant. ER, endoplasmic reticulum; HBsAg, hepatitis B surface antigen; NES, normalized enrichment score; TUDCA, tauroursodeoxycholic acid; WT, wild-type.

Discussion

An imbalance in protein homeostasis can lead to abnormal protein accumulation, disrupting normal cellular function and is associated with a variety of diseases, including cancer.[27] This study demonstrated that HBsAg accumulation induced ER stress, impaired autophagic flux and promoted proliferation in HBsAg-transgenic mice *in vivo* and in HBsAg-expressing cells *in vitro*. Mechanistically, HBsAg-induced ATF4 and ATF6 expression were identified to suppress *LAMP2* promoter activity. This led to a suppressed fusion of autophagosomes and lysosomes. Notably,

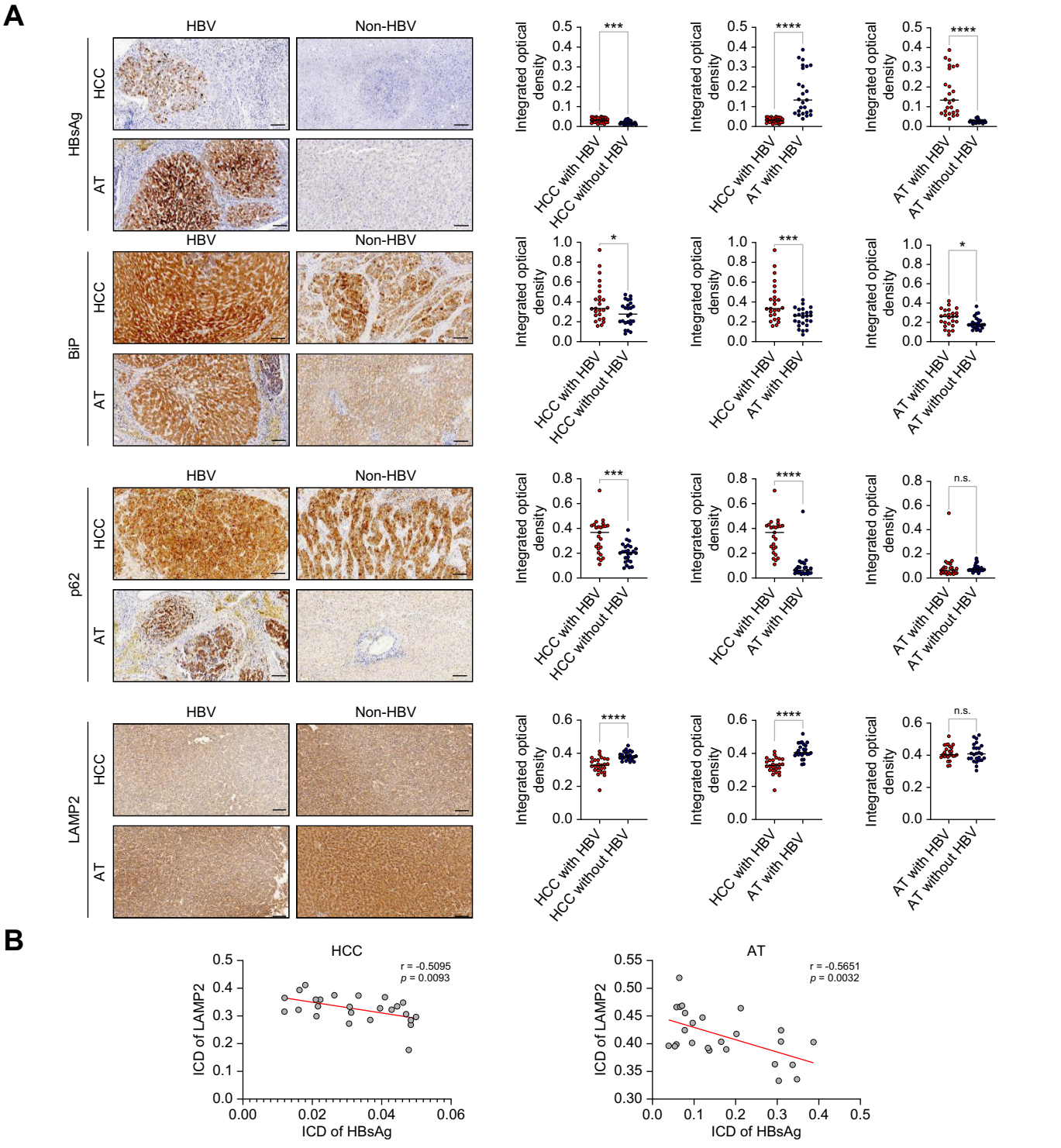

Fig. 8. HBsAg accumulation induces ER stress-associated autophagy impairment by suppressing LAMP2 in patients with HCC and HBV infection. (A) Expression of HBsAg, BiP, p62 and LAMP2 was verified by immunohistochemical staining in paraffin-embedded HCC specimens with (n = 5) or without (n = 5) HBV infection. Tumour and AT were analysed. Quantitative analysis of HBsAg, BiP, p62 and LAMP2 based on integrated optical density of five randomly selected areas for each patient. (B) Spearman's rank correlation was applied to the integrated optical density values of HBsAg and LAMP2. Scale bar, 100 μm. *p* values (unpaired t-test, unequal variances t-test) **p* <0.05; ***p* <0.01; ****p* <0.001; *****p* <0.0001; n.s., not significant. AT, adjacent tissue; ER, endoplasmic reticulum; HBsAg, hepatitis B surface antigen; HCC, hepatocellular carcinoma.

treatment with ER stress inhibitors partially restored LAMP2 expression, thereby normalising autophagic and proliferative activities. These findings provide a rational explanation for how HBsAg impairs autophagic flux in liver tumourigenesis.

Due to the complex and error-prone nature of protein folding, the capacity of the ER to fold proteins can be easily overwhelmed following physiological or pathological insults.[7] Therefore, the continuous accumulation of HBsAg in the ER induces ER stress,

thereby causing a series of intracellular events. Previous studies have demonstrated that ER stress plays a key role in liver disease progression.[28,29] PreS1 and PreS2 mutations have been shown to induce ER stress, associated with DNA damage, centrosome overduplication, and genomic instability.[28,30] In the present study, wild-type HBsAg was shown to induce sustained ER stress, thereby activating the UPR as in PreS mutation models.[28,30] Here, HBsAg-transgenic mice and HBsAg-transduced cells expressed all three HBsAg proteins (large, medium and small), with maximum levels for the L-HBsAg. However, expression of PreS1 (large), PreS2 (medium) and S (small) genes likely depends on cellular conditions and may differ in distinct settings. Possible differential effects of the different HBsAg versions on ER stress and autophagy were not investigated in this study. In conclusion, excessive ER stress and subsequent UPR can lead to hepatic inflammation, tissue damage and fibrosis, including cellular malignant transformation, ultimately contributing to various liver diseases.

Previous reports have shown that HBV can increase the autophagic process in hepatoma cells without increasing lysosomal protein degradation by HBsAg-mediated LC3-1 lipidation.[31] In the present study, an additional mechanism was described. LAMP1 and LAMP2 are essential components of the lysosome;[32] a recent study showed that LAMP2 reduction is associated with impaired autophagic flux in response to oxygen or nutrient deprivation therapy. A significant increase in autophagic flux is observed when LAMP2 is overexpressed.[33] It has been reported that ER stress can reduce autophagic flux by inhibiting LAMP expression, although the mechanism remains unclear.[34] The results of the present study led to the suggestion that HBsAg alters lysosomal pH by downregulating LAMP2 expression, thereby impairing autophagic flux in a transgenic mouse model. A previous study also showed that HCC tissues expressed significantly less LAMP2 than adjacent tissues, and its expression level correlated with HCC metastasis.[35] Their results are consistent with ours, but they did not explain the reason for the decrease in LAMP2, which is thought to be influenced by HBsAg expression. In this study, HBsAg-induced ATF4 and ATF6 were identified as negative transcriptional regulators of LAMP2. A related mechanism has been described for free fatty acid-induced ER stress in alcohol-related liver disease, where ATF4 represses LAMP2 expression and thereby impairs autophagy flux.[36] Another recent study further indicated that LAMP2A expression was downregulated in human HCC biopsies, and showed that HCC and human hepatocyte cell lines were more proliferative and migratory when LAMP2A expression was inhibited.[37] LAMP2A represents 25% of rat liver lysosomal LAMP2.[38] Their conclusion clearly supports our findings that LAMP2 downregulation due to long-term HBsAg accumulation induces incomplete autophagic flux, promotes proliferation and supports tumourigenesis. In this study, inhibition of HBsAg-induced UPR or PERK-eIF2α signalling partially restored LAMP2 protein expression. This finding further suggests that reducing ER stress or maintaining the autophagic flux may be effective strategies for inhibiting HBsAg-induced hepatocarcinogenesis. Further investigation is required to determine the specific molecular mechanisms by which large, medium and small HBsAg activate the PERK-eIF2α axis.

The role of autophagy in the degradation of HBsAg has previously been investigated, indicating that HBsAg is primarily degraded by autophagy.[11] Thus, impaired autophagic flux leads to accumulation of HBsAg. Interestingly, HBV uses distinct host trafficking machinery to assemble and release its particle types, including the endosomal sorting complexes required for the transport pathway and the autophagy process.[39] Therefore, HBsAg-induced suppression of LAMP2 may be a mechanism to protect viral particles from degradation while occupying multivesicular and autophagic processes. This study suggests that HBsAg inhibits autophagic flux by downregulating autophagosome-lysosome fusion, which in turn leads to a further imbalance in cellular homeostasis, which determines cell fate. The relationship between autophagy and cell fate is complex. The link between impaired autophagic flux and proliferation needs to be further validated.

Although the HBsAg-transgenic mouse model is widely used to study the mechanism by which high HBsAg expression induces HCC in chronic HBV infection, its limitations should be carefully considered. The Alb-HBs model clarified the pathogenic properties of HBsAg, but the intracellular accumulation did not fully reflect the natural distribution of HBsAg. Finally, as proof of principle, a small number of HCC samples confirmed the role of ER stress and impaired autophagy in HBV-associated HCC. However, a larger sample size and multicentre analysis of clinical samples are needed to develop a potential targeted therapeutic strategy.

In conclusion, this study demonstrated that HBsAg induced UPR and impaired autophagy not only in HBsAg-transgenic mice and HBsAg-overexpressing Hepa1-6 cells but also in patients with HBV-related HCC. These findings help to explain the intracellular events mediated by HBsAg. The present data suggest that restoring LAMP2 function in chronic HBV infection may have both antiviral and anti-cancer effects.

Abbreviations

ER, endoplasmic reticulum; GSEA, gene set-enrichment analysis; HBsAg, hepatitis B surface antigen; HCC, hepatocellular carcinoma; IF, immunofluorescence; IHC, immunohistochemical; LAMP1/2, lysosome-associated membrane protein 1/2; PMH, primary murine hepatocytes; TUDCA, tauroursodeoxycholic acid; UPR, unfolded protein response.

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Conflicts of interest

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Performed the experiments and drafted the manuscript: YL. Produced and analysed the data: XL, SS and LTM. Provided and performed clinical samples: HD and BW. Performed paraffin section: HAB. Discussed the manuscript: ML, HW and HS. Designed, revised and provided financial support: RB. All authors reviewed and approved the final manuscript.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2024.101012.

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Author names in bold designate shared co-first authorship

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