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Hepatitis B virus X protein promotes MAN1B1 expression by enhancing stability of GRP78 via TRIM25 to facilitate hepatocarcinogenesis

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BACKGROUND: GRP78 has been implicated in hepatocarcinogenesis. However, the clinical relevance, biological functions and related regulatory mechanisms of GRP78 in hepatitis B virus (HBV)-associated hepatoma carcinoma (HCC) remain elusive.
METHODS: The association between GRP78 expression and HBV-related HCC was investigated. The effects of HBV X protein (HBX) on GRP78 and MAN1B1 expression, biological functions of GRP78 and MAN1B1 in HBX-mediated HCC cells and mechanisms related to TRIM25 on GRP78 upregulation to induce MAN1B1 expression in HBX-related HCC cells were examined.
RESULTS: GRP78 expression was correlated with poor prognosis in HBV-positive HCC. HBX increased MAN1B1 protein expression depending on GRP78, and HBX enhanced the levels of MAN1B1 to promote proliferation, migration and PI3-K/mTOR signalling pathway activation in HCC cells. GRP78 activates Smad4 via its interaction with Smad4 to increase MAN1B1 expression in HBX-expressing HCC cells. TRIM25 enhanced the stability of GRP78 by inhibiting its ubiquitination. HBX binds to GRP78 and TRIM25 and accelerates their interaction of GRP78 and TRIM25, leading to an increase in GRP78 expression.
CONCLUSIONS: HBX enhances the stability of GRP78 through TRIM25 to increase the expression of MAN1B1 to facilitate tumorigenesis, and we provide new insights into the molecular mechanisms underlying HBV-induced malignancy.

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INTRODUCTION

The hepatitis B virus (HBV) is the leading etiologic agent responsible for the development of some liver disorders, including hepatitis and fibrosis, as well as cirrhosis and hepatoma carcinoma (HCC) [1, 2]. Multiple factors, such as the integration of the viral genome into host genomic DNA, epigenetic dysregulation caused by HBV and chronic inflammation stimulated by the virus, contribute to the occurrence and development of HCC [3, 4]. HBX, a vital nonstructural protein encoded by the virus, is also essential for HCC initiation and progression. Especially, accumulating evidence suggests that HBX utilises diverse cellular factors to modulate various cellular events, including growth, autophagy, metastasis, post-transcriptional modification and non-coding RNA regulation, to facilitate hepatocarcinogenesis [5–9]. However, the exact mechanisms that benefit HCC development induced by the viral protein remain unclear.

The 78 kDa glucose-regulated protein (GRP78) is an endoplasmic reticulum chaperone activated by endoplasmic reticulum stress to maintain organelle homoeostasis [10]. The increase in GRP78 expression has been confirmed in various malignancies and plays critical roles in cancer growth, migration, angiogenesis, apoptosis and therapeutic resistance [11, 12]. Evidence suggests that GRP78 is responsible for HBV infection [13–15]. Moreover, the role of GRP78 in HCC development has been reported [16, 17]. Particularly, Li et al. showed that HBX could interact with GRP78 to prevent cellular death and negatively regulate DNA repair [18]. Nevertheless, the information regarding the clinical relevance of GRP78 in HBV-associated HCC and the biological roles of GRP78 in HBX-induced HCC development induced by HBX remains elusive.

MAN1B1 is responsible for encoding α-1,2-mannosidase and is implicated in the development of different cancers [19, 20]. However, the potential role of MAN1B1 and related regulatory mechanisms in HBV-related HCC development has not been well examined. In this study, we assessed whether GRP78 participated in the modulation of MAN1B1 in HBX-expressing HCC cells and the molecular mechanisms that contributed to the increase in GRP78 induced by HBX. Our study provides new insights into the mechanisms of tumorigenesis induced by HBX and offers a novel opportunity to develop a potential molecular target for the treatment of HBV-associated HCC.

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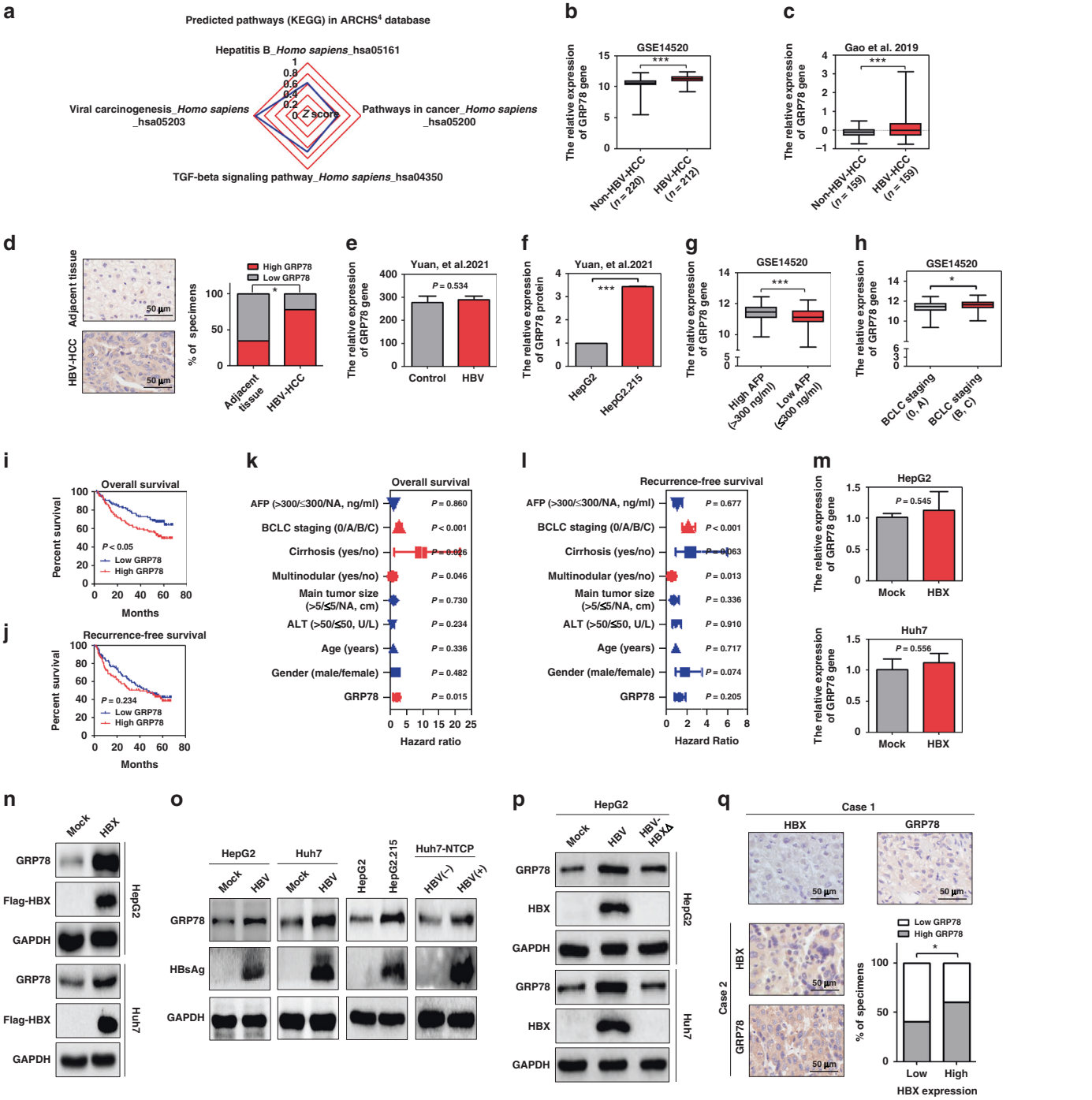
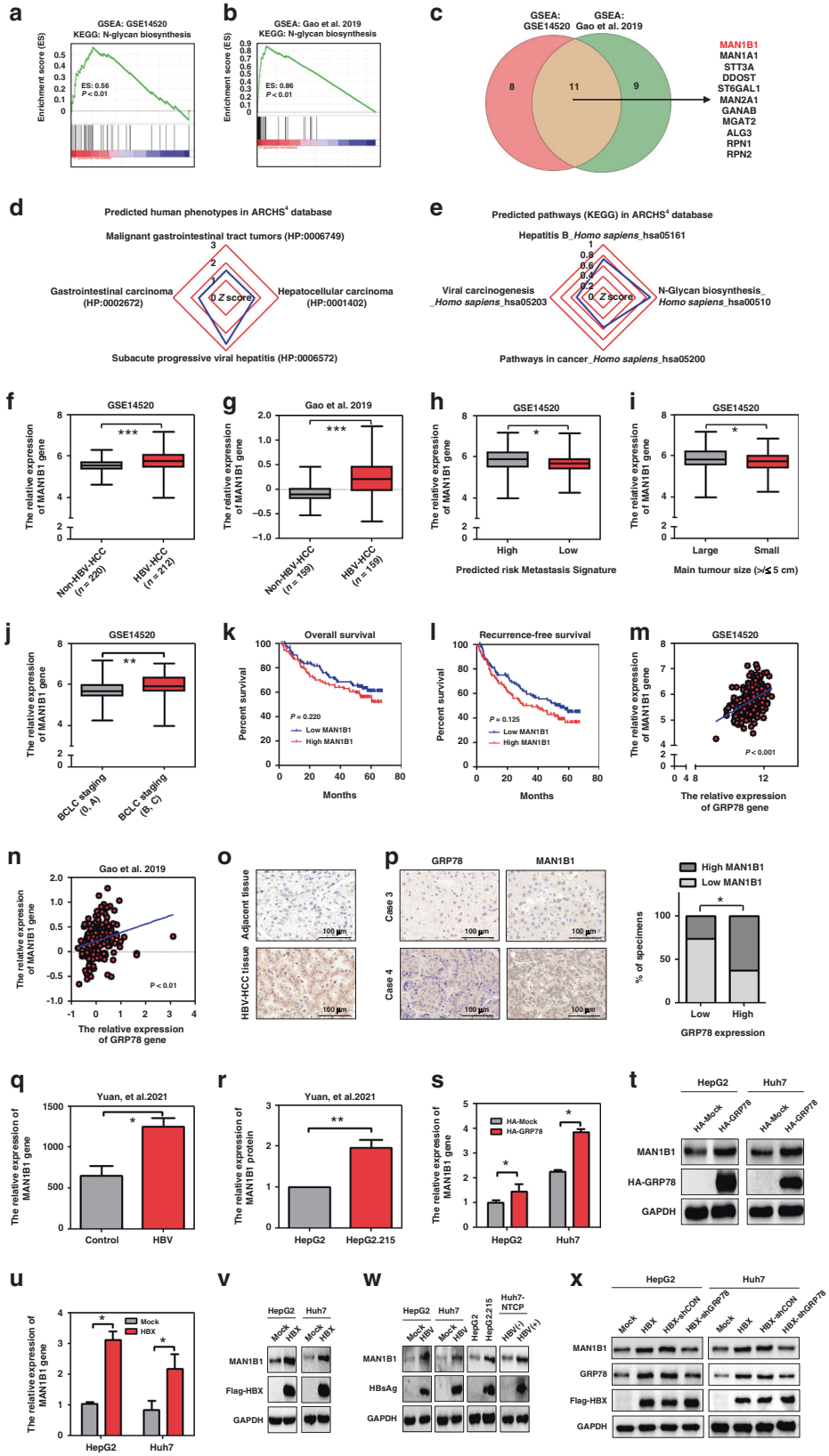


Fig. 1 Clinical importance of GRP78 in HBV-related HCC and HBX contribute to GRP78 expression. **a** The predicted pathway is related to GRP78 in the ARCHS[4] database. **b** Expression of the GRP78 gene in the GSE14520 cohort. **c** Expression of GRP78 gene in the Gao et al. cohort. **d** Expression of GRP78 in adjacent tissues and HBV-positive HCC tissues assessed by IHC. **e** Relative expression of the GRP78 gene in HBV-infected HCC cells and control cells extracted from the transcriptome data from Yuan et al. **f** Relative expression of GRP78 protein in HepG2 cells (HBV−) and HepG2.215 cells (HBV+) extracted from the proteomic data from Yuan et al. **g** The association between AFP and GRP78 in HBV-relative HCC tissues in GSE14520. **h** The relationship between BCLC stages and GRP78 in HBV-relative HCC tissues in GSE14520. **i** Based on the GSE14520 cohort, the association between GRP78 and the overall survival of HBV-positive patients with HCC was assessed, dependent on univariate survival analysis. **j** Dependent on the GSE14520 cohort, the association between GRP78 and the recurrence-free survival of HBV-positive patients with HCC relied on univariate survival analysis. **k** Dependent on the GSE14520 cohort, the association between multiple clinical parameters and the overall survival of HBV-associated patients with HCC was investigated, dependent on multivariate survival analysis. **l** Based on the GSE14520 cohort, an investigation of the relationship between multiple clinical parameters and the recurrence-free survival of HBV-expressing patients with HCC was conducted, dependent on multivariate survival analysis. **m** GRP78 gene expression induced by HBX assessed by real-time PCR. **n** GRP78 protein expression mediated by HBX assessed by western blot experiment. **o** Protein expression of GRP78 in HBV plasmid-transfected hepatoma cells and its control plasmid-transfected cells, in HepG2.215 and HepG2 cells, and in Huh7-NTCP cells with or without HBV particle infection. **p** Role of HBX in GRP78 protein expression induced by HBV in hepatoma cells. **q** Relationship of GRP78 with HBX expression in HBV-positive hepatoma tissues. Mock: cells transfected with control plasmid, HBV: cells transfected with HBV infection. HBX: cells transfected with HBX plasmid, HBV-HBXΔ: cells transfected with HBV plasmid with HBX gene deletion mutation. $^{*}P < 0.05$, $^{***}P < 0.001$.



MATERIALS AND METHODS

The materials and methods used for the reagents and cell transfection, clinical samples, real-time polymerase chain reaction (PCR) and other information, are provided in the Supplementary Materials.

RESULTS

HBX upregulates protein expression of GRP78, which is associated with the poor prognosis of HBV-associated HCC

Based on bioinformatic analysis using the ARCHS^4 database [21], we predicted the KEGG pathways associated with GRP78. The results

Fig. 2 Influence of GRP78 on the expression of MAN1B1 induced by HBX in HCC cells. a Association of GRP78 with N-glycan biosynthesis based on GSEA analysis in the GSE14520 cohort. **b** Association of GRP78 with N-glycan biosynthesis based on GSEA analysis in the Gao et al. cohort. **c** Common genes related to N-glycan biosynthesis between two cohorts. **d** Predicted human phenotypes related to MAN1B1 in ARCHS^4 database. **e** Predicted pathways related to MAN1B1 in the ARCHS^4 database. **f** Expression of MAN1B1 gene in the GSE14520 cohort. **g** Expression of MAN1B1 gene in the Gao et al. cohort. **h** Association of MAN1B1 with predicted-risk metastasis signature in the GSE14520 cohort. **i** Association of MAN1B1 with main tumour size in the GSE14520 cohort. **j** Association of MAN1B1 with BCLC stages was assessed in the GSE14520 cohort. **k** Association of MAN1B1 with the overall survival of HBV-positive patients with HCC was examined, dependent on univariate survival analysis. **l** Relying on univariate survival analysis, the association of MAN1B1 with the recurrence-free survival of HBV-positive patients with HCC was investigated. **m** Association of GRP78 with MAN1B1 gene in the GSE14520 cohort. **n** Association of GRP78 with MAN1B1 gene in the Gao et al. cohort. **o** Expression of MAN1B1 protein in adjacent tissues and HBV-related HCC tissues measured by IHC. **p** Association of GRP78 with MAN1B1 in HBV-associated HCC tissues tested by IHC. **q** Relative expression of MAN1B1 gene in HBV-infected HCC cells and control cells extracted from transcriptome data from Yuan et al. **r** Relative expression of MAN1B1 protein in HepG2 cells (HBV−) and HepG2.215 cells (HBV+) extracted from the proteomic data from Yuan et al. **s** Real-time PCR was used to assess expression of MAN1B1 gene mediated by GRP78. **t** The effect of GRP78 on MAN1B1 protein expression was detected by western blot. **u** Real-time PCR was utilised to examine the role of HBX in MAN1B1 protein expression. **v** Influence of HBX on MAN1B1 protein expression assessed by western blot. **w** Protein expression of MAN1B1 in HBV plasmid-transfected hepatoma cells and its control plasmid-transfected cells, in HepG2.215 and HepG2 cells and in Huh7-NTCP cells with or without HBV particle infection. **x** Role of GRP78 in the regulation of MAN1B1 expression induced by HBX. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

showed that GRP78 was related to hepatitis B, viral carcinogenesis, pathways in cancer and the TGF-β signalling pathway (Fig. 1a). These predictions imply that GRP78 is required for HBV-mediated tumorigenesis, and its role might be associated with the TGF-β signalling pathway. We assessed the gene expression levels of GRP78 in adjacent normal tissues and HBV-associated HCC tissues from the GSE14520 and Gao et al. cohorts [22–24]. The results revealed that compared with adjacent tissues, GRP78 expression was upregulated in HBV-positive HCC tissues (Fig. 1b, c). We detected GRP78 protein expression by using immunohistochemistry (IHC) in adjacent tissues, as well as in HBV-positive HCC tissues. As shown in Fig. 1d, GRP78 protein expression was higher in HBV-positive HCC tissues than in adjacent tissues. Dependent on transcriptomic and proteomic data from Yuan et al. [25, 26], HBV was found to enhance GRP78 protein expression, but not GRP78 gene expression, in HCC cells (Fig. 1e, f). The clinical relevance of GRP78 in HBV-related HCC was also investigated. Based on the GSE14520 cohort, we discovered that compared with the HBV-positive patients with HCC with low AFP levels, GRP78 expression was upregulated in patients with high AFP levels (Fig. 1g). Additionally, the levels of GRP78 were higher in HBV-positive patients with HCC with high BCLC stages (B and C) than in those with low BLCL stages (0 and A) (Fig. 1h). Furthermore, based on univariate and multivariate survival analysis and recurrence analysis, GRP78 was significantly correlated with poor overall survival in patients with HCC with HBV infection (Fig. 1i–l). The aforementioned results indicate that GRP78 was implicated in HBV-associated HCC development, the virus was responsible for its upregulation in HCC, and overexpression of GRP78 was correlated with the poor prognosis of the disease.

HBX has been shown to contribute to an increase in GRP78 in hepatocytes [18]. We detected the effect of HBX on GRP78 expression, and the results revealed that HBX accelerated GRP78 protein expression but not GRP78 gene expression (Fig. 1m, n). The role of HBX in GRP78 expression in HBV-mediated HCC cells was also examined. Consistent with the results of Yuan et al. [25, 26], our results demonstrated that HBV was capable of upregulating GRP78 protein in HCC cells (Fig. 1o). After the HBX gene is deleted in the viral genome, the role of HBV in GRP78 expression was suppressed in HCC cells (Fig. 1p). Moreover, as shown in Fig. 1q, in HBV-related HCC, the expression of GRP78 was higher in HBX-high expression tissues compared with its level in the HBX-low expression tissues. In summary, these results demonstrate that GRP78 is closely related to the development of HBV-associated HCC and HBX facilitates GRP78 upregulation at the protein level in HBV-positive hepatoma cells.

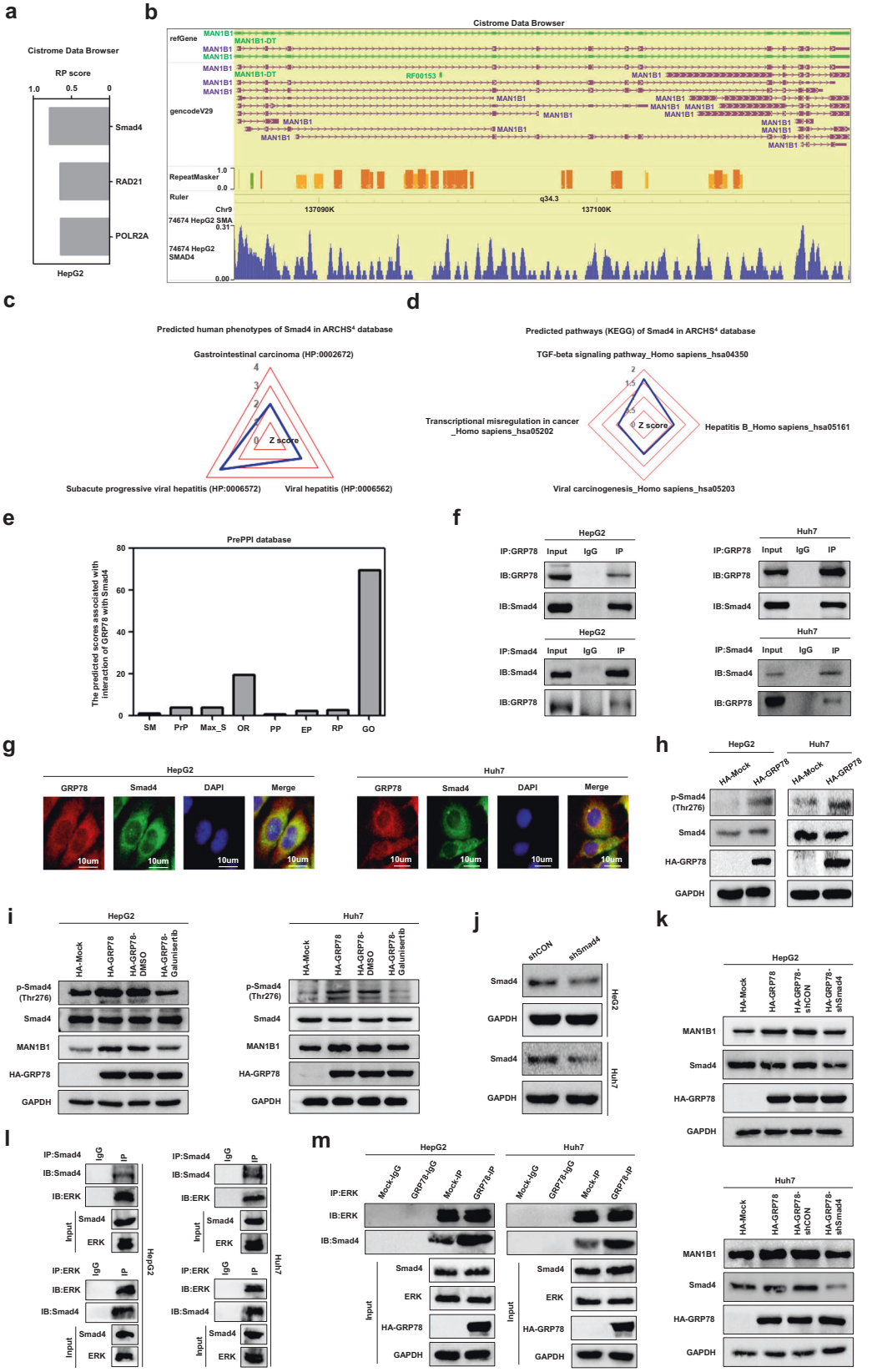
GRP78 facilitates the growth and migration of HBX-expressing hepatoma cells

In our prior work, we have shown that HBX can accelerate the proliferation and metastasis of hepatoma cells [27, 28]. In this study, we examined the biological role of GRP78 in HBX-expressing hepatoma cells. We constructed a special shRNA targeting GPR78, and as Supplementary Fig. 1A shows, this shRNA can suppress GRP78 protein expression in hepatoma cells. Based on the CCK-8 assay, HBX enhanced the growth of HCC cells (Supplementary Fig. 1B). Clone formation experiments also indicated that HBX increased HCC cell proliferation. After inhibiting GRP78 expression using its specific shRNA, the growth efficiency of HBX-related HCC cells was blocked in hepatoma cells (Supplementary Fig. 1C). We also examined the effect of GRP78 on the migration of HBX-expressing HCC cells. The results of the transwell assay showed that HBX enhanced HCC cell migration. When GRP78 was knocked down, the migration efficiency of HBX-expressing hepatoma cells decreased (Supplementary Fig. 1D). The results of the wound healing experiment also indicated that after the knockdown of GRP78, the cellular migration ability of HCC cells stimulated by HBX was restricted (Supplementary Fig. 1E). In addition, we investigated the role of GRP78 in HBX-induced HCC development in vivo. As Supplementary Fig. 1F shows, the volume and weight of the HBX-expressing tumours in nude mice were greater than those of control tumours. After the specific shRNA suppressed GRP78, the ability of HBX-positive hepatoma cells to form tumours was lower than that of HBX-expressing hepatoma cells treated with control shRNA (Supplementary Fig. 1F–H).

HBX enhances MAN1B1 expression via GRP78 in HCC cells

Based on GSEA analysis [29], we found that GRP78 was related to the gene set with N-glycan biosynthesis in HBV-associated HCC tissues in the GSE14520 and the Gao et al. cohorts (Fig. 2a, b). We identified the common genes related to N-glycan biosynthesis in these two cohorts. As shown in Fig. 2c, 11 common genes were identified in both cohorts. Among the identified common genes, we chose MAN1B1 for further investigation. Because based on the ARCHS^4 database [21], it was shown that MAN1B1 was related to the predicted human phenotypes of malignant gastrointestinal tract tumours, gastrointestinal carcinoma, hepatocellular carcinoma and subacute progressive viral hepatitis (Fig. 2d). Besides these, MAN1B1 was associated with predicted pathways in hepatitis B, viral carcinogenesis, N-glycan biosynthesis and pathways in cancer (Fig. 2e). These predictions implied that MAN1B1 may play important roles in HBV-mediated tumorigenesis.

We explored MAN1B1 gene expression in the GSE14520 and Gao et al. cohorts [22–24]. Compared with adjacent tissues, MAN1B1 gene expression was upregulated in HBV-associated HCC tissues (Fig. 2f, g). Based on the GSE14520 cohort, MAN1B1 expression was upregulated in patients with high predicted-risk metastasis signature, large tumour size and high BCLC stages (Fig. 2h, i, j). However, no significant relationship of overall survival



and recurrence-free survival with MAN1B1 was found in patients with HBV with HCC (Fig. 2k, l). We also assessed the association between GRP78 and MAN1B1 expression in HBV-associated HCC tissues. As shown in Fig. 2m, n, GRP78 was significantly positively correlated with MAN1B1 gene expression in HBV-associated HCC in these two cohorts. On the basis of our collected HBV-associated HCC tissues, MAN1B1 was also increased in HBV-associated HCC tissues compared with adjacent tissues (Fig. 2o). Additionally, in

Fig. 3 GRP78 interacts with and activates Smad4 to facilitate MAN1B1 expression in hepatoma cells. a Proteins can interact with the promoter of MAN1B1 in the Cistrome data browser database. **b** Detailed information on the interaction of Smad4 with the promoter of MAN1B1. **c** Predicted human phenotypes related to Smad4 in the ARCHS^4 database. **d** Predicted pathways related to Smad4 in the ARCHS^4 database. **e** The prediction of the interaction of GRP78 with Smad4 was based on the PrePPI database. **f** Co-IP assay was used to investigate the interaction between GRP78 and Smad4 in HCC cells. **g** The colocation of GRP78 with Smad4 in hepatoma cells was examined based on the immunofluorescence assay. **h** Activation of Smad4 was mediated by GRP78 in hepatoma cells. **i** Inhibition of Smad4 activation by Galunisertib on MAN1B1 expression induced by GRP78 in HCC cells. **j** Inhibition of Smad4 protein on its specific shRNA in HCC cells. **k** Suppression of Smad4 by its shRNA on MAN1B1 expression in GRP78-overexpressing HCC cells. **l** Interaction of ERK with Smad4 in HCC cells. **m** GRP78 promotes the interaction between ERK and Smad4 in HCC cells.

HBV-associated HCC, compared to GRP78-low expressing tissues, the expression of MAN1B1 protein was higher in GRP78-high expressing tissues (Fig. 2p). Furthermore, dependent on the data from Yuan et al. [25, 26], we found that HBV stimulated MAN1B1 expression at the gene and protein levels in HCC cells (Fig. 2q, r). In summary, these results indicate that MAN1B1 expression was increased and closely correlated with GRP78 in HBV-associated HCC, and the virus contributed to the upregulation of MAN1B1 expression.

We tested whether GRP78 promotes MAN1B1 expression in hepatoma cells. The results demonstrated that MAN1B1 expression was increased in GRP78-overexpressing HCC cells at the gene and protein levels (Fig. 2s, t). We examined whether HBX induced MAN1B1 expression in HCC cells as well. As shown in Fig. 2u, v, HBX enhanced the expression of MAN1B1 at the gene and protein levels. We also assessed the role of HBV in MAN1B1 expression. The results showed that HBV upregulated MAN1B1 in hepatoma cells. After the inhibition of GRP78 by specific shRNA, the expression of MAN1B1 in HBX-expressing HCC cells was significantly suppressed (Fig. 2x). These results demonstrate that the viral protein can increase MAN1B1 expression via GRP78 in HCC cells.

MAN1B1 facilitates the growth, migration and PI3-K/mTOR signalling pathway activation of HCC cells

Next, we investigated the effect of MAN1B1 on the biological processes of hepatoma cells. We constructed a MAN1B1 expression plasmid and transfected it into HCC cells. The expression of exogenous MAN1B1 was detected in HCC cells (Supplementary Fig. 2A). CCK-8 assay revealed that MAN1B1 enhanced the growth of hepatoma cells (Supplementary Fig. 2B). The results of the colony formation assay also suggest that MAN1B1 enhanced HCC cell proliferation (Supplementary Fig. 2C). The results from the transwell and wound healing assays suggest that MAN1B1 can elevate the migration of HCC cells (Supplementary Fig. 2D, E). Moreover, the results of the subcutaneous tumorigenesis experiment in nude mice revealed that MAN1B1 can promote the growth of HCC cells in vivo (Supplementary Fig. 2F–H). Until now, the activation of signalling pathways stimulated by MAN1B1 has not been well identified. Based on the ARCHS^4 database, a prediction was that MAN1B1 was associated with the PI3-K and mTOR signalling pathways (Supplementary Fig. 2I), which are responsible for cellular growth and migration [30]. We explored whether MAN1B1 could initiate PI3-K and mTOR signalling pathway activation in HCC cells. As expected, based on the detection of the phosphorylation levels of AKT and mTOR, the sensitisation levels of the PI3-K and mTOR signalling pathways were increased in MAN1B1-expressing hepatoma cells (Supplementary Fig. 2J). These results imply that PI3-K and mTOR signalling pathway activation may be closely correlated to the increased proliferation and motion induced by MAN1B1 in HCC cells.

MAN1B1 facilitates the growth, migration and PI3-K/mTOR signalling pathway activation of HCC cells induced by HBX

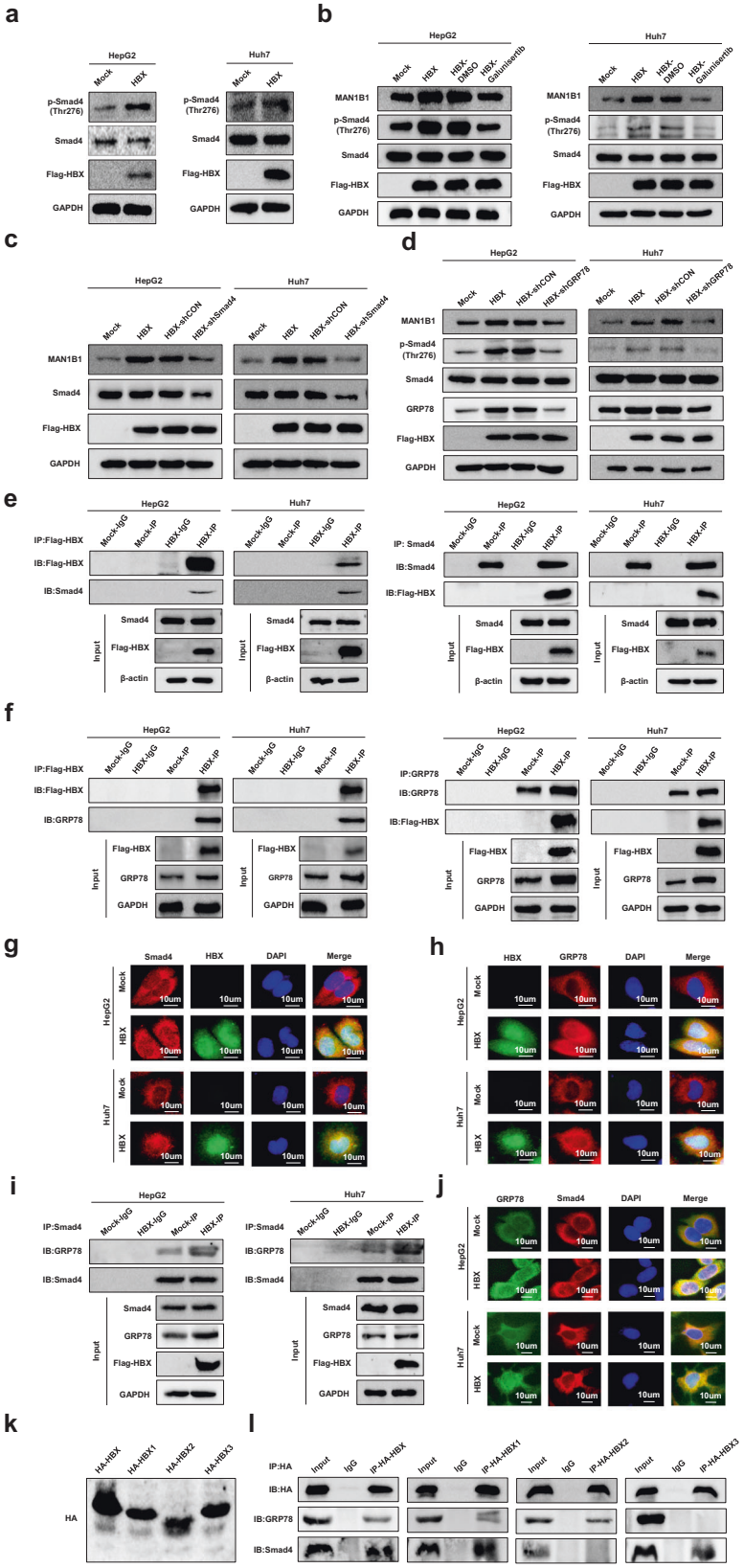
Subsequently, the effect of MAN1B1 on biological processes and signalling activation induced by HBX was assessed. A specific shRNA targeting MAN1B1 was constructed, and the ability of shRNA to inhibit MAN1B1 expression was assessed (Supplementary Fig. 3A). Consistent with the results of our published studies [27, 28], the results showed that the viral protein was capable of increasing HCC cell growth. After MAN1B1 suppression, enhanced cellular proliferation mediated by the viral protein was inhibited (Supplementary Fig. 3B, C). In addition to proliferation, HBX can elevate the migration ability of HCC cells. Furthermore, upon MAN1B1 inhibition, the increased migration efficiency mediated by HBX in HCC cells was downregulated (Supplementary Fig. 3D, E). As shown in Supplementary Fig. 3F–H, HBX also increased the growth of HCC cells in nude mice. After MAN1B1 was knocked down by its specific shRNA, the growth of hepatoma cells induced by the viral protein was downregulated in vivo. Studies have indicated that HBX can stimulate the activation of PI3-K, as well as mTOR signalling pathways [31, 32]. Our results showed that the viral protein also sensitised the activation of these signalling pathways (Supplementary Fig. 3I). With the knockdown of MAN1B1, the activation of these signalling pathways was downregulated (Supplementary Fig. 3J). These results indicate that PI3-K, as well as mTOR signalling, might be involved in the modulation of MAN1B1-mediated biological processes in HBX-stimulated HCC.

GRP78 interacts with Smad4 and promotes Smad4 activation to facilitate MAN1B1 expression in hepatoma cells

Based on the Cistrome data browser database, a resource of human cis-regulatory information obtained from ChIP-sequencing [33], we discovered that Smad4, RAD21 and POLR2A can interact with the promoters of GRP78 in HepG2 cells (Fig. 3a, b). We chose Smad4 for further study because, as aforementioned, GRP78 is predicted to be related to the TGF-β signalling pathway, and Smad4 is a vital downstream molecule of the TGF-β signalling pathway [34]. This implies that Smad4 may be necessary for the modulation of MAN1B1 gene expression mediated by GRP78. Moreover, based on the ARCHS^4 database [21], Smad4 is related to human phenotypes, including gastrointestinal carcinoma, subacute progressive viral hepatitis and viral hepatitis (Fig. 3c). Smad4 was also predicted to be involved in the TGF-β signalling pathway, transcriptional dysregulation in cancer, hepatitis B and viral carcinogenesis (Fig. 3d). These predicted human phenotypes and pathways imply that Smad4 is essential for HBV-mediated HCC development. In addition, based on the PrePPI database [35], GRP78 has the potential to interact with Smad4 (Fig. 3e). These findings suggest that GRP78 may interact with and activate Smad4 to facilitate MAN1B1 expression in HCC cells.

We also investigated the interaction between GRP78 and Smad4 in hepatoma cells. Co-immunoprecipitation (Co-IP) assays showed that GRP78 can bind to Smad4 in HCC cells (Fig. 3f). The immunofluorescence assay demonstrated that GRP78 and Smad4 were colocalized in hepatoma cells (Fig. 3g). Furthermore, the activation of Smad4 at Thr276 was increased in GRP78-overexpressing hepatoma cells (Fig. 3h). These results indicate that GRP78 interacts with and activates Smad4 in hepatoma cells.

Next, we assessed the effects of Smad4 on MAN1B1 expression in GRP78-overexpressing HCC cells. As aforementioned, Smad4 is a functional downstream molecule of the TGF-β signalling



pathway [34]. We used Galunisetib to suppress the sensitisation of the TGF-β signalling pathway to block the activity of Smad4. The results demonstrated that after incubating hepatoma cells with Galunisetib, the activation of Smad4 at the Thr276 site significantly declined. Additionally, the expression of MAN1B1 was down-regulated in GRP78-overexpressing HCC cells (Fig. 3i). In this study, a specific shRNA against Smad4 was also constructed, and the role of the specific RNA in the inhibition of Smad4 protein was

Fig. 4 Role of smad4 in the expression of GRP78 in HBX-expressing hepatoma cells. a Activation of Smad4 in HBX-expressing hepatoma cells. **b** Suppression of Smad4 activation by Galunisertib on MAN1B1 expression in HBX-associated HCC cells. **c** Suppression of Smad4 by its shRNA on the expression of MAN1B1 in HBX-expressing HCC cells. **d** Knockdown of GRP78 by its shRNA on Smad4 activation and MAN1B1 expression in HBX-associated HCC cells. **e** The interaction of HBX and Smad4 was assessed by the Co-IP experiment in HCC cells. **f** The interaction of HBX and GRP78 was assessed by Co-IP assay in hepatoma cells. **g** Relying on the immunofluorescence assay, the colocation of HBX with Smad4 was assessed in HCC cells. **h** Dependent on the immunofluorescence experiment, the colocation of HBX with GRP78 in HCC cells was investigated. **i** The interaction of Smad4 and GRP78 was assessed by Co-IP assay in HBX-positive HCC cells and control cells. **j** The colocation of Smad4 with GRP78 in HCC cells in HBX-positive HCC cells and control cells based on immunofluorescence assay. **k** Expression of HBX and its mutants in HEK293T cells. **l** Interaction of HBX and its mutants with GRP78 and Smad4 in HEK293T cells.

confirmed (Fig. 3j). Furthermore, when shRNA significantly suppressed Smad4 expression, the expression of MAN1B1 induced by GRP78 decreased (Fig. 3k).

In summary, we observed that via Smad4, GRP78 elevated MAN1B1 expression in HCC cells. Another study indicated that ERK was responsible for the activation of Smad4 at Thr276 [36]. Based on the Co-IP assay, we found that ERK interacted with Smad4 (Fig. 3l). Furthermore, GRP78 promoted the interaction between ERK and Smad4 (Fig. 3m). These results indicate that GRP78 promotes Smad4 activation by enhancing the interaction between ERK and Smad4 in HCC cells.

Smad4 contributes to the upregulation of MAN1B1 induced by GRP78 in HBX-expressing HCC cells

HBX has been reported to accelerate the activation of Smad4 to facilitate HCC development [37]. In this study, we assessed the effects of HBX on Smad4 activation. As shown in Fig. 4a, compared with the control cells, the phosphorylation levels of Smad4 were upregulated in HBX-expressing HCC cells. After the incubation of hepatoma cells with Galunisetib, the activation of Smad4 was restrained. MAN1B1 expression was also downregulated in HBX-expressing HCC cells (Fig. 4b). In addition, the treatment of HBX-expressing hepatoma cells with Smad4 specific shRNA, MAN1B1 expression was suppressed (Fig. 4c). We also assessed whether GRP78 contributes to the activation of Smad4 to induce MAN1B1 expression in HBX-expressing HCC cells. When GRP78 was suppressed by a specific shRNA, Smad4 activation decreased. The levels of MAN1B1 were also reduced in HCC cells (Fig. 4d).

HBX was shown to interact with Smad4 [38]. In addition, the binding of HBX to GRP78 has been reported [18]. We investigated whether the increased activation of Smad4 induced by HBX was related to the interaction of HBX with Smad4 and GRP78. As shown in Fig. 4e, f, the interaction of HBX with Smad4, as well as the interaction of the viral protein with GRP78, was observed in HCC cells. Additionally, the colocalization of HBX with Smad4, as well as the colocalization of HBX with GRP78, was observed in hepatoma cells (Fig. 4g, h). Furthermore, relying on Co-IP assay and compared with control HCC cells, increased interaction between GRP78 and Smad4 was observed in HBX-positive HCC cells (Fig. 4i). The colocalization of GRP78 with Smad4 was also observed in HBX-positive HCC cells and control cells (Fig. 4j). Next, we assessed whether HBX interacts with GRP78 and Smad4 in different regions of the viral protein. Based on the three HBX mutations constructed previously [28] (Fig. 4k), we discovered that HBX interacted with GRP78 at the C-terminal, and the viral protein bound to Smad4 at its N-terminal and C-terminal (Fig. 4i). These results suggest that HBX may form a protein complex with GRP78 and Smad4 in different regions, and dependent on GRP78, the HBX can elevate Smad4 activation to facilitate MAN1B1 expression in HCC cells.

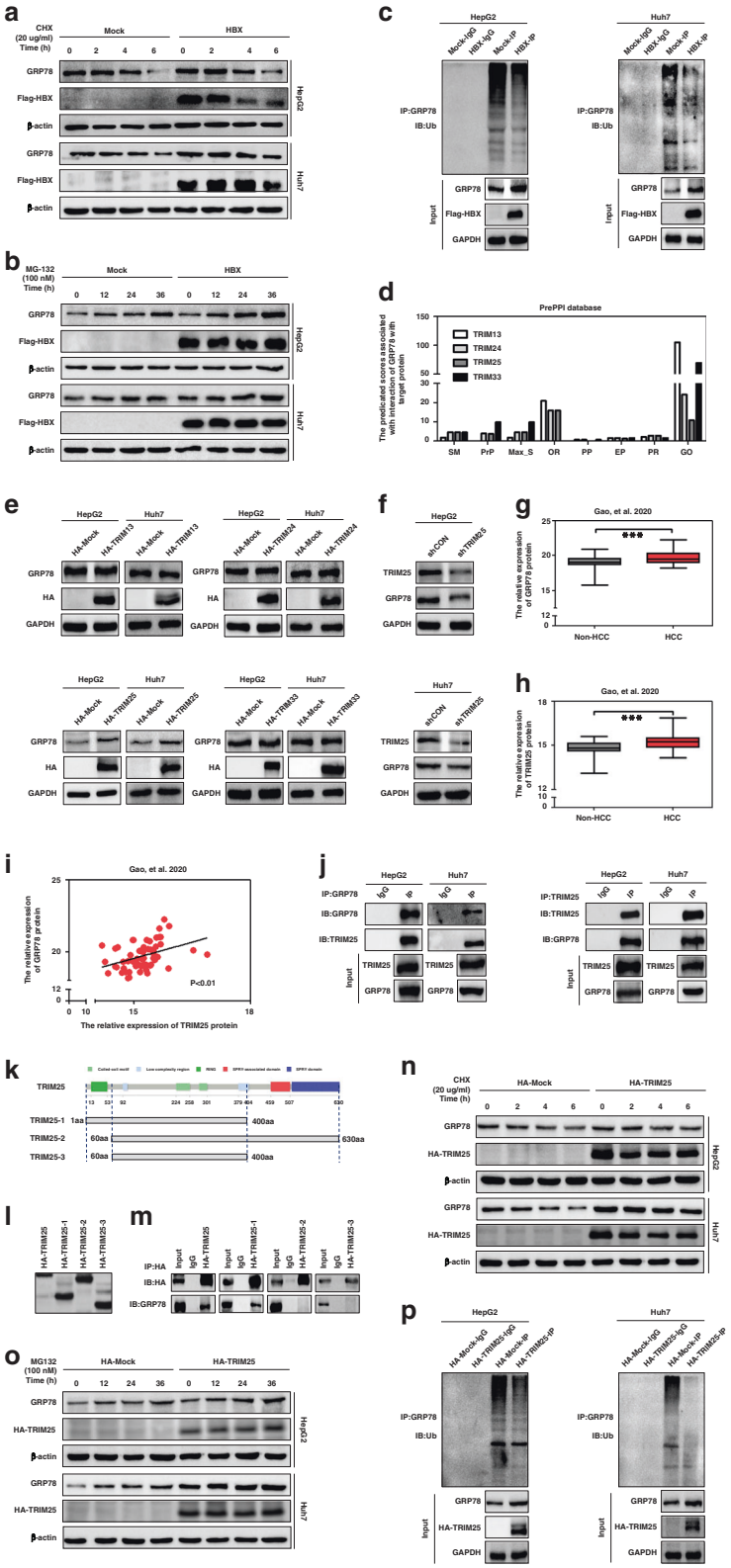
TRIM25 stabilises GRP78 protein by inhibiting its ubiquitination in HCC cells

As aforementioned, HBX promotes GRP78 expression at the protein level. However, the cellular factors involved in the modulation of GRP78 induced by the viral protein are unclear. The ubiquitin-proteasome system (UPS) has been demonstrated to be critical for protein degradation [39]. Depending on the UPS, various cellular proteins can be regulated by HBX [39]. We explored whether HBX could regulate the stability and ubiquitination of GRP78. The protein turnover rate of GRP78 was determined by treatment with cycloheximide (CHX), an inhibitor of protein synthesis. As shown in Fig. 5a, compared with control hepatoma cells, the turnover of GRP78 was significantly reduced in HCC cells overexpressing HBX. After treatment with MG132, a proteasome inhibitor, the increase in GRP78 in HBX-positive HCC cells was higher than that in the control HCC cells (Fig. 5b). However, the ubiquitination levels of GRP78 were lower in HBX-expressing HCC cells than in the control hepatoma cells (Fig. 5c). In summary, the evidence reveals that the viral protein can accelerate the stability of GRP78 by inhibiting its UPS-dependent degradation in HCC cells.

Evidence in the literature has indicated that multiple TRIM proteins participate in the modulation of cellular factors to promote HCC development [40–42]. TRIM proteins are also involved in various biological processes induced by HBX [43, 44]. We explored whether TRIMs contribute to the modulation of GRP78 protein induced by the viral protein. Based on the PrePPI database [35], TRIM13, TRIM24, TRIM25 and TRIM33 were predicted to interact with GRP78 (Fig. 5d). In our test of the role of these TRIMs on GRP78 expression, the TRIM expression plasmids were constructed and transfected into HCC cells. Among these four TRIMs, only TRIM25 enhanced GRP78 expression in HCC cells (Fig. 5e). A specific shRNA against TRIM25 was constructed, and silencing TRIM25 reduced the level of GRP78 protein substantially (Fig. 5f). We also examined the association between GRP78 and TRIM25 expression in HCC tissues. As shown in Fig. 5g, h, based on the proteomics data from Gao et al. [45], the expression of GRP78 and TRIM25 was increased in HCC tissues. Furthermore, a significant positive correlation between GRP78 and TRIM25 was observed (Fig. 5i). Based on the Co-IP assay, the interaction between TRIM25 and GRP78 was confirmed (Fig. 5j). In addition, we assessed which regions of the TRIM25 protein could bind to GRP78 and relied on the domain distribution in the TRIM25 protein (Fig. 5k), three TRIM25 mutants were constructed (Fig. 5l). Our results demonstrated that the N-terminus of TRIM25 interacts with GRP78 in HCC cells (Fig. 5m). We further tested whether TRIM25 affects GRP78 stability. We used CHX and MG132, which effectively inhibit the protein synthesis and proteolytic activity of the proteasome complex, as aforementioned, to evaluate the stability of GRP78 mediated by TRIM25. We found that TRIM25 was found to be responsible for GRP78 stability in hepatoma cells (Fig. 5n, o). Furthermore, the stability of GRP78 regulated by TRIM25 was related to its ability to inhibit GRP78 ubiquitination (Fig. 5p).

TRIM25 participates in the upregulation of GRP78 in HBX-expressing HCC cells

Based on the ARCHS[4] database, our prediction was that TRIM25 was associated with different human phenotypes, including recurrent viral infection and viral hepatitis (Fig. 6a). Additionally, TRIM25 was associated with pathways related to hepatitis B and



viral carcinogenesis (Fig. 6b). These predictions suggest that TRIM25 plays a critical role in HBV-associated HCC. Therefore, we investigated the effect of HBV on TRIM25 expression. Dependent on transcriptome data from Yuan et al. [26], we found that HBV had no significant effect on TRIM25 gene expression (Fig. 6c). Based on proteomic data from Yuan et al. [25], a slight upregulation of TRIM25 protein could be regulated by HBV (fold change less than 1.5-folds) (Fig. 6d). In this study, we tested whether HBX could promote the expression of TRIM25 to facilitate the expression of GRP78. Inconsistent with our expectations, the

Fig. 5 Role of TRIM25 in GRP78 stability in liver cancer cells. a HBX-positive hepatoma cells and control cells were given with CHX, and the levels of GRP78 were examined. **b** HBX-positive hepatoma cells, as well as control cells, were stimulated with MG-132, and GRP78 protein expression was investigated. **c** Role of HBX in GRP78 ubiquitination in HCC cells. **d** Predicted interaction of TRIMs with GRP78 based on the PrePPI database. **e** Role of different TRIMs in GRP78 expression in liver cancer cells. **f** Inhibition of TRIM25 by its specific shRNA on GRP78 expression in hepatoma cells. **g** Expression levels of GRP78 protein extracted from proteomic data from Gao et al. cohort. **h** Expression of TRIM25 protein extracted from proteomic data from Gao et al. cohort. **i** Association of TRIM25 with GRP78 protein in Gao et al. cohort. **j** Interaction of TRIM25 with Smad4 in HCC cells. **k** Sketch maps of TRIM25 mutants. **l** Expression of TRIM25 mutants in HEK293T cells. **m** Interaction of TRIM25 mutants with GRP78 in HEK293T cells. **n** TRIM25-positive hepatoma cells and control cells were treated with CHX, and the levels of GRP78 were assessed. **o** TRIM25-positive HCC cells and control cells were treated with MG132, and the levels of GRP78 were detected. **p** Effect of TRIM25 on GRP78 ubiquitination in hepatoma cells. ***$P < 0.001$.

results revealed that HBX had no significant effect on TRIM25 expression (Fig. 6e). We also examined the HBV-induced expression of TRIM25 in hepatoma cells. Consistent with the results of Yuan et al., our results demonstrated that an increase in TRIM25 expression could be modulated by HBV in HCC cells (Fig. 6f). These results indicate that although the virus was capable of increasing the expression of TRIM25, the effect of HBV on the expression of TRIM25 did not rely on HBX.

However, inhibition of TRIM25 expression in HBX-expressing HCC cells suppressed GRP78 levels (Fig. 6g). The literature indicates that HBX binds to TRIM25 in hepatoma cells [46]. In this study, we also investigated whether HBX was capable of interacting with both TRIM25 and GRP78. As expected that shown in Fig. 6h, HBX interacted with GRP78 and TRIM25. Furthermore, based on the Co-IP experiment, we found that HBX promoted the interaction between TRIM25 and GRP78 in hepatoma cells. Dependent on the immunofluorescence assay, the colocalization of GRP78 and TRIM25 was observed in HBX-expressing HCC cells and control cells (Fig. 6i). Furthermore, we found that the inhibition of TRIM25 was capable of reducing GRP78 stability mediated by HBX in HCC cells (Fig. 6j, k). Meanwhile, the ubiquitination levels of GRP78 induced by the viral protein were markedly upregulated when TRIM25 was suppressed in HBX-expressing HCC cells (Fig. 6l). Additionally, we tested whether the viral protein could interact with GRP78 and TRIM25 in different regions. As expected, HBX interacted with GRP78 through its C-terminus. However, the viral protein can bind to TRIM25 in its central domain (Fig. 6m). In summary, these results demonstrate that HBX can also bind to TRIM25 and GRP78 in different regions to promote GRP78 stability mediated by TRIM25 in hepatoma cells.

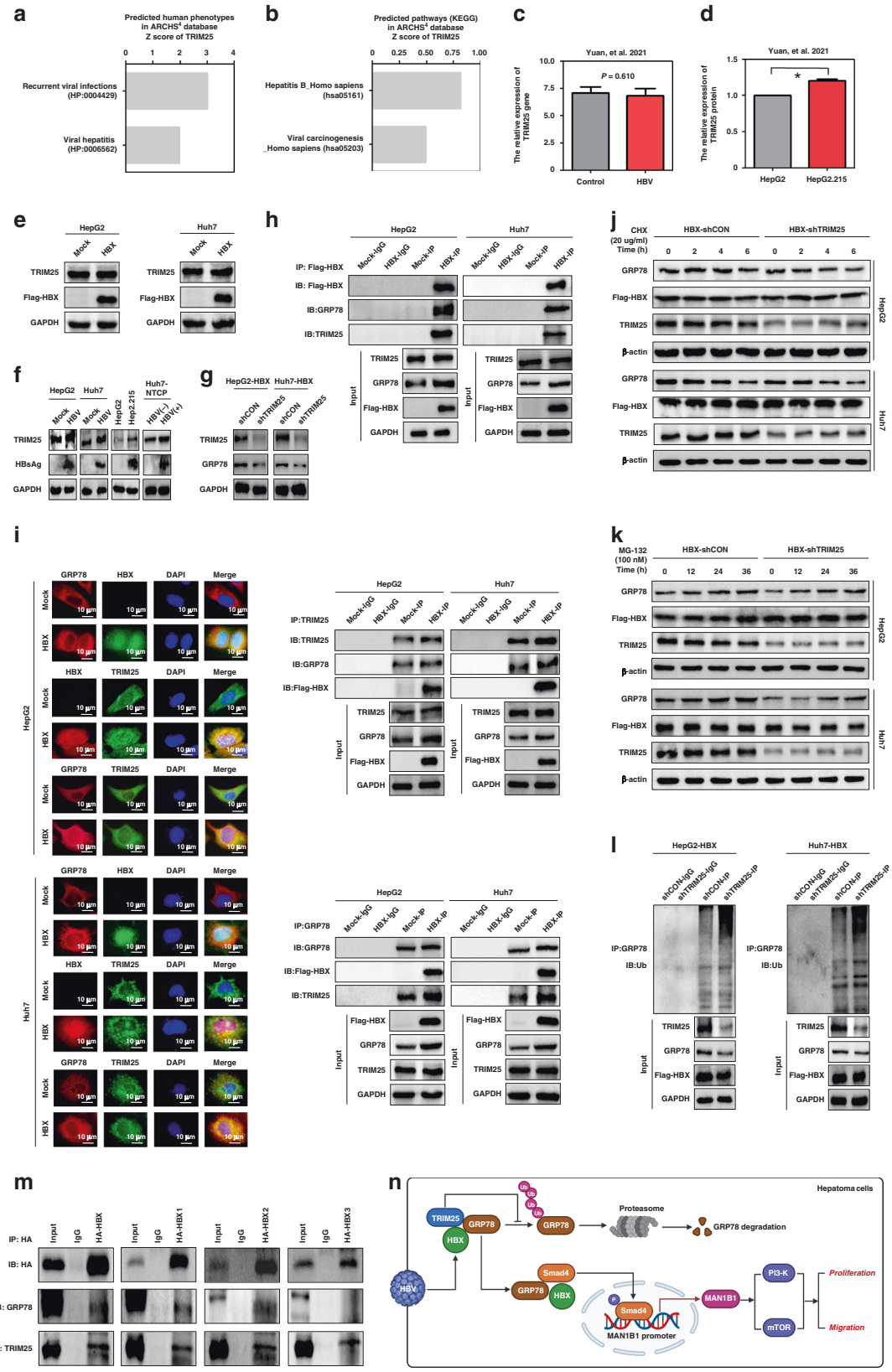
DISCUSSION

HBV-associated HCC is a common, highly lethal solid tumour. Despite the rapid development of multimodal treatments, the prognosis of HBV-associated HCC remains poor owing to its uncontrolled growth and invasiveness. It has been demonstrated that HBX is critical for the development of HCC [5]. However, the detailed mechanisms involved in HBX-mediated hepatocarcinogenesis have not been well assessed. Therefore, further exploration of the molecular basis for the initiation and progression of HCC mediated by the viral protein is of great value for the development of novel diagnostic and therapeutic strategies. Our results in this study revealed that HBX promoted the expression of GRP78 at the protein level. Mechanistically, HBX can bind to TRIM25 and GRP78 to inhibit the ubiquitination of GRP78 and enhance its stability in hepatoma cells. Furthermore, HBX interacts with GRP78, and Smad4, and based on GRP78, the viral protein enhances the activation of Smad4 to facilitate the expression of MAN1B1, which contributes to cellular growth and migration of HBX-expressing HCC cells (Fig. 6n).

As a multifunctional protein, GRP78 is critical for the development and pathogenesis of various tumours by regulating cellular growth, metastasis, differentiation, apoptosis and autophagy [11, 12]. Evidence indicates that GRP78 is essential for HBV infection [13, 15], and its biological functions are associated with HBX in HBV-HCC cells [18]. However, the effects and associated mechanisms of GRP78 on HBX-mediated hepatocarcinogenesis are largely unknown. In this study, we observed that the expression of GRP78 was significantly upregulated in HBV-associated HCC tissues. Furthermore, GRP78 was not only related to higher AFP levels and BCLC stages but was also responsible for the poor survival of HBV-positive patients with HCC. These results indicate that GRP78 can serve as a prognostic marker for patients with HCC with HBV infection. We examined the effect of HBX on GRP78 expression, and our results suggest that the viral protein only increased GRP78 expression at the protein level. GRP78 has been demonstrated to be essential for the proliferation and motility of HCC cells [16, 47]. Based on experiments to detect HCC cell growth and migration induced by HBX and GRP78, we found that HBX promoted cellular proliferation and movement through GRP78. Considering the importance of GRP78 in HCC, therapies are being developed to target GRP78 and represent a promising approach for combating HBV-associated HCC.

Next, we estimated the molecular basis responsible for the development of HCC mediated by GRP78. Dependent on GESA [29], we discovered that GRP78 was associated with the gene set related to N-glycan biosynthesis, and MAN1B1, a vital gene responsible for the biological process, was shown to be regulated by GRP78. Additionally, MAN1B1 has been shown to be upregulated and significantly related to the poor prognosis of patients with HCC [48], despite the importance of MAN1B1 in the development of HCC, the molecular mechanisms responsible for its regulation remain unclear. Particularly notable is that the involvement of MAN1B1 in HBV-triggered tumorigenesis has not been well investigated. Based on the bioinformatics analysis, MAN1B1 was found to be related to HBV infection and viral carcinogenesis. Furthermore, MAN1B1 expression was confirmed to be upregulated in HBV-positive HCC tissues and virus-infected hepatoma cells. In addition, our study revealed a vital role for GRP78 in increasing MAN1B1 expression to facilitate HCC cell proliferation and migration. Furthermore, we discovered that PI3-K and the mTOR signalling pathway could be activated by MAN1B1. Because the PI3-K and mTOR signalling pathways participate in modulating multiple cellular processes, including cellular proliferation, metabolism, motility and survival [30], we can reasonably speculate that these two signalling pathways play a critical role in the regulation of the biological processes of HCC cells mediated by MAN1B1.

The molecular mechanisms responsible for the increase in MAN1B1 mediated by GRP78 were not clear so far. According to the Cistrome data browser database [33], a downstream molecule of the TGF-β signalling pathway, Smad4 [34], was observed to interact with the promoters of MAN1B1. Based on the evidence from bioinformatics analysis and Co-IP assay, our results suggest that GRP78 can interact with Smad4 and GRP78 can activate Smad4 to enhance MAN1B1 expression. Recent studies have shown that HBX can interact with GRP78 to promote cellular survival [18]. In addition, the ability of HBX to interact with Smad4 and activate Smad4 to promote HCC development has been reported [37, 38]. Consistent with the results of these reports, we found that HBX not only binds to Smad4 but also interacts with



GRP78. Furthermore, the viral protein promotes the interaction between GRP78 and Smad4. More importantly, by relying on GRP78, HBX was capable of enhancing the activation of Smad4 to facilitate MAN1B1 expression in HCC cells.

We also explored cellular factors that contributed to the alteration of GRP78 expression mediated by HBX. Because multiple TRIM proteins have been reported to affect different biological processes mediated by HBX [43, 44], we were interested

Fig. 6 Effect of TRIM25 on the stability of GRP78 in HBX-positive HCC cells. a Predicted human phenotypes related to TRIM25 in the ARCHS[4] database. **b** Predicted pathways related to TRIM25 in the ARCHS[4] database. **c** Relative expression of TRIM25 gene in HBV-infected HCC cells and control cells extracted from the transcriptome data from Yuan et al. **d** Relative expression of GRP78 protein in HepG2 cells and HepG2.215 cells extracted from the proteomic data from Yuan et al. **e** Role of HBX in TRIM25 expression in HCC cells. **f** Protein expression of TRIM25 in HBV plasmid-transfected hepatoma cells and its control plasmid-transfected cells, in HepG2.215 and HepG2 cells, and in Huh7-NTCP cells with or with HBV particle infection. **g** Effect of TRIM25 knockdown by its specific shRNA on GRP78 expression in HBX-expressing HCC cells. **h** The interaction of HBX with Smad4 and TRIM25 was detected by the Co-IP experiment in HCC cells. **i** The colocation of HBX with Smad4 and TRIM25 was detected by immunofluorescence assay. **j** TRIM25 shRNA-positive HBX-HCC cells and HBX-HCC cells were treated with CHX, and the levels of GRP78 were detected. **k** TRIM25 shRNA-positive HBX-hepatoma cells and HBX-hepatoma cells were given with MG132, and the levels of GRP78 were assessed. **l** Effect of TRIM25 on GRP78 ubiquitination in HBX-expressing HCC cells. **m** Interaction of HBX and its mutants with GRP78 and TRIM25 in HEK293T cells. **n** Sketch map of the mechanisms responsible for the increase in GRP78 mediated by HBX via TRIM25 to upregulate the expression of MAN1B1 and then facilitate HCC cell proliferation and migration. $^*P < 0.05$.

in investigating whether TRIM proteins are involved in the expression of GRP78 induced by the viral protein. Bioinformatics analysis and associated experiments revealed that TRIM25 can bind to GRP78 and elevate its expression in hepatoma cells. Reports indicate that TRIM25 is crucial for the development of HCC [40, 49, 50]. Therefore, we assessed whether TRIM25 contributes to hepatocarcinogenesis by enhancing the expression of GRP78. Our results showed that TRIM25 contributes to the stability of GRP78 and inhibits its ubiquitination. We also examined whether HBX regulates TRIM25 expression in hepatoma cells. Although our findings suggest that HBX does not influence TRIM25 expression, the viral protein is capable of enhancing the interaction between TRIM25 and GRP78. More importantly, HBX interacts with GRP78 and TRIM25 in different regions to facilitate GRP78 upregulation. Furthermore, based on TRIM25, HBX can promote GRP78 stability by suppressing its ubiquitination. These results indicate that TRIM25 is essential for HBX-induced HCC development by accelerating expression levels of GRP78.

In conclusion, in this study, we comprehensively analysed the relationship between GRP78 and HBV-related HCC and the effect of HBX on GRP78 and associated molecular mechanisms responsible for the development of HBV-associated HCC. Our findings revealed that GRP78 is correlated with poor prognosis in HBV-associated HCC. Relying on GRP78, HBX increased MAN1B1 expression. Mechanistically, HBX accelerated the levels of GRP78 by promoting its stability through TRIM25, which interacts with the viral protein. Particularly notable is that our study further elucidates the role of the HBX/GRP78/MAN1B1 regulatory axis in tumorigenesis and provides a promising therapeutic target for HBV-associated HCC. However, in this study, the investigation of the effect of HBX on GRP78, MAN1B1, and associated molecular mechanisms was mainly based on HBX plasmid-transfected cell models. Thus, to further explore the biological function of HBX, GRP78, MAN1B1, and downstream molecules in HBV-associated hepatocarcinogenesis, natural infection models, such as those for primary hepatocytes that can support HBV replication, are necessary. Additionally, clinical samples and associated animal models are required for further research.

DATA AVAILABILITY

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

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AUTHOR CONTRIBUTIONS

HY, NZ and TY performed the experiments, analysed the experimental data and contributed equally to the present study. HY and FK wrote the manuscript. QL, DY, XW, LM, DK and XL performed laboratory work. WH and DL contribute to data analysis. KZ, FK and RT designed the work and checked the revised manuscript.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The present study was approved by the ethics committee of Xuzhou Medical University and the Animal Care and Use Committee at Xuzhou Medical University.

CONSENT TO PUBLISH

Not applicable.

ADDITIONAL INFORMATION

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