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Original Article

# RP11-40C6.2 Inactivates Hippo Signaling by Attenuating YAP1 Ubiquitylation in Hepatitis B Virus-associated Hepatocellular Carcinoma

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## Abstract

***Background and Aims:*** Chronic hepatitis caused by hepatitis B virus (HBV) infection is a leading cause of hepatocellular carcinoma (HCC). We investigated the roles of oncogenic HBV infection-associated long noncoding RNAs in HCC. ***Methods:*** Bioinformatics analysis of data from the Cancer Genome Atlas (TCGA) was performed to screen potential oncogenic HBV-related lncRNAs. Next, we assessed their expression in clinical samples and investigated their correlation with clinical characteristics. The detailed oncogenic effects were analyzed by performing *in vitro* and *in vivo* studies. ***Results:*** RP11-40C6.2, an HBV infection-related lncRNA, was identified by analysis of the TCGA–Liver Hepatocellular Carcinoma database. Gene Set Enrichment Analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of differentially expressed genes revealed a strong association of RP11-40C6.2 with the Hippo signaling pathway. RP11-40C6.2 was overexpressed in HCC patients with HBV infection compared to those without HBV infection. RP11-40C6.2 transcription showed a positive association with HBV-X protein (HBx), but not HBV core protein (HBc) expression, both of which are carcinogenic proteins. Luciferase gene reporter and ChIP assays revealed that YAP1/TAZ/TEADs complex enhanced RP11-40C6.2 transcription by binding to its promoter area. RP11-40C6.2 showed oncogenic characteristics in HCC cell lines and in animal models that were mediated via activation of YAP1. *In vitro* ubiquitylation assay revealed that RP11-40C6.2 can promote the stabilization of YAP1 by stopping phosphorylation at its s127 residue and further stopping its degradation through binding to 14-3-3. ***Conclusions:*** RP11-40C6.2 is an HBV infection-related lncRNA that exerts its oncogenic effects by targeting the Hippo signaling pathway.

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## Introduction

Chronic hepatitis caused by hepatitis B virus (HBV) infection is a leading cause of hepatocellular carcinoma (HCC).[1] Epidemiological studies have demonstrated a strong association between HBV infection and HCC; however, the exact molecular mechanisms underlying this association have not been elucidated in detail.[2] Studies have demonstrated the contribution of HBx and HBc oncoproteins of HBV to carcinogenesis owing to their effect on the cellular biological processes,[3] especially by their participation in various tumor-associated pathways including p53 and DR5.[4,5] For example, HBx protein was shown to promote a variety of growth factors and stimulate the regulatory subunits p85 and the catalytic subunits p110 of PI-3K to form the p85-p110 complex, enhancing the activity of the PKB/Akt pathway.[6] Moreover, HBx protein was reported to suppress the function of p53 and to be involved in several tumor-related pathways including JAK/STAT,[7] JNK,[8] and NF-kB[9] to promote cell proliferation, invasion, and migration. HBc protects hepatocytes against TRAIL-induced apoptosis by inhibiting DR5 expression, contributing to the development of chronic hepatitis and HCC.[5] In addition to the regulatory effect of HBx/HBc on encoded genes, a few groups have focused on the effect of HBx/HBc protein on the expression of long noncoding RNAs (lncRNAs). For instance, Guerrieri F *et al.*[10] recently reported that HBx binds to the promoter area of lnc-DLEU2, strengthens the transcription and causes the accumulation of lnc-DLEU2 in infected hepatocytes.

**Keywords:** Hepatocellular carcinoma; Hippo signaling; lncRNA; YAP1; HBV.
**Abbreviations:** ChIP, Chromatin immunoprecipitation; EZH2, enhancer of zeste homolog 2; HBc, HBV core protein; HBV, hepatitis B virus; HBx, HBV-X protein; HCC, hepatocellular carcinoma; KEGG, Kyoto Encyclopedia of Genes and Genomes; lncRNAs, long noncoding RNAs; TCGA, the Cancer Genome Atlas.
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They observed that HBx and the histone methyltransferase enhancer of zeste homolog 2 (EZH2), the catalytic active subunit of the polycomb repressor complex 2 (PRC2) complex, are directly bound by nuclear lnc-DLEU2. EZH2 is displaced from the viral chromatin by HBx and lnc-DLEU2 corecruitment on the cccDNA to drive transcription and viral replication in HBV-associated HCCs. Moreover, Xiao *et al*.[11] revealed that HBV-associated HCC is promoted by HBx/ERα complex-mediated linc01352 downregulation via the miR-135b-APC axis. In this study, we found overexpression of HBV infection-related lncRNA, RP11-40C6.2 in HBV-positive HCC. Our study indicated that this lncRNA was not only a HBx protein-related lncRNA, but also closely related to YAP/TAZ activation. Moreover, we explored its effect on the promotion of cell proliferation and invasion and the related molecular mechanisms.

## Methods

### *Patients*

A total of 40 patients with HCC were enrolled in this hospital-based case-control research. All were enrolled from the Hepatobiliary Center, The First Affiliated Hospital of Nanjing Medical University between January 2016 and January 2019. All patients had undergone surgery for primary HCC. Patients with a history of cancer or chemotherapy were excluded. Cancer-free controls were from the same geographic region and had no evidence of a genetic connection with the HCC patients. The ethics committee of The First Affiliated Hospital of Nanjing Medical University approved this research, and all patients provided written informed consent.

### *Bioinformatics analysis*

http://ibl.mdanderson.org/tanric/_design/basic/download.html provided RNA sequencing data for the Cancer Genome Atlas (TCGA) human liver cancer (LIHC) program. According to Tang *et al*.,[12] we obtained 18 non-HBV-infected samples and 55 samples with HBV infection from the TGCA-LIHC database. R packages including *limma, pheatmap, clusterprofiler* were used to identify differentially expressed lncRNAs and generate heatmaps. Gene Set Enrichment Analysis (GSEA) (http://software.broadinstitute.org/gsea/index.jsp) was used to analyze the gene sets.

### *Cell lines and reagents*

Human HCC cell lines SMMC-7721, Huh7, MHCC-97H, and Hep3B were obtained from the American Type Culture Collection. All cells were cultured in Dulbecco's Modified Eagle's Medium purchased from Gibco (Waltham, MA, USA) with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and kept in a humidified atmosphere containing 5% $CO_2$ at 37°C. Lysophosphatidic acid (LPA) was obtained from ENZO (BML-LP100-0025). YAP1/TAZ Inhibitor was purchased from Selleckchem (s8661).

### *Real-time polymerase chain reaction (RT-PCR)*

TRIzol reagent was used to isolate total RNA from human HCC tissues. The expression of RP11-40C6.2 was detected using the sybr-green-based real-time PCR. The following primers were used for PCR: forward primer: GCATGGGATTCTGACTGAG, reverse primer: AGAGAAGAGATCTATCGCT, amplicon size 157bp for RP11-40C6.2, and forward primer: TGTGGGCATCAATGGATTTGG, reverse primer: ACACCATGTATTCCGGGTCAAT, amplicon size 110bp for GAPDH.

### *RNA pull-down, in vitro ubiquitination, and Co-IP assay*

Thermo Scientific Pierce Magnetic RNA-Protein Pull-Down Kits (ThermoFisher, Waltham, MA, USA) were used for the RNA pull-down assay. Briefly, total RNA was extracted from SMMC-7721 and Hep3B cells, and magnetic beads were incubated with probes for biotin-labeled RP11-40C6.2 (Genescript Co., Nanjing, China) (sequence: ACAGGCTGATGCATGGGATTCT) and U6 control (sequence: TTGGAACGATACAGAGAAGATT). For *in vitro* ubiquitination detection, whole cell lysates were obtained using a lysis buffer containing 10 mM N-Ethylmaleimide. YAP1 was immunoprecipitated and anti-ubiquitin antibody (1:1,000, ab7254; Abcam, Cambridge, UK) was used to detect ubiquitination. To examine the interaction of TAZ with HBx or HBc, Hep3B cells were transduced with HBx or HBc. Cellular TAZ was immunoprecipitated with its antibody (ab652; Abcam) using Dynabead Protein A Immunoprecipitation Kits (ThermoFisher). The presence of HBx and HBc in the immune complex was assayed by western blotting and anti-HBx (ab203540; Abcam) or anti-HBc antibodies (ab8637; Abcam).

### *Chromatin immunoprecipitation (ChIP) assay*

Chromatin immunoprecipitation assays were performed with a commercially available kit (Millipore, Temecula, CA, USA) with anti-TEAD1, anti-TEAD2, anti-TEAD3, anti-TEAD4, and p65 antibodies; IgG antibody was the negative control. A 200-bp fragment corresponding to the core RP11-40C6.2 promoter was amplified by ChIP primers: 5′-CGAGGGGGCGGTGACAGCA-3′ and 5′-CCTCGCTAGCTTTGATGCGCAGA-3′. Analysis of samples included at least three independent assays.

### *IHC and multiple color IHC*

After deparaffinization and rehydration process of tissue sections, the endogenous peroxidase activity was blocked by a 30-minute treatment with 1% $H_2O_2$ in methanol. Then the tissue sections were rinsed twice in phosphate-buffered saline for 5 m each, followed by blocking with 10% normal goat serum. After rinsing, the samples were incubated with a primary anti-YAP1 (#4912; Cell Signaling Technology, Danvers, MA, USA), anti-YAP1 (s127) (#13008, Cell Signaling Technology). Then the sections were incubated with secondary antibodies, followed by staining with 3,3′-diaminobenzidine based on the manufacturer's protocol. The sections were then mounted on slides, and photographed with a digital microscope camera (Nikon, Tokyo, Japan). Multiple color staining was performed with Opal 4-anti-rabbit automation IHC kits (NEL830001KT, AKOYA science) following the manufacturer's instructions. The sections were incubated overnight with primary antibodies to AXL (#8661; Cell Signaling Technology), HBx (ab203540; Abcam) and HBc (ab8637; Abcam) at 4°C. Subsequently, photographs were obtained with a digital microscope camera (Nikon).

### *Western blotting*

For western blotting, proteins were extracted from cultured

cells using RIPA buffer with phenylmethanesulfonylfluoride (PMSF; Beyotime, Nantong, China) and 100 μg samples of total protein were resolved by 7.5%/12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to polyvinylidene fluoride (PVDF) membranes for staining. Primary polyclonal antibodies targeting anti-YAP1 (#4912; Cell Signaling Technology), anti-YAP1 (s127) (#13008; Cell Signaling Technology), LATS1 (#9153; Cell Signaling Technology), 14-3-3 (#8312; Cell Signaling Technology), anti-HBx (ab203540; Abcam), anti-HBc antibodies (ab8637; Abcam), and anti-TAZ (ab652; Abcam) were used. The secondary antibody was anti-rabbit HRP-conjugated IgG (Abcam), and an enhanced chemiluminescence reagent (Millipore) was used to develop the blots. A beta actin antibody was used to confirm equal amounts of protein loading in each lane. Integrated band densities were read using ImageJ software (La Jolla, CA, USA).

### *Cell counting kit (CCK)-8 and Transwell cell migration assays*

Cells treated differently were seeded into the upper surface of 8 μm Transwell chamber (Millipore) membranes coated with Matrigel (BD Biosciences, San Jose, CA, USA) for the invasion assay or without Matrigel for the migration assay. After incubation for 24 h, cells on the upper membrane of the Transwell chambers were removed with cotton wool and the cells that had migrated or invaded the membrane were fixed for 10 m with methanol and stained with 0.1% crystal violet for 30 m at 37°C. Three randomly chosen microscopic fields were counted in each study group. The assays were independently repeated three times.

### *Luciferase reporter assay*

The promoter region of RP11-40C6.2 with the wild-type or mutant potential target site for TEADs was designed and synthesized by Genescript Co. and inserted into the pGL4 Vector (Promega, Madison, WI, USA). For luciferase assay, C33A was transfected with pGL4-RP11-40C6.2 wild-type (WT) pro or pGL4-RP11-40C6.2 MU pro, with pCMV-HBx, pCMV-HBc or control (GenePharma, Shanghai, China) using Lipofectamine 2000 (ThermoFisher). The LPA-treated cells were harvested 48h after transfection for luciferase assay with a Dual-Luciferase® Reporter Assay System (Promega) based on the manufacturer's protocol.

### *Animal experiments*

To further investigate the biological role of RP11-40C6.2 on tumor development *in vivo*, 6-week-old male nude mice (BALB/c background) were given subcutaneous xenografts of HCC cells. Transfected SMMC-7721 cells ($5\times10^6$) were injected into the left or right flanks of mice. The mice were sacrificed mice 4 weeks later, the calculation of the volume of every tumor was performed (length×width$^2$×0.5). For the lung metastasis model, all lungs were stained with hematoxylin and eosin, and the number of metastases was determined. The animal procedures were performed following the Institutional Animal Care and Use Committee guidelines of The First Affiliated Hospital of Nanjing Medical University.

### *Statistical analysis*

Data were reported as means±standard deviations (SDs). Between-group differences in demographic and clinical characteristics were assessed with Student's *t*-tests and analysis of variance. All *in vitro* expression assays were repeated at least three times. The significance of correlations between two factors was analyzed by Pearson's correlation coefficient. STATA 9.2 was used for the statistical analysis, and data were presented using GraphPad Prism software (San Diego, CA, USA). *P*-values <0.05 were considered statistically significant.

## Results

### *RP11-40C6.2 is an HBV infection-specific lncRNA in HCC*

To explore human HBV infection-specific lncRNAs, we screened hepatocellular carcinoma RNA sequencing (RNA-seq) data from the TCGA database. As reported by Tang *et al*.,[12] we obtained data from 18 samples from patients with HCV infection, which also promotes the development of liver cancer, and 55 samples of patients with HBV infection from the TGCA_LIHC database. After statistical analysis (*t*-test), we identified 13 lncRNAs that were significantly different expression in HBV and non-HBV infection. Four were down-regulated and nine were upregulated lncRNAs (adjusted *p*-value <0.05, Fig. 1). Of the 13 lncRNAs, only RP11-40C6.2 (ENSG00000219928.2) was found to be significantly up-regulated in tumors after comparison of normal tissues following a search of the online TCGA using the Gepia tool (Fig. 1B). After obtaining genes that were differentially expressed between the top and low 20 RP11-40C6.2 expression HCC samples in the TCGA database, Gene Ontology (GO), and KEGG enrichment analyses were carried out. The GO enrichment results (Biological Process, BP) using up-regulated differentially expressed genes (DEGs) are shown in Fig. 1C. The BPs associated with high expression of RP11-40C6.2 included "DNA replication," "chromosome segregation," and "DNA-dependent DNA replication." On KEGG enrichment analysis, the signaling pathways associated with high expression of RP11-40C6.2 included "cell cycle," "cellular senescence," and "Hippo signaling pathway" (Fig. 1D). We speculated that the cell cycle or DNA replication related features of RP11-40C6.2 were associated with activation of the Hippo signaling pathway. We also performed GSEA of the DEGs. Interestingly, high expression of RP11-40C6.2 had a positive correlation with the Hippo signaling pathway (Fig. 1E). Next, we performed a single sample GSEA (ssGSEA) adopting RP11-40C6.2 top20 and low 20 samples using C6 gene sets. The results also found significant differences in Hippo signaling pathway between RP11-40C6.2 high and low expression samples (Fig. 1F). Next, the upregulated genes were mapped in the sketch of Hippo signaling. We found that RP11-40C6.2 enhanced the transcription of YAP1, which is a core protein in Hippo signaling (Fig. 1G).

### *The clinical relevance of RP11-40C6.2 was discussed in terms of early diagnosis of HCC as well as prognostication after treatment of HCC as a biomarker*

In the current study, 120 HCC samples pathologically diagnosed (including tumor tissues, matched adjacent non-cancerous liver tissues and preoperative/ postoperative serum) acquired between September 2013 and December 2014 at the First Affiliate Hospital of Nanjing Medical University (Nanjing, Jiangsu, China) were extra collected. We used the qRT-PCR assay to detect the expression level of RP11-40C6.2 in HCC tumor tissues and adjacent ones. As

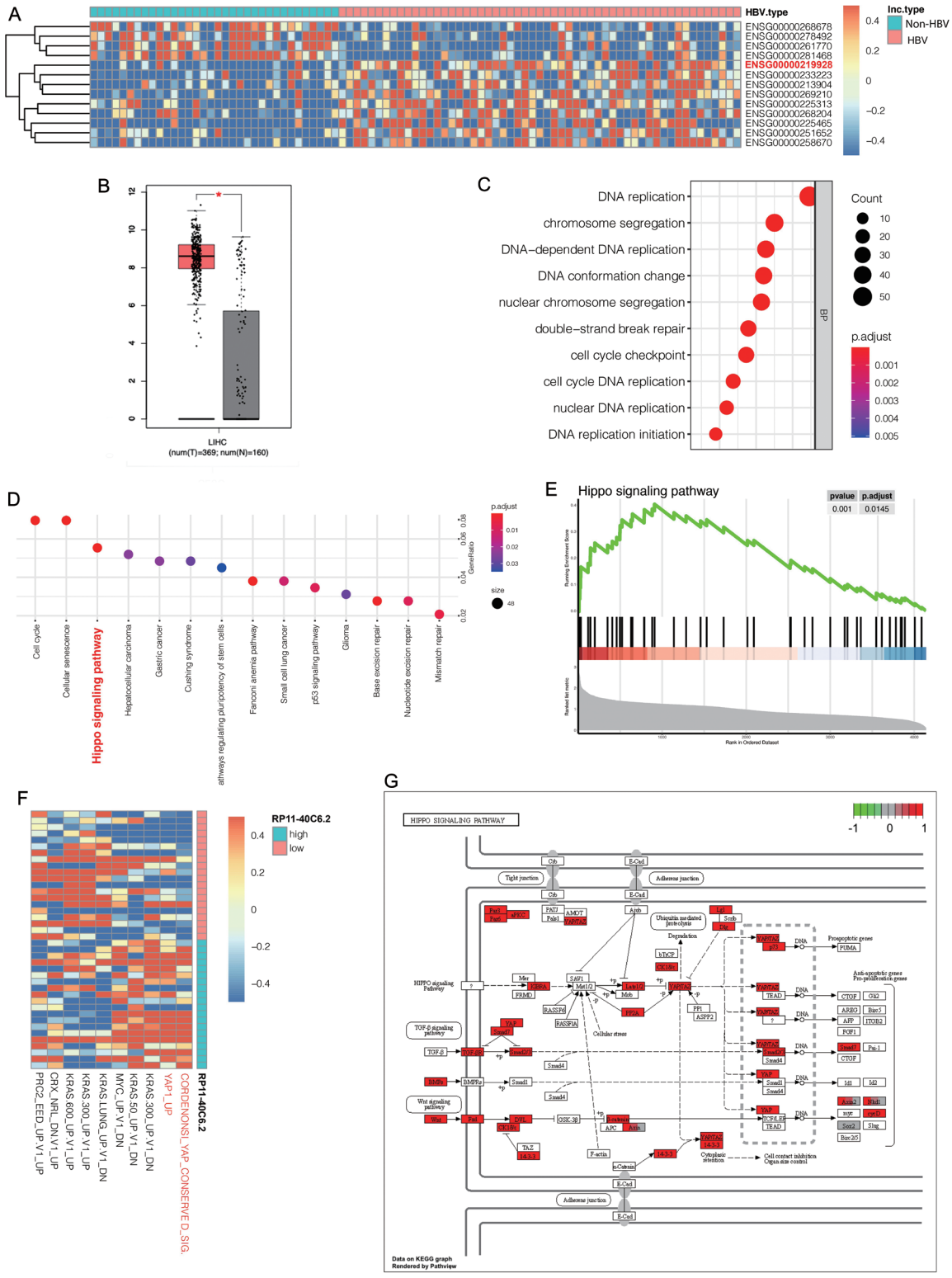


**Fig. 1. RP11-40C6.2 is specifically overexpressed in human HBV-infected HCC.** (A) Heatmap showing 13 differentially expressed lnc-RNAs in HBV-positive and HCV-positive human hepatocellular carcinoma (HCC) tissues in the Cancer Genome Atlas (TCGA) database. (B) Comparison of the expression of RP11-40C6.2 in tumor and normal tissues with the Gene Set Enrichment Analysis (GSEA) dataset in TCGA. GO (C) and KEGG (D) enrichment analysis was performed using differentially expressed genes (DEGs) by comparing top20 and low20 linc000925 expression HCC samples in TCGA database. (E) GSEA targeting high and low expression of RP11-40C6.2 displayed that the expression of RP11-40C6.2 was related to Hippo signaling pathway. (F) A single sample GSEA (ssGSEA) was performed using RP11-40C6.2 top20 and low20 samples using C6 gene sets. g) DEGs identified on comparing top20 and low20 linc000925 expression HCC samples were mapped in human Hippo signaling pathways.

presented in Supplementary Figure 1A, RP11-40C6.2 in tumor tissues was obviously higher than that in adjacent ones (P<0.05). We also used the qRT-PCR analysis to detect serum RP11-40C6.2 in each group and found that RP11-40C6.2 in preoperative serum was higher than that in in postoperative serum among 120 HCC patients (Supplementary Fig. 1B, P<0.01). The results of Supplementary Figure 1C also demonstrated that serum RP11-40C6.2 expression was increased in preoperative serum compared with other benign liver diseases and healthy controls (P<0.01). The findings above suggested that serum RP11-40C6.2 expression could distinguish HCC patients from people with benign liver diseases.

Next, to assess whether the aberrant expression of RP11-40C6.2 was associated with HCC progression, the clinical and pathological information of HCC patients mentioned above was collected and analyzed. As presented in Supplementary Table 1, 120 patients were fallen into RP11-40C6.2 high group and RP11-40C6.2 low group in accordance with the median value of RP11-40C6.2. Statistical analysis shown that up-regulation of RP11-40C6.2 was positively associated with tumor size (P=0.011), microvascular invasion (P=0.044), portal vein tumor thrombus (P=0.016) and TNM stage (P=0.004).

To explore the diagnostic value of RP11-40C6.2, we performed the ROC curve. The results in Supplementary Figure 2A showed an AUC of 0.669 with a sensitivity of 72.1% and a specificity of 62.3%. The ideal cut-off value was 0.227.

To study the prognostic value of RP11-40C6.2 in HCC, we performed Kaplan-Meier and Cox regression analyses. The results of Kaplan-Meier analysis showed that overall survival of HCC patients with low RP11-40C6.2 expression was longer than the high RP11-40C6.2 expression group (Supplementary Fig. 2B). Log-rank testing showed the result possessed statistical significance (P=0.004). Additionally, multivariate analysis was conducted through Cox regression analysis. High expression of RP11-40C6.2 (HR=2.195, 95CI%=1.165 -4.134, P=0.015) appear to be related to the poor prognosis of HCC (Supplementary Table 2).

### *RP11-40C6.2 is an HBx protein-associated lncRNA*

As HBV has two vital oncogenic proteins, HBx, and HBc, we evaluated AXL, the downstream target gene for YAP1 activation,[13] HBx, and HBc protein expression levels in HCC tissues using multicolor staining. The 40 HCC patients were further categorized into HBx+HBc+ (nine cases), HBx+ HBc− (six cases), HBx−HBc+ (five cases), and HBx−HBc− (20 cases) groups (Fig. 2A). RP11-40C6.2 had a significant positive correlation with AXL protein expression level (Fig. 2B). We divided the 40 patients with HCC based on RP11-40C6.2 expression (Fig. 2C) and found a significant association of RP11-40C6.2 level with HBx but not HBc (Fig. 2D, E).

### *HBx-associated RP11-40C6.2 is closely associated with the expression and nuclear localization of YAP1*

We investigated the relationship between HBV infection and activation of YAP1 protein in HCC by immunohistochemistry (IHC) and found that YAP1 expression in HBx+ groups (HBx+ HBc− and HBx+ HBc+) was significantly higher than that in HBx− groups (HBx− HBc− and HBx− HBc+) (Fig. 3A). The nuclear localization of YAP1 in HBx + groups was much greater than that in HBx− groups (Fig. 3A). As the nuclear localization of YAP1 is closely related to its activation,[13] the findings suggest that HBx expression was associated with the activation of YAP1. To confirm that observation, we assayed YAP1(s127), YAP1 degradation-related phosphorylation residue, and found it to be much lower in HBx+ HCC than in HBx− HCC (Fig. 3B). We also analyzed the correlation of nuclear YAP1 or YAP1(s127) with RP11-40C6.2 in HCC. The expression of RP11-40C6.2 was positively correlated with nuclear YAP-1 but negatively correlated with YAP1(s127) (Fig. 3C–E).

A previous study has shown that HBx protein promoted YAP1/TAZ translational activity by binding to the promoter of YAP1.[14] We found that the HBx level was positively correlated with RP11-40C6.2. We investigated the potential binding site of TEADs, cotranscriptional factors of YAP1/TAZ, in the promoter region of RP11-40C6.2 and found a potential binding site (Fig. 3F). We mutated the binding site and found a significant decrease in the promoter activity compared with the WT promoter following treatment with HBx, LPA (YAP-1 activator), HBx + LPA, or HBc + LPA. HBc protein alone had no apparent effect on the promoter activity of RP11-40C6.2 (Fig. 3G). To further confirm that TEADs bind to the promoter region of RP11-40C6.2, we performed ChIP assays using antibodies to TEAD1-4 and p65. The results demonstrated that TEAD1-4 bound to the promoter region of RP11-40C6.2 (Fig. 3H).

Co-immunoprecipitation (co-IP) with Hep3B and Hep3B, HBx/HBc (Hep3B cells overexpressing HBx and HBc) cells with TAZ antibody revealed that HBx, but not HBc, bound to TAZ (Fig. 3I). The results show that the enhanced expression of RP11-40C6.2 in HCC was attributed to the increase in the transcriptional activity of YAP1/TAZ/TEADs promoted by HBx. We speculate that RP11-40C6.2 may serve as an HBx -specific lncRNA.

### *RP11-40C6.2 enhances the proliferation and invasion of HCC cells by YAP1 activation*

To examine the biological functions of RP11-40C6.2, we studied the expression of RP11-40C6.2 in different HCC cell lines, including Hep3B, SMMC-7721, MHCC-97H, and Huh7. SMMC-7721 and MHCC-97H had very low HBx and RP11-40C6.2 expression levels, but Huh7 and Hep3B cells had significantly higher expression of HBx and RP11-40C6.2.

Proliferation and invasion were significantly lower in SMMC-7721 and MHCC-97H cells than in Hep3B and Huh7 cells (Fig. 4B, C). We induced overexpression of RP11-40C6.2 in SMMC-7721 cells (SMMC-7721-RP11-40C6.2) and knocked down its expression in Hep3B cells (Hep3B-RP11-40C6.2 short-hairpin RNA [shRNA]) (Fig. 4D). The proliferation and invasion abilities were significantly increased in SMMC-7721-RP11-40C6.2 cells but decreased in Hep3B -RP11-40C6.2 shRNA cells (Fig. 4E, F). We also found that CA3, a specific inhibitor of YAP1/TAZ, significantly decreased the proliferation and invasiveness of SMMC-7721-RP11-40C6.2 and Hep3B cells (Fig. 4G, H). In addition, mouse subcutaneous xenograft models were established, and SMMC-7721 cells with stable overexpression of RP11-40C6.2 were injected subcutaneously in nude mice. Tumor xenografts originating from cells with overexpression of RP11-40C6.2 were larger and weighed more than control cells (Supplementary Fig. 3A–C). To further investigate the effect of RP11-40C6.2 on HCC metastasis *in vivo*, we knocked down RP11-40C6.2 expression in Hep3B cells with shRNA. Treated cells were injected into mouse tail veins to construct lung metastasis models. Four weeks later, the mice were killed, and lung tissues were harvested. Because of the short time after injection, we did not find visible metastases on the surface of the lung tissues. However, hematoxylin and eosin staining confirmed that overexpression of RP11-40C6.2 significantly increased the number of lung of metastatic nodes (Fig. S3D, E).

On investigating the activation of YAP1 by western blot-

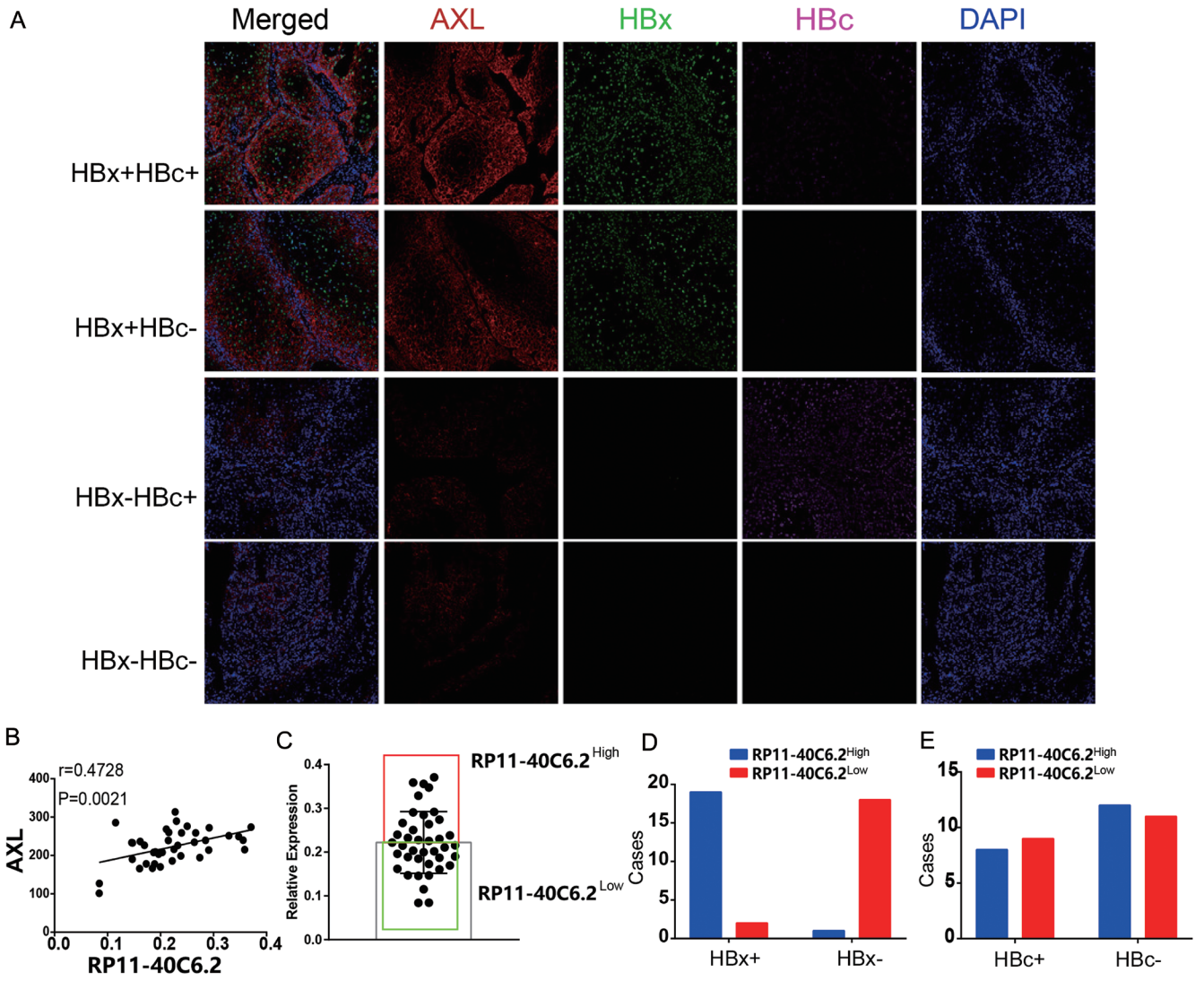


**Fig. 2. RP11-40C6.2 is an E6 protein-related lncRNA.** (A) Representative images of multiple color staining for AXL, HBx, and HBc expression in human HCC samples were grouped as shown in the figure (×100). (B) Linear correlation analysis between transcription of RP11-40C6.2 and AXL protein expression assessed by Integrated Optical Density Value (IOD) in human HCC tissues. ImageJ was used to analyze the figures. (C) The chosen human HCC tissues were subcategorized into RP11-40C6.2 high ($n$=20) and low ($n$=20) expression groups based on the median expression level. (D and E) Chi-square tests were used to assess the association between RP11-40C6.2 expression and HBx or HBc expression in human HCC. Mean±SEM values of at least three independent assays are shown. *$p$<0.05, **$p$<0.01.

ting analysis, the level of total YAP1 was found to be the highest and that of YAP1(s127) was the lowest in Hep3B cells (Fig. 4I). Furthermore, we activated YAP1 by lysophosphatidic acid (LPA) (0.1 μM), and found a decrease in YAP(s127) level and an increase in total YAP1 level in all cell lines, especially Hep3B cells (Fig. 4J). Total YAP1 activation increased in SMMC-7721-RP11-40C6.2 cells but decreased in Hep3B-RP11-40C6.2 shRNA cells with or without treatment with LPA (Fig. 4K, L). The results suggest that RP11-40C6.2 promoted the proliferation and invasion of HCC cells by YAP1 activation.

### *RP11-40C6.2 stabilizes YAP-1 by binding to YAP1 (s127)*

We used the online tool catRAPID to predict the possibility of binding between RP11-40C6.2 and YAP1. The results suggested that RP11-40C6.2 (201–252 nt) has the greatest potential to directly bind to YAP1 protein at the region of amino acids 102–154 (Fig. 5A, B). Next, we found that the sense probe, but not the antisense probe, of RP11-40C6.2 could pull-down YAP-1 (Fig. 5C). To verify the specific binding site of RP11-40C6.2 on YAP1, we constructed a mutated form (201–252 deletion) of RP11-40C6.2 (RP11-40C6.2 MU) and found that the 201–252 fragment of RP11-40C6.2 was essential for its binding to YAP1 (Fig. 5D).

To investigate the effect of RP11-40C6.2 on YAP1 nuclear translocation, we performed immunofluorescence (IF) staining and found that overexpression of RP11-40C6.2, but not RP11-40C6.2 MU, in SMMC-7721 and Hep3B cells enhanced the nuclear translocation of YAP1 (Fig. 5E). Next, we investigated the role of YAP-1 and RP11-40C6.2 integration in promoting cell proliferation and invasion. We found that RP11-40C6.2 MU overexpression significantly attenuated the positive effects of RP11-40C6.2 on cell proliferation and invasion (Fig. 5F, G).

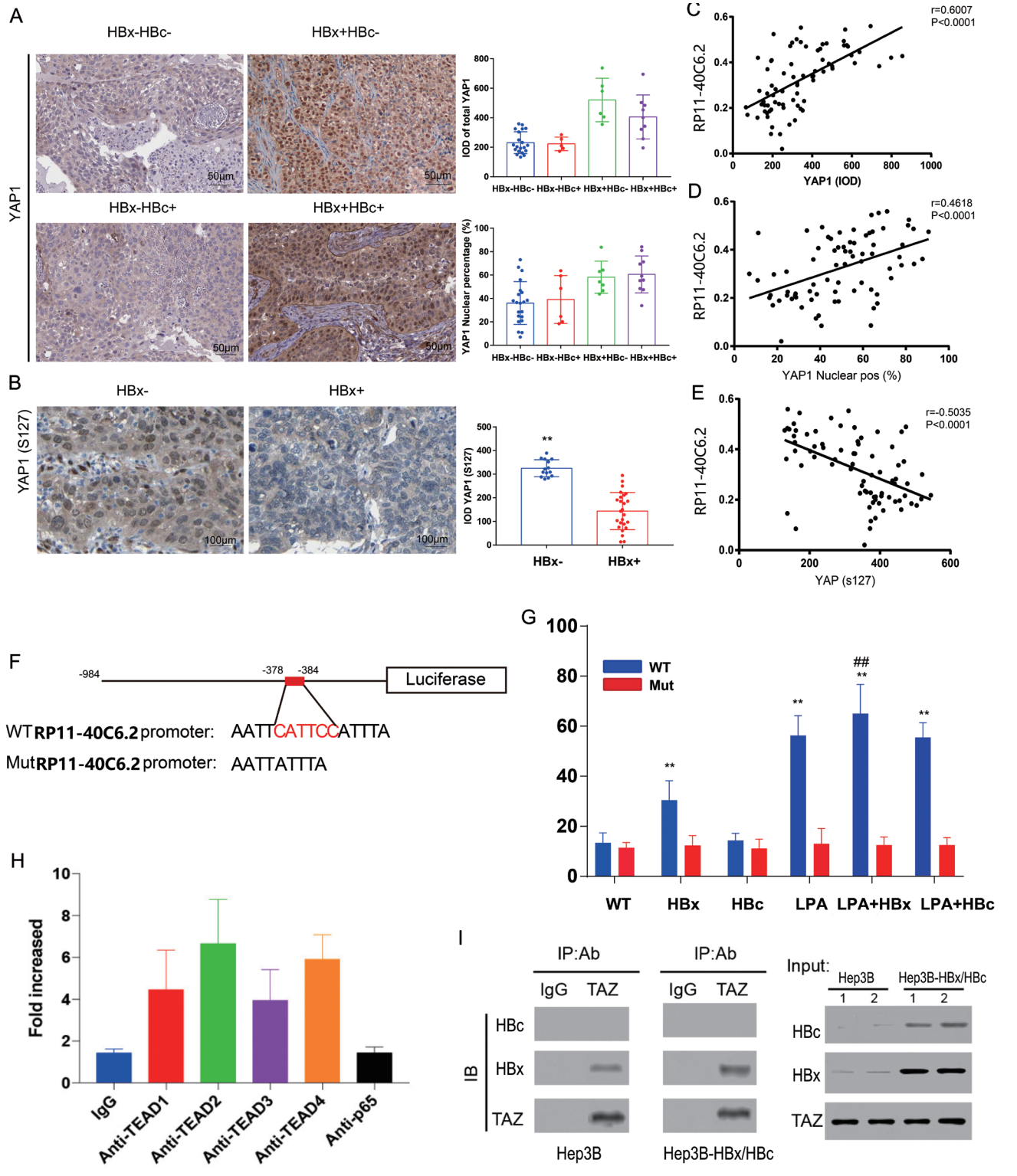


**Fig. 3. HBx-associated RP11-40C6.2 is closely associated with the expression and nuclear localization of YAP1.** (A and B) Representative images and analysis of immunohistochemistry staining for YAP1 (×100) and YAP1(s127) (×200) in human HCC subgrouped as shown in the figure. ImageJ was used to analyze the images. (C–E) Linear regression analysis of the expression of RP11-40C6.2 and YAP1 (IOD value) or percentage of YAP1 nuclear translocation and YAP1 (s127) in human HCC. (F) Diagram of potential TEADs binding sites and mutation design within the RP11-40C6.2 promoter region. (G) Luciferase reporter assay of HBx, HBc, and LPA treatment on the activity of wild-type (WT) and TEADs binding site mutation (Mut) RP11-40C6.2 promoter. (H) Antibody to TEAD 1-4 was used in chromatin immunoprecipitation (ChIP) assays; p65 and IgG were negative controls. (I) Co-IP assays were performed to study the binding between TAZ and HBx or HBc; the protein complex was immuno-precipitated with TAZ antibody and further examined by HBx or HBc antibody. Mean±SEM values from at least three independent experiments are presented. *$p$<0.05, **$p$<0.01.

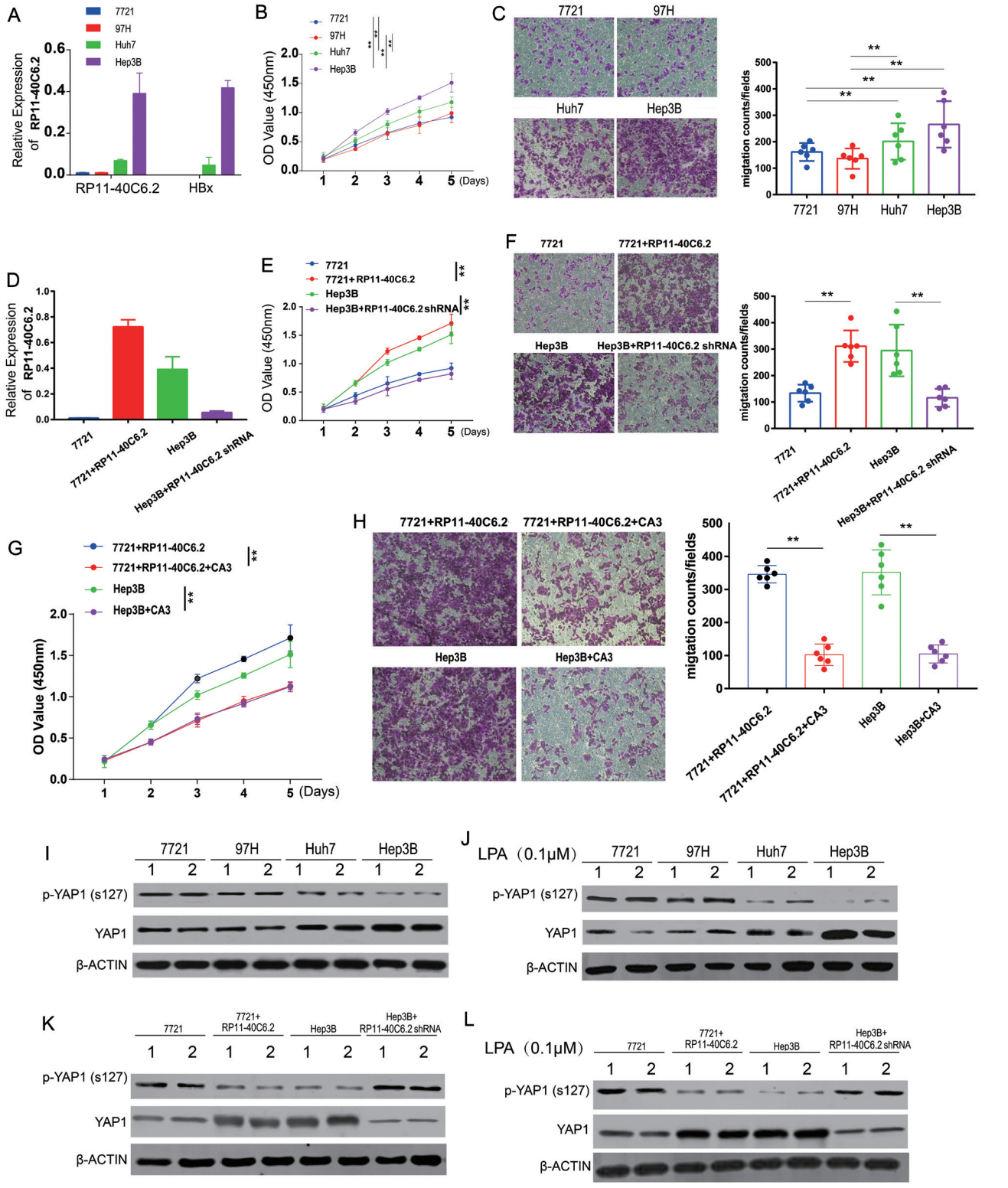


**Fig. 4. RP11-40C6.2 enhances the proliferation and invasiveness of HCC cells by YAP1 activation.** (A) Real-time PCR in SMMC-7721, MHCC-97H, Huh7, and Hep3B cells shows the expression of RP11-40C6.2. (B) CCK8 assays show the proliferation of SMMC-7721, MHCC-97H, Huh7, and Hep3B cells. (C) Transwell assays show the invasiveness of SMMC-7721, MHCC-97H, Huh7, and Hep3B cells. (D–F) The expression of RP11-40C6.2 and proliferation and invasiveness of Hep3B, SMMC-7721-RP11-40C6.2, Hep3B, and Hep3B-RP11-40C6.2 shRNA. (G, H) Results of CCK8 and Transwell assays of proliferation and invasiveness of SMMC-7721-RP11-40C6.2 and Hep3B cells treated with or without CA3. (I–J) HCC cell line was treated with or without LPA (0.1 μM) and protein expression of YAP1(s127) was assayed by western blotting with beta actin as an internal control. Mean±SEM values from at least three independent experiments are shown. *$p$<0.05, **$p$<0.01.

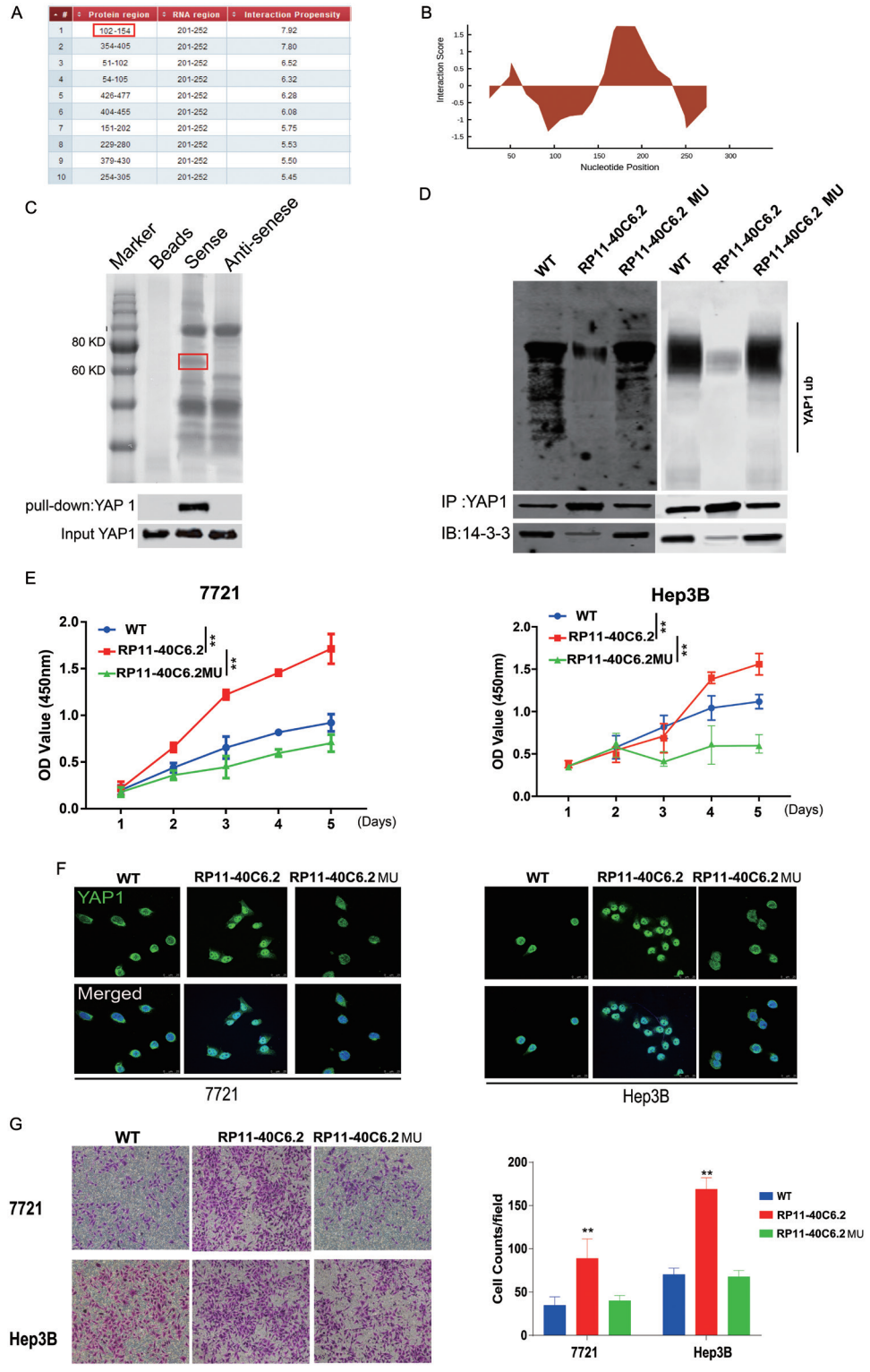


**Fig. 5. RP11-40C6.2 stabilize YAP-1 protein and activate the downstream pathway by binding to YAP1 (s127) residue.** (A, B) catRAPID was used to predict hidden binding sites of RP11-40C6.2 and YAP1. The potential binding regions and interaction marks are shown. (C) RNA pull-down performed with sense and antisense probes specific for RP11-40C6.2 and controlled by beads. Western blotting detected precipitated and input total proteins with YAP1 antibody. (D) Ubiquitination detection of YAP1 in SMMC-7721 and Hep3B cells transfected with WT and MU RP11-40C6.2. (E) Proliferation of SMMC-7721 and Hep3B cells transfected with WT and MU RP11-40C6.2. (F) IF staining assay was performed to detect YAP1 nuclear translocation in SMMC-7721 and Hep3B cells transfected with WT and MU RP11-40C6.2. (G) Transwell cell migration assay was used to assess invasion of SMMC-7721 and Hep3B cells transfected with WT and MU RP11-40C6.2. Mean±SEM values from at least three independent experiments are presented. *$p$<0.05, **$p$<0.01.

## Discussion

This study identified an lncRNA, RP11-40C6.2, which enhanced nuclear translocation of YAP1 by binding to amino acids 101–125 amino acids, which carry an important phosphorylation residue of YAP1 (s127). The s127 residue of YAP1 caused proteasome-induced degradation by 14-3-3-derived ubiquitination. Overexpression of RP11-40C6.2 blocked the binding of 14-3-3 and YAP1 by facilitating its binding to the s127 region, thereby reducing protein degradation. YAP1 is a core protein of the Hippo signaling pathway that plays an important role in cell proliferation, apoptosis, and organ development.[15] YAP1 itself has no transcriptional activity, but is a transcriptional co-activator when combined with its TAZ and TEADs.[16] All of these three components are indispensable for downstream transcripts boost; therefore, the nuclear translocation of YAP1 is the key regulatory event, and the process is blocked by the Hippo pathway when it is in the "on" state. The "on" state of the Hippo pathway is considered to have tumor suppressive activity. Several studies have revealed that its "on" state is capable of stopping cell proliferation, drive apoptosis, and regulating stem/progenitor cell expansion.[17]

We also found that the positive effects of RP11-40C6.2 on cell proliferation and invasion were attributed to YAP1 activation, and that the YAP/TAZ activation inhibitor CA3 completely blocked the carcinogenic effects of RP11-40C6.2. Several other lncRNAs have been reported to exert tumorigenic effects by targeting Hippo signaling. Li *et al*.[18] reported that the crosstalk between ROR1-HER3 and the Hippo signaling pathway promoted bone metastasis of breast cancer. Zhu *et al*.[19] also reported that lncBRM associated with BRM initiates the BRG1/BRM switch and that the BRG1-embedded BAF complex triggers the activation of YAP1 signaling.

We investigated the expression of RP11-40C6.2 in HCC and found that the transcription of RP11-40C6.2 was associated with HBV infection and driven by HBx. HBx protein is a multifunctional regulatory protein of approximately 17 kDa. It contains 154 amino acids including nine cysteine residues, which are required for four main functions. (1) HBx protein is known to have both pro-apoptotic and anti-apoptotic roles. The anti-apoptotic activity of HBx protein may be attributable to its association with p53, a tumor suppressor gene.[20] (2) HBx acts as a transcriptional activator by interacting with the nuclear transcription element and controlling cytoplasmic signal transduction pathways such as Ras, Raf, mitogen activated protein kinase, Janus family tyrosine kinase-signal transducer and activators of transcription, focal adhesion kinase, and protein kinase cascade pathways.[21] (3) HBx regulates hepatocyte proliferation, apoptosis, and cell development.[22] (4) HBx has a regulatory effect on nucleotide excision repair (NER).[23] We found that RP11-40C6.2 is one of the downstream products of HBx/TAZ/TEADs complex in HBV-infected human HCC cells. Similar results have also been reported by other research groups. Thus, RP11-40C6.2 was driven by HBx/YAP1/TAZ/TEADs and promoted proliferation and invasion of HCC cells by preventing YAP1 degradation by the E3 ubiquitination ligase 14-3-3. We believe that high expression of RP11-40C6.2 is an important cause of HCC and that RP11-40C6.2 may serve as a potential therapeutic marker of HCC.

In summary, on exploring the TCGA database, we discovered an HBV infection-related lncRNA, RP11-40C6.2 in HCC. High expression of RP11-40C6.2 was associated with TMN stage and HBx protein expression. Further study revealed that RP11-40C6.2 was driven by HBx/YAP1/TAZ/TEADs, and that it promotes proliferation and invasion of HCC cells by binding to YAP1 in the region containing s127, preventing YAP1 degradation by E3 ubiquitination ligase 14-3-3. We believe that high expression of RP11-40C6.2 is an important cause and potential therapeutic target for HCC.

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## Conflict of interest

The authors have no conflict of interests related to this publication.

## Author contributions

ZT and YT designed the research; HZ and CW wrote the paper; HZ, JT performed the in vitro and in vivo experiments; YT, HZ, FZ, ZX and DS analyzed data.

## Ethical statement

The ethics committee of The First Affiliated Hospital of Nanjing Medical University approved this research, and all patients provided written informed consent.

## Data sharing statement

Data are available on request to the authors.

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