

ORIGINAL ARTICLE

Wnt8B, transcriptionally regulated by ZNF191, promotes cell proliferation of hepatocellular carcinoma via Wnt signaling

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Funding information

Natural Science Foundation of China, Grant/Award Number: 31671461, 31301162, 81871929, 81572298 and 81972621

Abstract

Dysregulation of wingless-type (Wnt) signaling is implicated in hepatocellular carcinoma (HCC). Wnt family member 8B (Wnt8B), one of the canonical Wnt ligands, is implicated in oncogenesis. However, the role of Wnt8B in human HCCs and its transcriptional regulation mechanism are presently unknown. Here, we report that Wnt8B expression was frequently increased in HCCs and was significantly associated with poorer patient prognosis. Wnt8B knockdown suppresses HCC cell growth both in vitro and in vivo via inhibiting the canonical Wnt signaling. Zinc finger transcription factor 191 (ZNF191) can positively regulate Wnt8B mRNA and protein expression, and promoter luciferase assay indicated that ZNF191 can increase the transcription activity of the 2-Kbps *WNT8B* promoter. Chromatin immunoprecipitation-qPCR and electrophoretic mobility shift assay showed that ZNF191 protein directly binds to the *WNT8B* promoter, and the binding sites are at nt-1491(ATTAAATT) and nt-1178(ATTCATT). Moreover, Wnt8B contributes to the effect of ZNF191 on cell proliferation, and Wnt8B expression correlates positively with ZNF191 in human HCCs. Our findings suggested that Wnt8B, directly transcriptionally regulated by ZNF191, plays a pivotal role in HCC proliferation via the canonical Wnt pathway and may serve as a new prognostic biomarker and a potential therapeutic target for HCC patients.

Abbreviations: ALT, alanine aminotransferase; CTNNB1, β -catenin; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; LEF, lymphoid enhancer factor; MMTV, mouse mammary tumor virus; mRNA, messenger RNA; qRT-PCR, quantitative real-time polymerase chain reaction; RNAi, RNA interference; SD, standard deviation; shRNA, short hairpin RNA; siRNA, small interfering RNA; TCF, T cell factor; TCGA, The Cancer Genome Atlas; TMA, tissue microarray; Wnt, wingless-type MMTV integration site family; Wnt8B, Wnt family member 8B; ZNF191, zinc finger transcription factor 191.

Yufeng Liu and Di Wu contributed equally to the work.

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KEYWORDS

Wnt8B, zinc finger transcription factor 191, hepatocellular carcinoma, cell proliferation, canonical wntless-type (Wnt) pathway

1 | INTRODUCTION

Hepatocellular carcinoma (HCC) is a primary cancer of the liver and the fifth most common cancer worldwide with an estimated death rate of 781 600 deaths/year, which represents the second most common cause of cancer death worldwide.¹ The interaction of genetic predisposition, environmental factors (metabolic syndrome, alcohol, aflatoxin B1, aristolochic acid), and viruses (HBV, HCV) has been reported to drive HCC development.² Several cellular signal transduction pathways, which are activated and involved in HCC, and aberrant activation of the canonical wntless-type (Wnt)/ β -catenin signaling pathway in HCC have been frequently reported in the pathogenesis of the development and progression of HCC.³⁻⁶

The canonical Wnt/ β -catenin signaling is activated following Wnt ligand binding to frizzled receptors (FZD). Then, the β -catenin destruction complex (Axin-APC-GSK-3 complex) is inhibited, leading to a block in β -catenin phosphorylation by GSK-3. Hypophosphorylated β -catenin (active- β -catenin) accumulates in the cytoplasm and then translocates to the nucleus, where it regulates target genes expression such as Cyclin D1 and c-Myc through an elevated level of constitutive transcriptional activation by β -catenin/TCF complexes.^{4,6,7} Aberrant activation of the signaling in HCC includes mutations in β -catenin, Axin, Axin2, or APC genes,^{8,9} or upregulation of ligands Wnt1/3/10B, frizzled-7, β -catenin,^{5,10,11} or altered expression of several antagonists of Wnt signaling such as sFRP, WIF1, and Dkk, etc.⁸ Identifying novel dysregulated Wnt pathway constituents and exploring their underlying biological mechanisms in HCCs may provide the rationale for developing novel diagnostic markers or therapeutic targets for HCCs.

Wnt family member 8B (Wnt8B), one of the canonical Wnt ligands,⁶ is implicated in oncogenesis, several developmental processes, including regulation of cell fate and patterning during embryogenesis,¹²⁻¹⁷ and other pathophysiological processes.¹⁸⁻²⁰ Expression of human Wnt8B mRNA was detected in embryoid body derived from ES cells, hepatocyte progenitors derived from ES cells, and fetal brain.^{21,22} In several tumors including gastric cancer, pancreatic cancer, colorectal cancer, breast cancer, and embryonal tumors, Wnt8B mRNA expression was also found upregulated.^{23,24} Two studies have observed the molecule expression in hepatoma cell lines, that is, Wnt8B mRNA was selectively expressed in well-differentiated hepatoma cell lines,²⁵ and HCV core protein could upregulate the mRNA and proteins of several molecules upstream of β -catenin including Wnt8B in hepatoma cell line SMMC-7721.²⁶ However, the role of Wnt8B in human HCCs and the transcriptional regulation mechanism of Wnt8B are presently unknown.

In this work, we demonstrated for the first time that Wnt8B is upregulated in human HCCs, and zinc finger transcription factor 191 (ZNF191) can directly bind to the *WNT8B* promoter, transactivate the *WNT8B* gene, increase Wnt8B mRNA and protein expression, and subsequently activate the canonical Wnt signal pathway to promote the cell proliferation of HCC. The findings suggest that Wnt8B may serve as a new prognostic biomarker for predicting patient outcome and as a potential therapeutic target for improving the efficacy of accurate treatment.

2 | MATERIALS AND METHODS

2.1 | Tumor samples and tissue microarrays (TMAs)

Fresh surgical tissues of HCC (n = 41), including tumor tissues and the neighboring pathologically nontumoral liver tissues, were obtained from patients with liver cancer at Zhongshan Hospital (Shanghai, China). All the samples were immediately frozen in liquid nitrogen after surgery and then later stored at -80°C before further analysis. TMAs containing paired (n = 160) HCC and adjacent nontumoral tissues were also collected from Zhongshan Hospital from February to December 2008. Informed consent was obtained from each subject or subject's guardian after approval by Zhongshan Hospital Ethics Committee. The mean age of the patients was 51.7 years (range, 22-77 years). The last follow-up was in June 2014, with the median follow-up of 21.53 months (range, 0.2-76 months).

2.2 | Immunohistochemistry (IHC) staining

HCC tissues or orthotopic tumors were sectioned as 4- μ m-thick slices and were dewaxed in xylene and rehydrated using 95% ethanol. The endogenous peroxidase activity was quenched by immersing the slides in a 0.3% H₂O₂ solution for 30 minutes at room temperature. Sections were incubated with primary antibodies overnight at 4°C. The horseradish peroxidase (HRP)-conjugated secondary antibody was applied at 37°C for 60 minutes and incubated with diaminobenzidine solution, and nuclei were then counterstained with Harris' hematoxylin. The relevant primary antibodies we used included anti-Wnt8B (Abcam), anti-ZNF191 (Abcam), anti- β -catenin (Santa Cruz Biotechnology), anti-active- β -catenin (Merck Millipore), anti-Cyclin D1 (Santa Cruz Biotechnology), anti-c-Myc (Cell Signaling), and Ki-67 (Abcam).

The stained tissue slices were scored by two different pathologists blinded to patients' clinical characteristics. The intensity of

IHC staining was scored as 0 (negative), 1 (weak), 2 (medium), and 3 (strong). The percentage of positive cells in the whole tissue slice was recorded. Intensity score and positive rate score were then multiplied to calculate the overall score.

2.3 | Luciferase reporter assays

For luciferase assay, the reporter construct (a 2100-bp section of the *WNT8B* promoter, wild type or mutants, was cloned into the pGL3-luciferase vector) was cotransfected into HEK-293T cells with pCMV-ZNF191 or the control pCMV-Myc vector alone using LipofectAMINE 2000 agents (Invitrogen). At 24 hours after transfection, cell lysates

were prepared and luciferase activity was measured by using a Dual Reporter Assay System (Promega) according to the manufacturer's instructions. The plasmid pRL-SV40 (Promega) encoding Renilla luciferase was used for internal normalization in each transfection. All transfections were performed three times in triplicate.

2.4 | Chromatin immunoprecipitation (ChIP) qPCR assay

Chromatin immunoprecipitation from Hep3B cells was performed as described previously using a validated rabbit polyclonal anti-ZNF191 antibody (from Abcam, ab176589).²⁷ The crosslinked protein-DNA

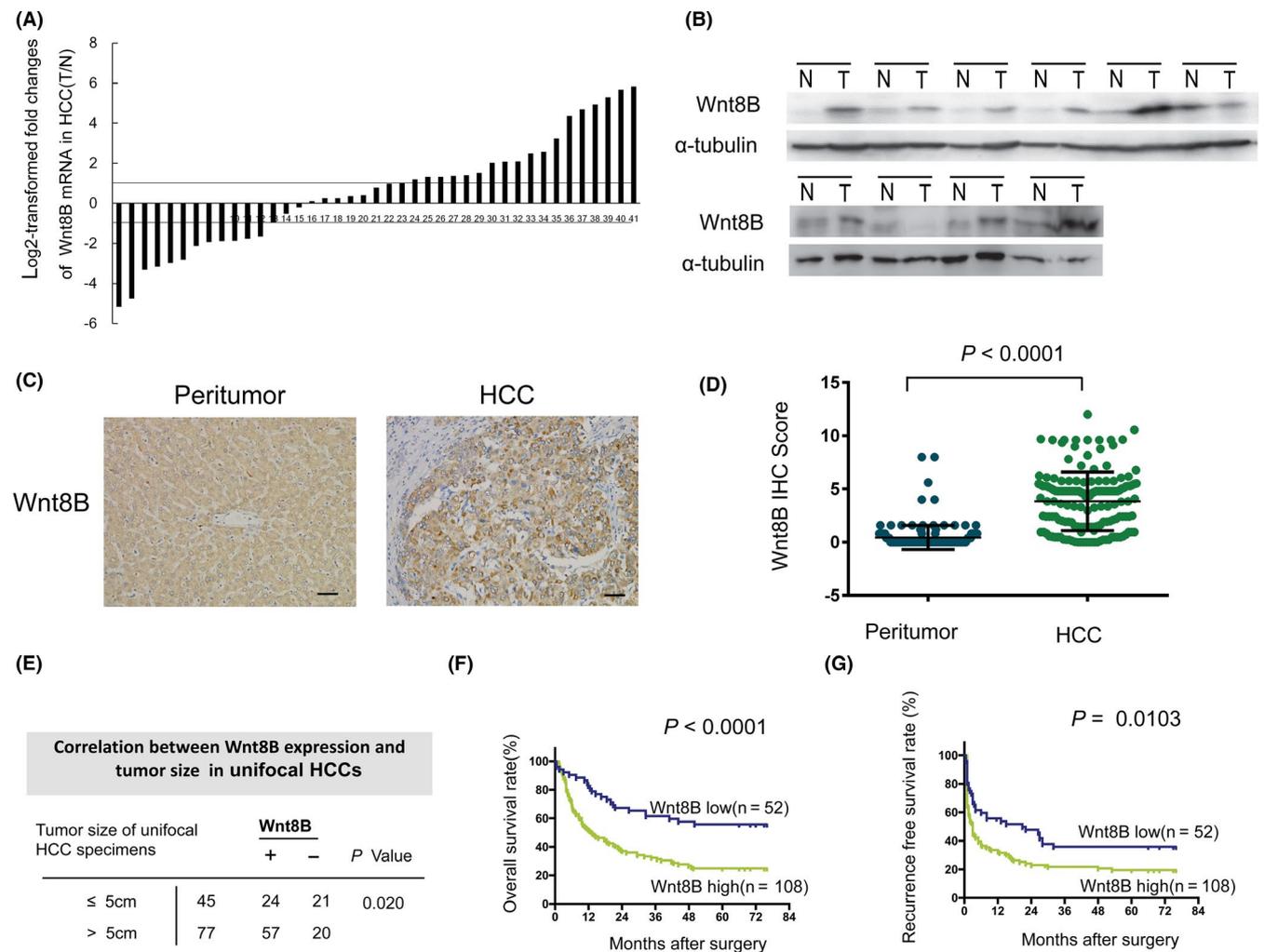


FIGURE 1 Upregulation of Wnt8B in hepatocellular carcinoma (HCC) is correlated with poor patient survival. A, Wnt8B mRNA expression levels were analyzed in 41 paired HCCs (nos. 1-41, numbered according to relative expression level from low to high) by real-time PCR and the $2^{-\Delta\Delta C_t}$ method. Values are expressed as log₂ transformed relative fold decrease or fold increase in the mRNA expression with respect to the adjacent nontumoral tissues after normalization to β 2-MG. A twofold change threshold was set for identifying significant changes in gene expression. B, Wnt8B protein expression was detected by Western blotting in ten pairs of in HCC tumor (T) and adjacent nontumoral tissues (N). C, Representative immunohistochemistry (IHC) staining with Wnt8B in HCC tissue (right) and adjacent normal tissue (left). Scale bar = 50 μ m. D, Scatter plots for corresponding evaluated IHC score in tumor and peritumor HCC specimens (n = 160). IHC scores were compared by Student's *t*-test. E, The relationship between Wnt8B staining status and tumor size of unifocal HCCs. Statistical significance was determined by χ^2 -test. Kaplan-Meier plots indicate (F) the overall survival rate and (G) recurrence-free survival rate (RFS) for HCC patients categorized by Wnt8B expression. *P*-value is determined by log-rank test

complexes were immunoprecipitated with corresponding antibodies. Isolated DNA was subjected to q-PCR. The primers used in the amplification are listed in Table S1.

2.5 | Electrophoretic mobility shift assay (EMSA)

5' biotin-labeled oligonucleotides were synthesized and labeled by Invitrogen. Purification of ZNF191 recombinant proteins was described previously.²⁸ DNA binding activity was detected using a LightShift Chemiluminescent EMSA kit (Pierce). For competition assays, unlabeled oligonucleotides were included in the binding reaction. The sequences of the oligonucleotides used for these binding studies are listed in Table S1.

2.6 | Statistical analysis

In real-time PCR, relative gene expression levels were calculated as described previously.⁵ A twofold change threshold was set for identifying significant changes in gene expression. The other data have been presented as the mean \pm standard deviation (SD). The significance of the differences was determined by Student's *t*-test. The statistical significance was set at a *P*-value of $<.05$. Statistical analyses were performed with the SPSS 12.0 (SPSS Inc) statistical software package and GraphPad Prism 6.0 (GraphPad).

Additional experimental methods are described in Appendix S1.

3 | RESULTS

3.1 | Wnt8B is upregulated in HCC tissues and correlated with survival

To investigate the expression status of Wnt8B in HCCs, Wnt8B mRNA expression between 41 pairs of fresh HCC tissues (T) with their adjacent nontumor tissues (N) was compared by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) assay and the $2^{-\Delta\Delta Ct}$ method. As shown in Figure 1A, 22 of 41 (53.6%) cases showed significant upregulation of Wnt8B in HCC tissues, while 19 of 41 (46.4%) cases showed no alteration or reduction. In agreement with the higher mRNA level of Wnt8B in HCC, the Wnt8B protein was also upregulated in seven out of ten HCC samples.

Next, TMAs, which contained 160 pairs of HCC specimens and corresponding adjacent noncancerous tissues, and detailed pathologic information with survival prognosis of patients were examined by IHC staining (Figure S1). As shown in Figure 1C, Wnt8B was expressed diffusely in the cytoplasm of tumor cells (Figure 1C), while it was only expressed at low levels in normal liver tissues. The positive staining of HCC was significantly higher in tumors (108/160, 67.5%) than in nontumor tissues (52/160; 32.5%; $P < .0001$) (Figure 1D).

The clinicopathological analysis revealed that high Wnt8B expression was closely correlated with liver cirrhosis ($P = .017$, χ^2 -test),

high ALT level ($P = .033$, χ^2 -test) (Table S2), and large tumor size in unifocal HCCs ($P = .020$, χ^2 -test) (Figure 1E). Importantly, Kaplan-Meier survival analysis showed that patients with high expression of Wnt8B had significantly worse overall survival (mean of 29.0 vs 50.4 months, log-rank test $P < .0001$; Figure 1F) and recurrence-free survival (RFS; mean of 19.7 vs 33.15 months, log-rank test $P = .0103$; Figure 1G) than those with low Wnt8B expression. The multivariate Cox regression analysis revealed that Wnt8B overexpression was an independent prognostic factor for overall survival (hazard ratio = 2.689; 95% confidence interval, 1.680-4.304; $P < .001$) and RFS (hazard ratio = 1.809; 95% confidence interval, 1.190-2.750; $P = .006$) (Table S3 and Table S4). Taken together, the data suggest that overexpression of Wnt8B is frequently detected in HCCs and that Wnt8B expression is a significant and independent index for HCC outcomes.

3.2 | Wnt8B knockdown suppresses HCC cell growth both in vitro and in vivo

To obtain insight into Wnt8B function, we employed a loss-of-function approach to assess the role of Wnt8B in HCC cell growth. Wnt8B was transient or stable knockdown with transfection of siRNA or lentivirus shWnt8B in human hepatic L02 cells and hepatoma Hep3B cells (Figure 2A, Figure S2A). Wnt8B stable knockdown induced the reduction of a number of colonies (Figure 2B). Consistent with the colony formation result, Cell Counting Kit-8 (CCK-8) assay, which allows sensitive colorimetric assays for the determination of cell viability in cell proliferation, showed that Wnt8B knockdown inhibited cell proliferation as well (Figure 2C). The results of transient Wnt8B knockdown were similar to stable knockdown as shown in Figure S2.

In xenograft mouse models, as shown in the left panels of Figure 2D,E, Wnt8B knockdown resulted in a significant decrease in the volume of L02 and Hep3B tumors. Consistently, the size and weight of Wnt8B knockdown tumors were much smaller than those of control tumors (Figure 2D,E, middle and right panels), accompanied by commensurate reduction in ki67 staining (Figure 2F). Taken together, these data suggest that Wnt8B knockdown suppresses cell proliferation and that Wnt8B is associated with HCC cell growth.

3.3 | Wnt8B activates the canonical Wnt signal pathway in HCC cells

Because Wnt8B is one of the Wnt family members which can activate canonical Wnt signaling cascades,²⁵ we hypothesized that Wnt8B regulates HCC cell proliferation by this signaling pathway. Not surprisingly, transient ectopic overexpression of Wnt8B increased in β -catenin, active- β -catenin, Cyclin D1, and c-Myc, the crucial proteins in canonical Wnt signaling cascades²⁹ in L02 and Hep3B cells transfected with pRNTER-His-Wnt8B (Figure 3A).

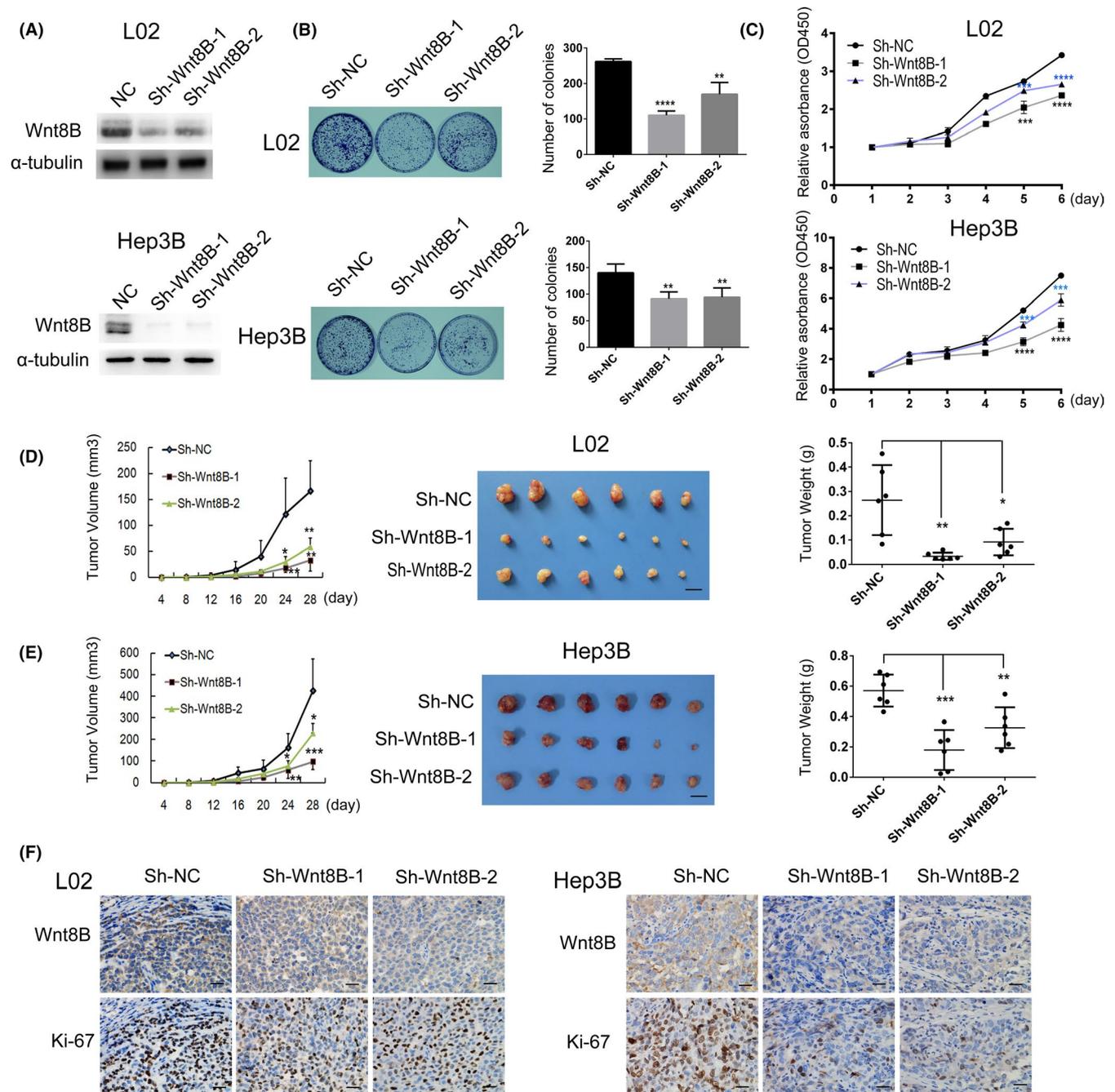


FIGURE 2 Wnt8B is associated with hepatocellular carcinoma (HCC) cell growth. A, Western blotting analysis showing stable endogenous ablation of Wnt8B in L02 (left panel) and Hep3B (right panel) cells infected with lentivirus expressing Wnt8B-specific short hairpin RNA (shRNA). The effect of Wnt8B stable knockdown on L02 or Hep3B cells' growth was measured with colony formation assay (B) and with CCK-8 assay (C). D, Effect of Wnt8B on the growth of L02 xenograft in nude mice. The left panel shows the tumor volume of subcutaneous xenografts at the indicated days after injection. The middle panel shows the resected tumors from individual nude mice. Scale bar = 1 cm. Weight of the resected tumors is shown in the right panel. E, Effect of Wnt8B on growth of Hep3B xenograft in nude mice. * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$. F, Expression of Wnt8B and expression of Ki67 were positively correlated. Immunohistochemistry (IHC) staining for indicated proteins in tumor tissues from mice with subcutaneous HCC implantation. Scale bar = 50 μm

While Wnt8B knockdown (with siRNA or shRNA) downregulated the above Wnt signaling crucial proteins (Figure S3 and Figure 3B). Moreover, in Wnt8B knockdown xenograft tumors, these crucial proteins were decreased as well (Figure 3C,D). Thus, the results suggest that Wnt8B can regulate HCC cell proliferation by activating the canonical Wnt signal pathway as well.

3.4 | Identification of transcription factor ZNF191 which positively regulates Wnt8B expression in HCC

The finding that Wnt8B is upregulated in HCCs and positively promotes HCC cell proliferation prompted us to determine the mechanism of the upregulation of Wnt8B. To this end, we first studied the

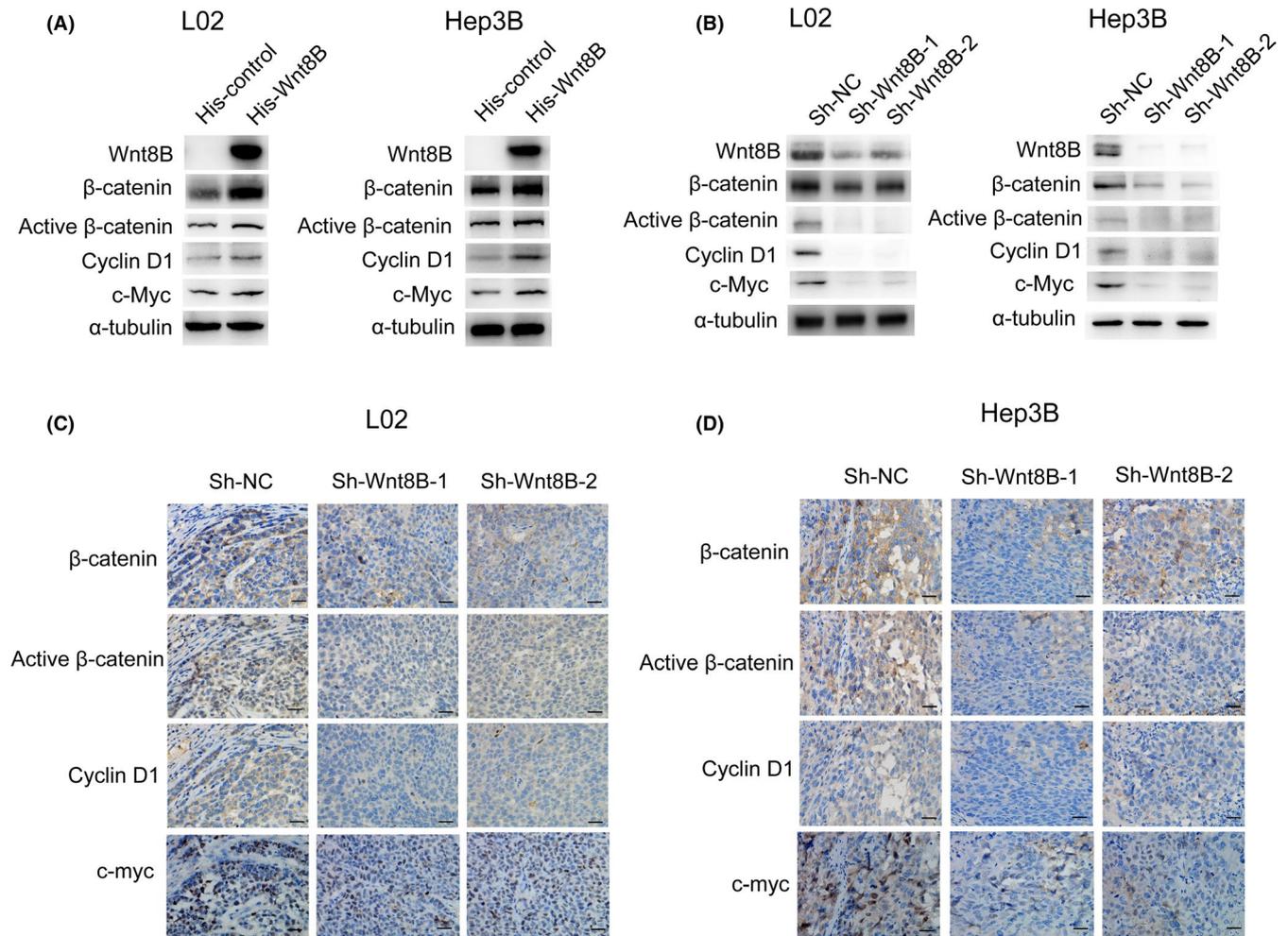


FIGURE 3 Wnt8B regulated the expression of the crucial proteins of the canonical Wnt pathway. Western blotting analysis of Wnt8B, β-catenin, active-β-catenin, Cyclin D1, c-myc, and α-tubulin (loading control) in (A) L02 (left panel) and Hep3B (right panel) cells transfected with pENTER-His-Wnt8B and mock pENTER-His vectors; and (B) L02 (left panel) and Hep3B (right panel) cells with Wnt8B stable knockdown. Immunohistochemistry staining of β-catenin, active-β-catenin, Cyclin D1, and c-myc in xenograft tumor tissues from mice with subcutaneous implantation of L02 (C) and Hep3B (D) cells with Wnt8B stable knockdown. Scale bar = 50 μm

status of mutation or copy number alteration of the *WNT8B* gene in HCCs. However, only one case (0.16%) of 625 HCC samples in The Cancer Genome Atlas (TCGA) database has a G77R missense mutation (Figure S4A,B). Thus, we believed that the upregulation of Wnt8B mRNA is not due to *WNT8B* gene mutation or copy number alteration.

Our previous studies have shown that transcription factor ZNF191, which is upregulated in HCCs, can activate Wnt/β-catenin signaling pathway by directly binding to the *CTNNB1* promoter and activate the expression of β-catenin and its downstream target genes such as Cyclin D1 in hepatoma cell lines.⁵ We doubted whether other members of the signaling pathway are also regulated by ZNF191 and involved in activating the Wnt pathway process. By mining transcript profiling data of ZNF191 knockdown or overexpression in HEK-293T cells and in our previous work,^{5,30} we found that several molecules of Wnt family members were potential targets of ZNF191. Then, Wnt8B and Wnt2 mRNA were confirmed to be significantly downregulated when ZNF191 was ablated with siRNA in HEK-293T cells (Figure S5A) with

qRT-PCR assay. Furthermore, when ZNF191 was ectopically overexpressed, Wnt8B mRNA was upregulated accordingly (Figure S5B).

To investigate the relationship of ZNF191 and Wnt8B in HCC, we analyzed the Wnt8B mRNA and protein levels in L02 and Hep3B cells with transient overexpressed or knockdown ZNF191. As expected, Wnt8B mRNA and protein levels increased with ectopic overexpression of ZNF191 protein (Figure 4A). Consistently, in transient ZNF191 knockdown L02 and Hep3B cells, the expression of Wnt8B was downregulated as the endogenous ZNF191 protein depleted, compared with controls (Figure 4B). Moreover, in stable ZNF191 knockdown L02 and Hep3B cell lines, Wnt8B, β-catenin, active-β-catenin, Cyclin D1, and c-Myc proteins were downregulated as well (Figure 4C).

To further confirm the correlation of ZNF191 and Wnt8B in HCC in vivo, the correlations of the ZNF191 mRNA expression level with Wnt8B were further studied in 41 human HCC tissues. Fold changes of ZNF191 and Wnt8B mRNA compared with nontumor

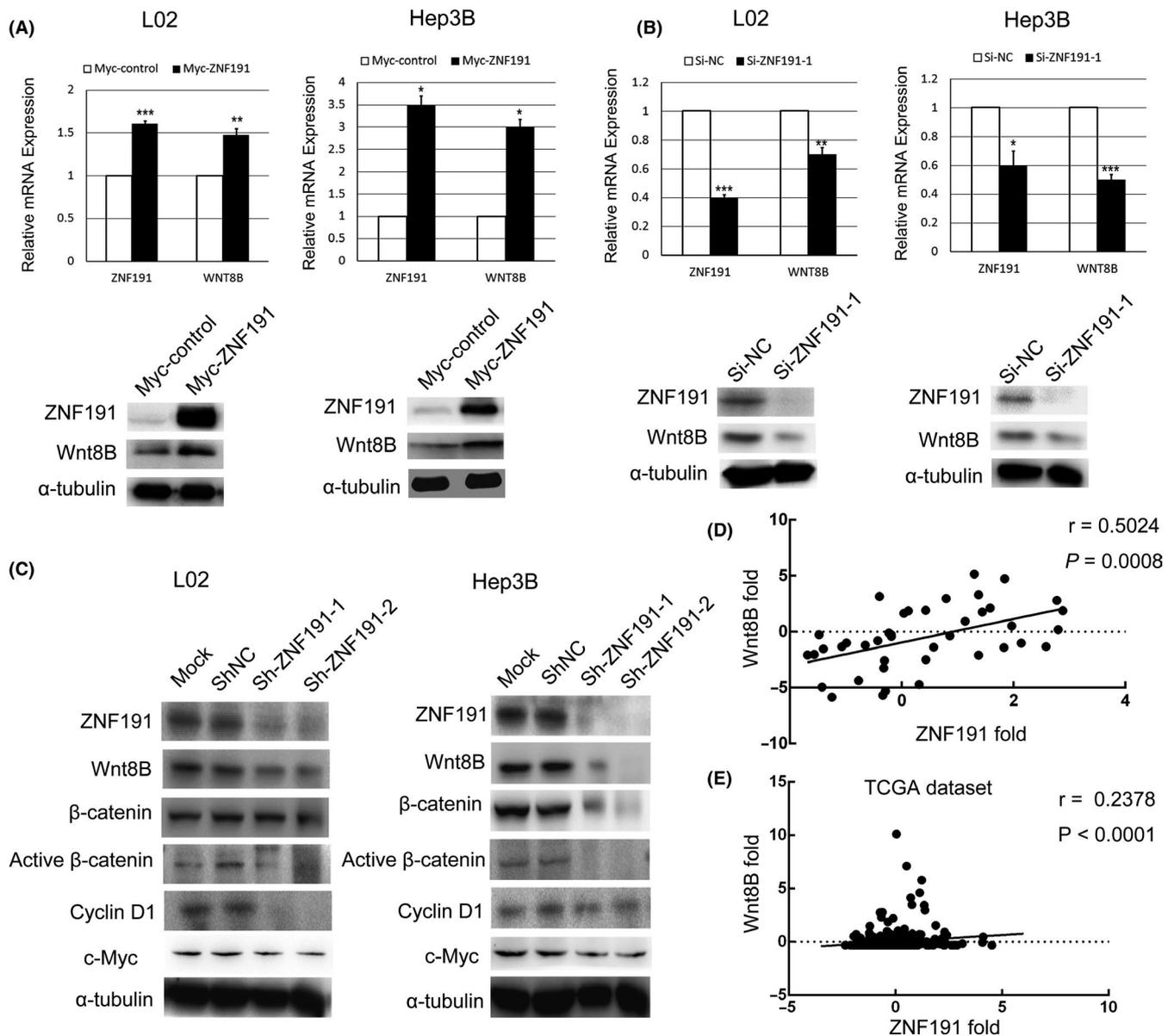


FIGURE 4 Zinc finger transcription factor 191 (ZNF191) can positively induce Wnt8B expression in hepatocellular carcinoma (HCC). A, Ectopic overexpression of ZNF191 increased Wnt8B mRNA and protein expression in L02 (left panel) and Hep3B (right panel) cells measured with qRT-PCR (top panel) and Western blotting analysis (bottom panel). B, Transient knockdown of ZNF191 downregulated Wnt8B mRNA and protein expression. * $P < .05$, ** $P < .01$, *** $P < .001$. C, Wnt8B, β -catenin, active- β -catenin, Cyclin D1, and c-myc protein expression were analyzed with Western blotting analysis in stable knockdown of ZNF191 in L02 (left panel) and Hep3B (right panel) cells with indicated lentivirus. D, The mRNA level of ZNF191 and Wnt8B in 41 pairs of human HCC and paracancerous tissues was examined by qRT-PCR. Correlation analysis was performed with Pearson's correlation test. E, ZNF191 and Wnt8B mRNA expression levels were correlated in HCC specimens ($P < .001$). Data from 373 HCC specimens were downloaded from The Cancer Genome Atlas (TCGA) database at the cbiportal website (<http://www.cbiportal.org>). Unit of axis: RNA-sequencing V2 RSEM. Spearman's correlation coefficients are listed in the panels

adjacent tissues were measured using qRT-PCR. Correlation analysis was performed with Pearson's correlation test. The results confirmed that Wnt8B expression was significantly correlated with the expression of ZNF191 ($r = 0.5024$; $P < .001$, Figure 4D). Moreover, the correlation of their mRNA levels was further supported in a cohort of 373 HCC samples of TCGA dataset ($r = 0.2378$; $P < .001$, Figure 4E). Taken together, these results suggested that Wnt8B is associated with ZNF191 in HCC, which likely contributes to tumor proliferation.

3.5 | ZNF191 increases the transcription activity of the WNT8B promoter and directly binds to the promoter

As ZNF191 can positively regulate mRNA and protein levels of Wnt8B, we wondered whether ZNF191 can directly bind to the WNT8B promoter and activate its transcription activity. With delicate analysis of the nucleotide sequences of the 5'-flanking region (-2000/+100) of the WNT8B gene, we found there are

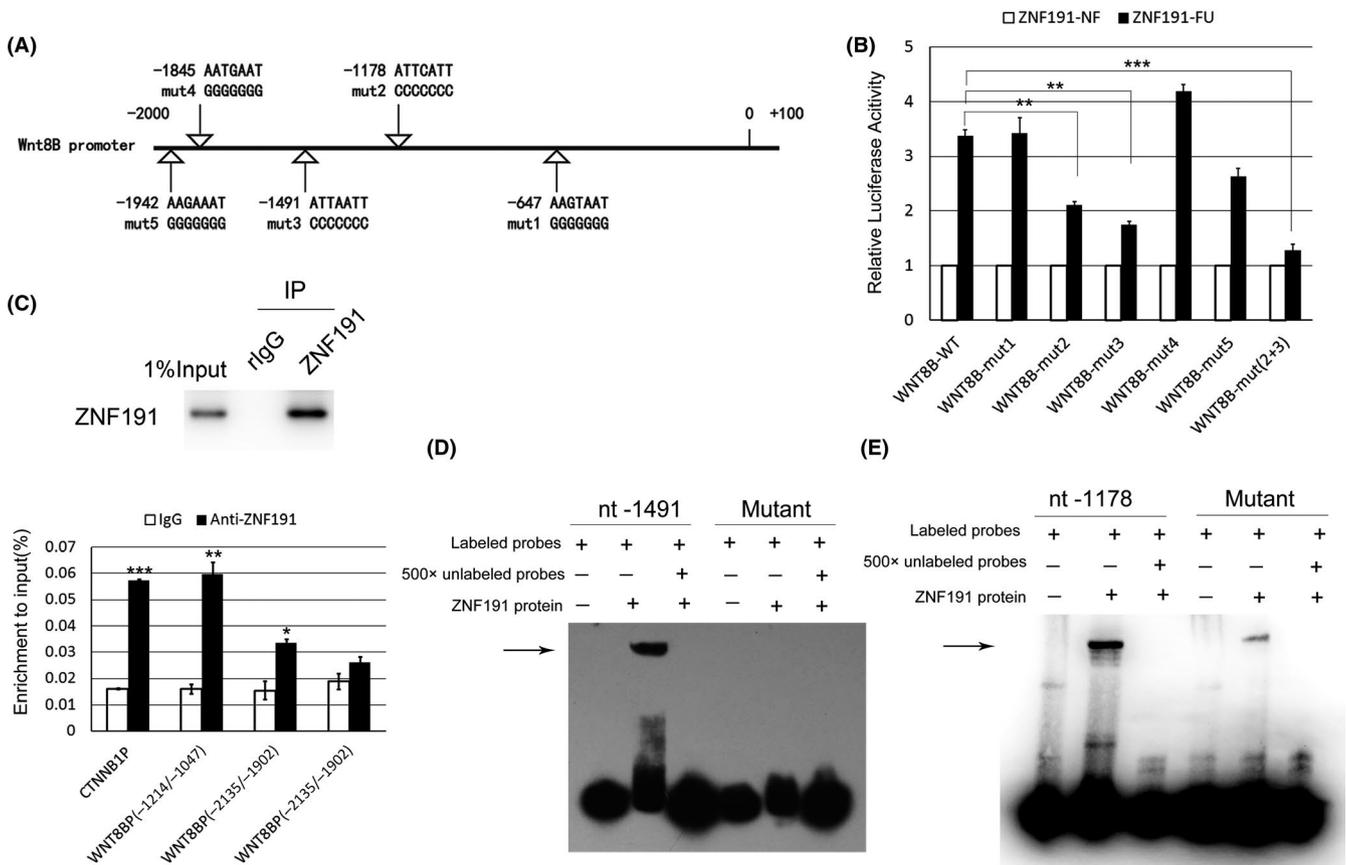


FIGURE 5 Zinc finger transcription factor 191 (ZNF191) activates *WNT8B* transcription and directly binds to the *WNT8B* promoter. A, The sketch of the 5'-flanking region (-2000/+100) of the *WNT8B* gene, and potential binding sites and their mutated sequences for further analysis. B, The effect of ZNF191 on the transcriptional activity of *WNT8B* promoters. Activity of wild-type *WNT8B* promoter and the mutated types (single mutations or double mutations) were measured by luciferase reporter assays. C, ChIP qPCR was performed to confirm the binding of ZNF191 in the *WNT8B* promoter. ZNF191 containing DNA-protein complexes were immunoprecipitated as verified by immunoblotting using rabbit polyclonal anti-ZNF191. Rabbit IgG (Sigma-Aldrich) was used as a negative control (top panel). The primers specific to the regions WNT8BP(-1047/-1241) and WNT8BP(-1305/-1511) containing two potential ZNF191 binding sites at nt-1491 (ATTAATT) and nt-1178(ATTTCATT), and WNT8BP(-2135/-1902) containing site 5 at nt (AAGAAAT) were included. CTNNB1P (CTNNB1 promoter containing ZNF191 binding site) was used as positive control (bottom panel). * $P < .05$, ** $P < .01$, *** $P < .001$. D-E, DNA binding activities of purified ZNF191 protein to two 30-bp annealed oligonucleotides of the *WNT8B* promoter WNT8BP(nt-1178), WNT8BP(nt-1491), and their mutants were detected by EMSA. Arrows indicate specific bands of purified ZNF191 binding to the oligonucleotides

two potential ZNF191 binding sites at nt-1491(ATTAATT) and nt-1178(ATTTCATT) (Figure 5A, Figure S4C), and the sequences are the same as or similar to the key binding sequence of ZNF191 in vivo (ie, ATTAATT), as we previously reported.⁵ Promoter luciferase assay indicated that ZNF191 can increase the transcription activity of the 2 Kbps full-length *WNT8B* promoter about 3.4-fold compared with transfecting control vector. While the activation ability of mutated *WNT8B* promoters (with ATTAATT and ATTTCATT being mutated to CCCCCC) by ZNF191, especially that of *WNT8B* promoters with mutation of both potential binding sites, was markedly reduced compared with that of wild-type promoters (Figure 5B).

Furthermore, ChIP qPCR confirmed that endogenous ZNF191 was directly binding to the promoter in Hep3B cells containing

the two sites (Figure 5C). EMSA further verified that recombinant ZNF191 directly bound to these sequences with high affinity. When the binding sequences were mutated, however, the binding capacity of ZNF191 was substantially mitigated (Figure 5D,E). Taken together, these data suggest that ZNF191 can directly bind to the *WNT8B* promoter, and the key sequences of binding sites are at nt-1491 (ATTAATT) and nt-1178 (ATTTCATT).

3.6 | Wnt8B contributes to the effect of ZNF191 on HCC cell proliferation, and they are clinically relevant

Our previous research demonstrated that ZNF191 promotes HCC cell growth by activating CTNNB1 via the canonical Wnt pathway,⁵

and now we proved that ZNF191 can directly bind to the *WNT8B* promoter and transactivates *WNT8B* in HCC cells. We wondered how Wnt8B affects ZNF191 function on cell proliferation via the pathway. To this end, we observed the effect of ZNF191 on HCC cells growth with or without endogenous Wnt8B protein expression. As shown in Figure 6A,B, when endogenous Wnt8B was depleted with siRNA in L02 and Hep3B, ZNF191 knockdown inhibited cell proliferation to a larger extent compared with knockdown ZNF191 alone without Wnt8B depletion beforehand. Correspondingly, in Wnt8B knockdown cells, depleting ZNF191 decreased β -catenin, active- β -catenin, Cyclin D1, and c-Myc more remarkably (Figure 6C). Moreover, as shown in Figure 6D, the depletion of Wnt8B inhibited ZNF191-induced Wnt-responsive TCF/LEF reporter Topflash-Luc activities.⁵ The results demonstrated that the cell proliferative function and the canonical Wnt pathway activation of ZNF191 is partially Wnt8B-dependent.

Next, we examined the correlation between the expression of ZNF191 and Wnt8B proteins in consecutive sections in a cohort of 80 HCC specimens using IHC (Figure 6E). Wnt8B expression was positively correlated with ZNF191 expression in HCC tissues (χ^2 -test, $P = .038$, Figure 6E). These data confirmed that Wnt8B was correlated with ZNF191 expression and they were clinically relevant in HCCs.

Taken together, ZNF191 may function through upregulating Wnt8B, in addition to β -catenin,⁵ to promote tumor cell proliferation at an early stage of HCC because ZNF191 can activate both *CTNNB1* and *WNT8B* to promote HCC cell proliferation, as shown in the sketch of Figure 6F.

4 | DISCUSSION

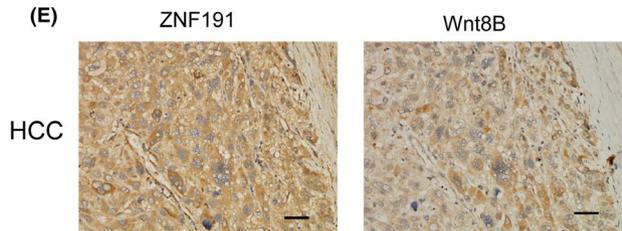
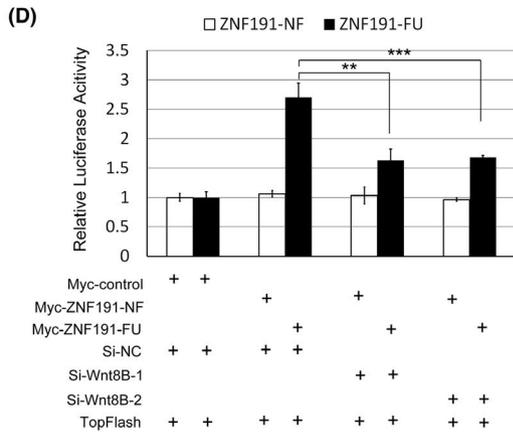
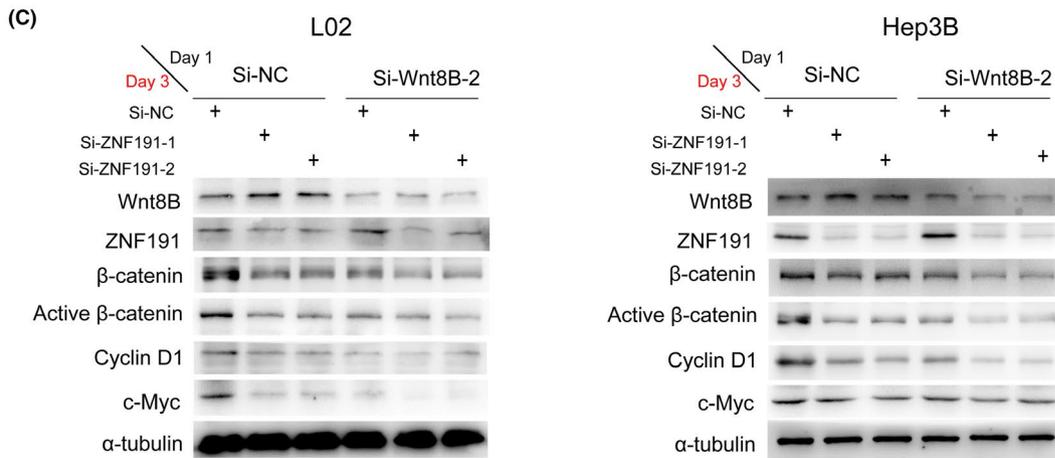
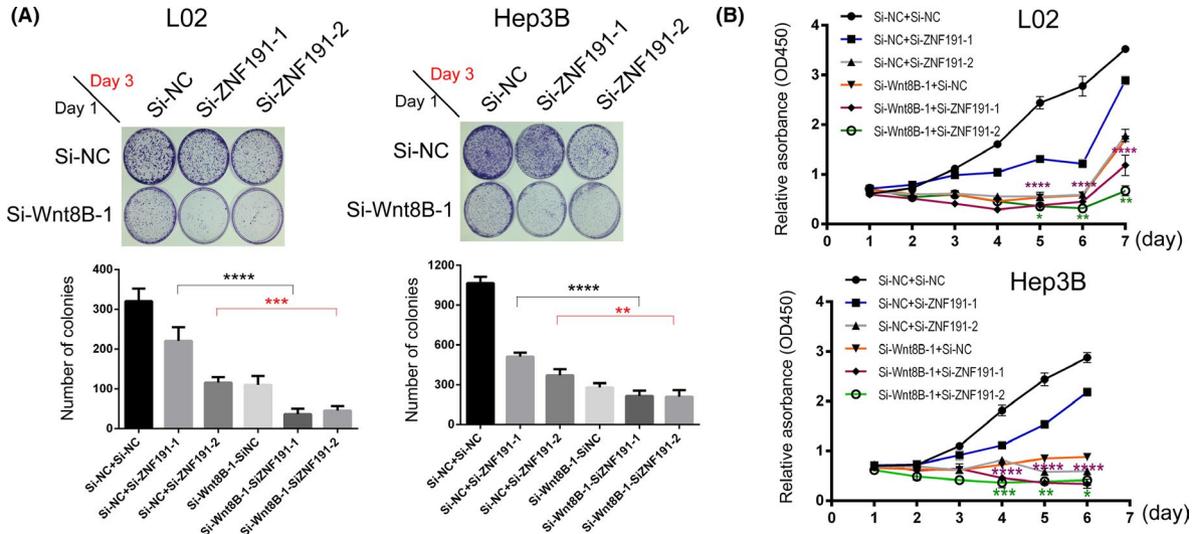
The human *WNT8B* gene is located at 10q24 and encodes the Wnt8B protein which shows 95%, 86%, and 71% amino acid identity to mouse, zebrafish, and *Xenopus* Wnt8B proteins, respectively.¹³ The expression patterns of human and mouse genes appear identical and are restricted to the developing brain. Wnt8B mRNA is not expressed in adult human liver tissue (<https://www.ncbi.nlm.nih.gov/gene/7479>). In this study, we demonstrate for the first time that Wnt8B mRNA and protein is highly expressed in human HCC tissues (Figure 1A-D). Furthermore, the analysis of TMAs validated the frequent overexpression of Wnt8B (102/160; 63.75%) in HCCs, which was significantly correlated with large tumor size in unifocal HCCs (Figure 1E). Moreover, sustained expression of Wnt8B in HCC tissue was correlated with accelerated tumor progression and worse patient outcome (Figure 1F,G). All of these data support the tumorigenic role of Wnt8B, and Wnt8B expression could be used as a potential prognostic predictor for HCCs. Furthermore, we proved that Wnt8B, as a canonical Wnt molecule,⁶ regulates HCC cell growth via activating the canonical Wnt signal pathway as well (Figures 2 and 3). Since Wnt8B is a secreted signaling protein,²⁹ and it functions in paracrine fashion rather than endocrine fashion,³¹ we surmise that it should be secreted and act locally in HCC

tissues rather than widely circulating by blood to promote HCC cell proliferation.

Given that there are rare gene mutations and copy number alterations of the *WNT8B* gene in liver cancer (Figure S4), and Wnt8B mRNA expression is also upregulated in several tumors,^{23,24} we concentrated on the transcriptional regulation of Wnt8B. Here, we identified that ZNF191 positively induces Wnt8B expression in HCC. With a series of methods (Figures 4 and 5), ZNF191 is proved to directly bind to the *WNT8B* promoter and activate the expression of the *WNT8B* gene in hepatoma cells. Moreover, the two molecules were both highly expressed and clinically relevant in HCCs (Figure 6D). Thus, although ZNF191 directly transactivates *WNT8B* and promotes cell proliferation of HCCs, the mechanism of ZNF191 high expression in HCCs needs further study. Besides, we proved that the transactivation of Wnt8B expression partially contributes to the effect of ZNF191 on the cell growth of HCCs (Figure 6E) because ZNF191 can also directly transactivate *CTNNB1*.⁵ Taken together, we identified a novel mode of mechanism involved in the control of Wnt8B abundance in HCCs.

Certainly, in addition to ZNF191, it is possible that other transcription factors may regulate Wnt8B expression as well. Katoh *et al* reported that conserved POU/OCT-binding and GATA-binding sites exist in the 5'-flanking promoter region of mammalian *WNT8B* orthologs. They revealed that Wnt8B expression in hepatocyte progenitors derived from human ES cells is due to POU5F1 (OCT3/OCT4) and GATA3, and that Wnt8B expression in diffuse-type gastric cancer is due to POU5F1 and GATA6.²¹ However, whether these factors directly bind to the *WNT8B* promoter to activate the gene was not shown in their research. Thus, whether besides ZNF191, POU5F1 and GATA3/6 or other transcription factors participate in the regulation of Wnt8B expression in hepatoma cells remains unknown and warrants further investigation.

Several studies have reported the regulation of Wnt8B expression in embryo development, including transactivation by transcription factors binding to its promoter or enhancer, or autoregulation mechanism. Two transcription factors, sine oculis-related homeobox 3 (Six3) and forkhead box G1 (Foxg1), have been reported to directly bind to the Wnt8B promoter, downregulate its activity, and repress Wnt8B expression in vivo during mouse or zebrafish embryo development.^{12,32,33} In the development of the mouse cerebral cortex, fibroblast growth factor (Fgf) signaling activates Ets transcription factors binding to a Wnt8B dorsomedial telencephalon enhancer, and then activates Wnt8B expression.¹⁷ The Wnt8B enhancer also contains an essential binding site for Lef/Tcf transcription factors which implies that Wnt8B can also autoregulate its own expression.³⁴ These reported regulation mechanisms of Wnt8B in zebrafish and mouse may be involved in the regulation human Wnt8B expression in embryo development and HCC tumorigenesis as well. As ZNF191/Zfp191 mRNAs have been reported to be expressed in embryo brain and are necessary to maintain neural cells as cycling progenitors,^{35,36} we suppose that the regulation of Wnt8B expression by ZNF191 may be also involved in embryo brain development, which warrants further study.



Relationship between ZNF191 and Wnt8B staining patterns in HCC

HCC specimens	Wnt8B		P Value
	+	-	
ZNF191 Higher	44	19	0.038
ZNF191 Lower	36	17	

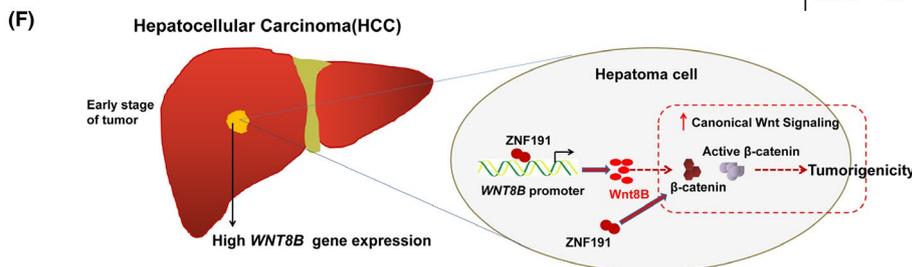


FIGURE 6 Wnt8B mediates the effect of zinc finger transcription factor 191 (ZNF191) on hepatocellular carcinoma (HCC) cell proliferation, and they are clinically correlated. The effect of double knockdown of Wnt8B and ZNF191 on the cell growth of L02 (left panel) and Hep3B (right panel) compared with knockdown ZNF191 alone was measured with the colony formation assay (A) and CCK-8 assay (B). C, Western blotting analysis of Wnt8B, β -catenin, active- β -catenin, Cyclin D1, and c-Myc in L02 and Hep3B cells with double knockdown of Wnt8B and ZNF191. D, The effect of Wnt8B knockdown on ZNF191-induced Topflash-Luc activities was assessed by luciferase activity assays. HEK-293T cells were transfected with Wnt8B siRNA, pCMV-Myc-ZNF191-FU (full length), and Top-flash-Luc. pRL-SV40 was used as the internal control in each transfection. pCMV-Myc-ZNF191-NF (without C2H2 zinc finger domain) was used as a negative control. $P < .05$, $**P < .01$. E, Correlation between the expression of ZNF191 and Wnt8B in human HCC tissues. Representative IHC staining of ZNF191 and Wnt8B in consecutive sections of HCC specimens (top panel). Scale bar = 50 μm . The relationship between ZNF191 and Wnt8B staining status. Statistical significance was determined by χ^2 -test (bottom panel). F, The hypothetical working model. This model diagram indicates ZNF191 can activate both CTNNB1 and WNT8B to promote HCC cell proliferation

Thus, we observed a new aberrant activation Wnt pathway constituent Wnt8B, and identified a novel mode of mechanism involved in the control of Wnt8B abundance in HCC. Wnt8B may serve as a clinically useful diagnostic and prognostic biomarker and a potential therapeutic target in HCCs.

ACKNOWLEDGEMENTS

This work was financially supported by the Natural Science Foundation of China (NSFC 31671461, NSFC 31301162, NSFC 81871929, NSFC 81572298, NSFC 81972621).

DISCLOSURE

The authors have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Liu Y, Wu D, Cheng H, et al. Wnt8B, transcriptionally regulated by ZNF191, promotes cell proliferation of hepatocellular carcinoma via Wnt signaling. *Cancer Sci*. 2021;112:629-640. <https://doi.org/10.1111/cas.14738>