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HBV core protein enhances WDR46 stabilization to upregulate NUSAP1 and promote HCC progression

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Abstract

Background: The HBV core protein (HBC) is crucial for the progression of HCC. WD repeat-containing (WDR) 46 (WDR46) is implicated in the development of different tumors. Nevertheless, whether WDR46 is controlled by HBC to drive hepatocarcinogenesis remains unclear.

Methods: Different HCC cohorts, immunohistochemical staining, and bioinformatics analysis were utilized to estimate the clinical correlation between WDR46 and HBV-associated HCC. Western blotting, co-immunoprecipitation, chromatin immunoprecipitation, and oncology functional assays were performed to evaluate the effect of HBC on WDR46 in upregulating nucleolar spindle-associated protein 1 (NUSAP1), the influence of WDR46 on HBC-mediated HCC cell biological functions, and the mechanisms of WDR46 upregulation mediated by HBC to increase NUSAP1.

Results: WDR46 expression was elevated in HBV-related HCC in a HBC-dependent manner. Overexpression of WDR46 is closely linked to severe prognosis of tumors. Functionally, WDR46 contributes to HBC-induced cell growth and migration in vitro and in vivo. Furthermore, HBC enhanced WDR46 protein stabilization by hampering the interaction between WDR46 and TRIM25, thereby decreasing WDR46 ubiquitination. NUSAP1, a DNA replication-related molecule, is a vital downstream target of WDR46. Relying on WDR46, HBC promoted NUSAP1 upregulation to modulate the biological functions of HBC in HCC cells. Importantly, HBC enhanced the interaction

Abbreviations: AFP, alpha-fetoprotein; CCK-8, cell counting kit-8; ChIP, chromatin immunoprecipitation; CHX, cycloheximide; Co-IP, co-immunoprecipitation; GSEA, gene set enrichment analysis; H&E, hematoxylin–eosin; HBC, HBV core protein; IF, immunofluorescence; IHC, immunohistochemistry; MS, mass spectrometry; NUSAP1, nucleolar spindle-associated protein 1; OS, overall survival; RFS, recurrence-free survival; shRNA, short-hairpin RNA; WDR, WD repeat-containing; WDR4, WD repeat-containing 46.

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between WDR46 and the transcription factor c-Myc to facilitate c-Myc recruitment to the NUSAP1 promoter, leading to the increase of NUSAP1 transcription.

Conclusions: Our comprehensive data provides new insights into the mechanisms responsible for HBC-induced hepatocarcinogenesis. WDR46 and its downstream molecule, NUSAP1, may act as novel therapeutic targets for HBV-related tumors.

Keywords: c-Myc, HBV core protein, NUSAP1, TRIM25, WDR46

INTRODUCTION

The infection of HBV is a significant cause of HCC.[1,2] Due to the lack of effective molecular targets in HBV-positive HCC, patients with tumors often have an adverse prognosis. HBV core protein (HBC), a virus-encoded molecule, not only contributes to viral replication but is also involved in the pathogenesis induced by HBV.[3] In particular, HBC facilitates HCC development by regulating the expression or activity of C5AR1,[4] NEU1,[5] Src,[6] and RANGAP1.[7] Considering the significance of HBC in hepatocarcinogenesis, further elucidation of the mechanisms driving tumor progression induced by the viral protein may provide novel therapeutic strategies targeting HCC with HBV infection.

WD repeat-containing (WDR) proteins, which have the characteristic of WD-repeat domains,[8] are implicated in a wide spectrum of cellular processes, and perturbations in many of the family proteins are closely related to a variety of human disorders,[9–11] including HCC.[12] WDR46 belongs to the WDR protein family.[13] Current investigations indicate that WDR46 is associated with the development of gastric carcinoma[14] and colorectal cancer.[11] However, the correlation between WDR46 and HCC, particularly HBV-associated HCC, remains unclear.

Nucleolar spindle-associated protein 1 (NUSAP1) is a critical regulator of DNA replication and mitosis.[15,16] In recent years, aberrant overexpression of NUSAP1 has been discovered with the development of prostate cancer,[17] glioma,[18] and renal cell carcinoma.[19] The upregulation of NUSAP1 has also been reported in HCC,[20] particularly in HBV-associated HCC.[21] Despite HBX, a non-structural protein of HBV, being discovered to benefit NUSAP1 upregulation in HCC cells with HBV infection,[22] our understanding of NUSAP1 elevation induced by the virus still needs to be explored.

In the present study, the effects of HBC on WDR46 in regulating NUSAP1 expression, and the influence of WDR46 and NUSAP1 on the biological roles of HBC in HCC were examined. We provided evidence that HBC can increase WDR46 stabilization by destroying the interaction of WDR46 with TRIM25 to suppress WDR46 ubiquitination. Depending on c-Myc, WDR46 enhances NUSAP1 gene transcription to facilitate cell growth and migration controlled by HBC. In summary, our findings revealed novel mechanisms that contribute to WDR46 upregulation mediated by HBC to elevate NUSAP1 and facilitate hepatocarcinogenesis.

METHODS

Reagents and cell culture

The short-hairpin RNA (shRNA) plasmid against TRIM25, HBV plasmid, TRIM25 plasmid, cycloheximide (CHX), MG132, plasmids with HBV-encoded genes, antibodies against HA-Tags, Flag-Tags, HBsAg, HBC, and other reagents were obtained as previously described.[5,7,23] The antibodies targeting ubiquitin, WDR46, GAPDH, β-Tubulin, TRIM25, and c-Myc were purchased from Abconal, Proteintech, ZEN-BIOSCIENCE, and Huabio. Protein G Sepharose beads were from Santa Cruz Biotechnology. The BeyoClick EdU Cell Proliferation Kit was obtained from Beyotime. Bafilomycin A1 (BafA1) was purchased from MedChemExpress (MCE). EpiQuik Chromatin Immunoprecipitation (ChIP) Kit was obtained from Epigentek (Epigentek Group Inc.). Plasmids containing shRNA against WDR46 (sequences information: TCGAT GGCCTGGAGAGTAATC), c-Myc (sequences information: GCAGTTGAAACACAAACTTGAA), and NUSAP1 (sequences information: GGAAGAAACGCGAGCAAGA), and vectors containing WDR46, c-Myc, WDR46 mutants, and HBC mutant genes were constructed by YouBio. HEK293T and hepatoma cell lines (PLC/PRF/5, HepG2, SK-Hep1, and Huh7) were cultured, and transfected with different vectors as previously described.[5,24]

Animal studies

Four-week-old BALB/c nude mice were purchased and fed as previously described.[7] To establish a subcutaneous transplanted tumor model, the nude mice were

randomly distributed in each group, HepG2 cell suspensions (0.1 mL, 2×10^7/mL per mouse), combined with Matrigel solution (0.1 mL, BD Biosciences), were injected into the shoulder of the nude mice. After feeding for 2–3 weeks, tumor samples were obtained from nude mice. Tumor weight and volume in each group were examined.

Lung metastasis models were established via intravenous tail vein injection of HepG2 cells (0.1 mL, 2×10^7/mL per mouse) into nude mice. Animals were dissected during the 4th week of the experiment. The lungs of nude mice in each group were fixed and stained with hematoxylin–eosin (H&E) solution to examine the number of lung metastatic nodules. Animal experiments were approved by the Animal Care and Use Committee of Xuzhou Medical University (Approval number: 202307T011).

RNA sequencing and bioinformatics analysis

RNA sequencing was performed at the Gene Denovo Biotechnology Co. The threshold for differentially expressed genes (DEGs) was a log 2-fold change of 2 ($p < 0.05$). Gene set enrichment analysis (GSEA), the performance of which has been described by our group previously,[7,23] was used to examine the significantly enriched pathways associated with WDR46, depending on the RNA-sequencing data and HCC cohort from the ICGC database.[25] The significant signaling pathways relevant to WDR46 and NUSAP1 were also predicted using the ARCHS^4 database.[26] The expression of the WDR46 gene was obtained from the Gao cohort[27], and information on NUSAP1 protein expression in the Gao cohort was from CPTAC database. Furthermore, based on the median value, the low or high WDR46 and NUSAP1 groups were divided to perform Kaplan–Meier survival analysis by using the ICGC cohort and Gao cohort;[25,27] the acquisition of clinical information of those HCC cohorts was mentioned previously.[7,28] Potential E3 ligases of WDR46 were obtained from the UbiBrowser database.[29] Gene information on WDR46 and NUSAP1 expression in HCC tissues was extracted from ICGC and 7 different HCC cohorts from the HCCDB database.[25,30] Additionally, the expression levels of these 2 genes in HBV-related HCC tissues were obtained from the Gao and GSE94660 datasets.[27,31]

HCC samples, H&E, immunohistochemistry (IHC) staining, ChIP assay, Co-immunoprecipitation (Co-IP), western blotting, and other methods used in the study were presented in the Supplemental Materials, http://links.lww.com/HC9/B945.

Statistical analysis

Data presented as the mean ± SD were obtained from at least 3 independent experiments. Statistical significance was determined using an unpaired *t* test or one-way ANOVA. Kaplan–Meier survival analysis with a log-rank test or Gehan–Breslow–Wilcoxon test was used to define the association between WDR46 or NUSAP1 expression and overall survival (OS) or recurrence-free survival (RFS). The chi-square test was performed to test the IHC staining results. *p* values are shown in the figures with asterisks: $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

RESULTS

WDR46 overexpression links to the unfavorable progress of HCC, in which HBC enhances WDR46 expression

To determine the correlation between WDR46 and HCC, the expression levels of WDR46 in HCC and adjacent tissues collected by us were analyzed by IHC. Compared to adjacent normal tissues, higher WDR46 expression was observed in cancer tissues (Figure 1A). For further verification, the HCC cohort from the ICGC database,[25] and 7 individual HCC cohorts from the HCCDB database were used.[30] In agreement with the IHC staining results, increased WDR46 expression was observed in these HCC cohorts (Figures 1B, C). Additionally, based on the ICGC cohort, elevated expression of WDR46 was found in HCC patients with TNM stages > II (compared to ≤ II) and with portal vein invasion (compared to those without portal vein invasion) (Figures 1D, E). Furthermore, HCC patients with high levels of WDR46 exhibited a poorer OS (Figure 1F). Together, these results suggest that the increase in WDR46 is significantly related to liver cancer progression and adverse clinical prognosis.

Importantly, relying on the ARCHS^4 database,[26] WDR46 was shown to be significantly relevant to viral carcinogenesis and hepatitis B (Figure 1G), implying that WDR46 might participate in the development of HCC relevant to HBV infection. Dependent on the Gao cohort[27] and GSE94660,[31] the elevated expression of WDR46 was identified in HBV-positive HCC tissues, compared to adjacent tissues (Figures 1H, I). The clinical relevance of WDR46 in the Gao cohort has also been explored. The expression levels of WDR46 in persons aged ≤ 50 years and alpha-fetoprotein (AFP) > 20 ng/mL were higher than those in HBV-associated HCC patients aged > 50 years and AFP ≤ 20 ng/mL (Figures 1J, K). Moreover, a significant association of WDR46 overexpression with OS and RFS was observed in patients with HBV-positive tumors (Figures 1L, M). Depending on the ICGC HCC cohort, we also investigated the expression of WDR46 in HBV-positive HCC, HCV-related HCC, and non-HBV/non-HCV (NBNC)-associated HCC (Figure 1N). The results showed that, compared to adjacent normal tissues, the

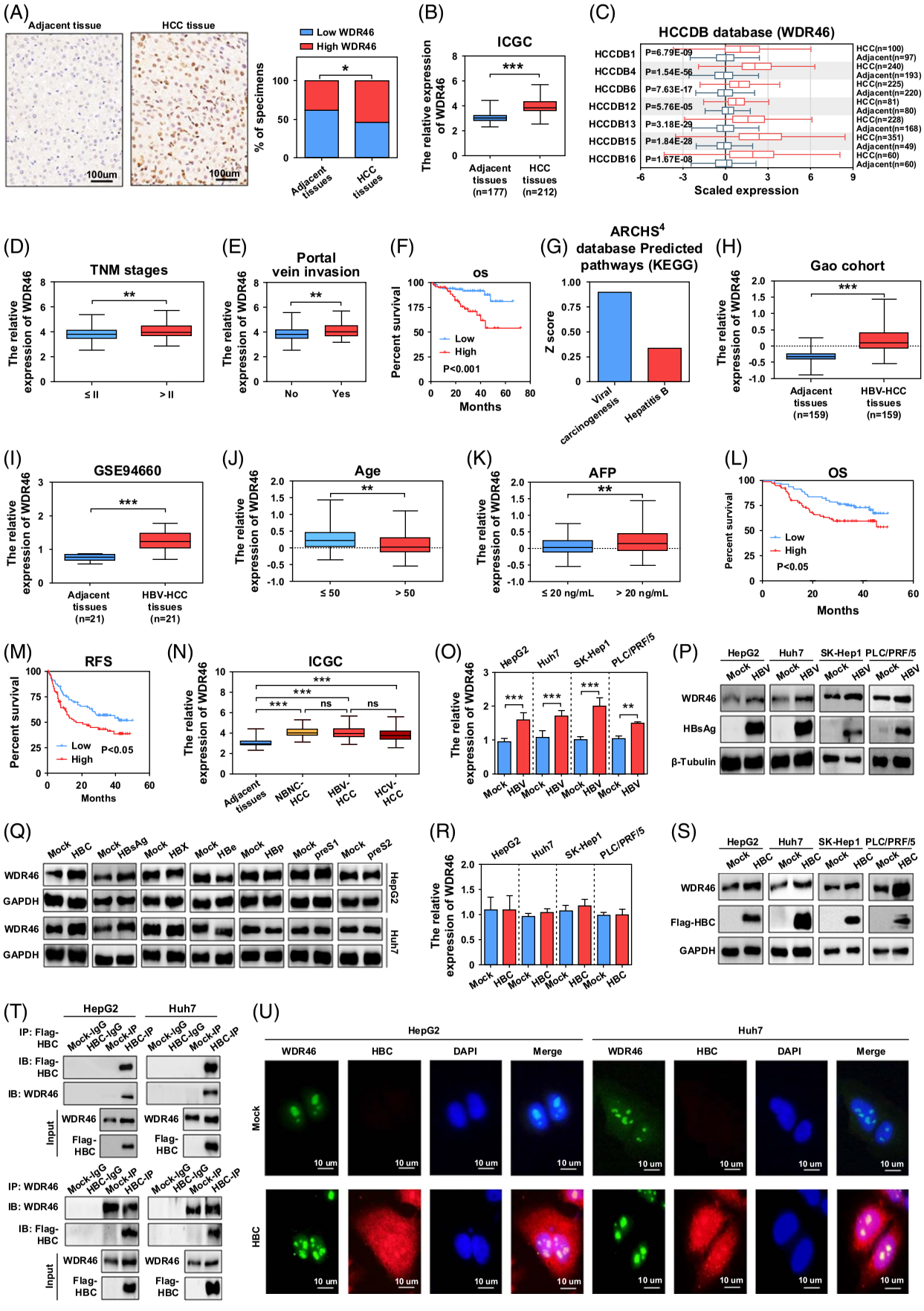

(A)
Adjacent tissue
HCC tissue
100um
100um
Low WDR46
High WDR46
% of specimens
Adjacent tissues
HCC tissues
(B)
ICGC
The relative expression of WDR46
Adjacent tissues (n=177)
HCC tissues (n=212)
(C)
HCCDB database (WDR46)
HCCDB1 P=6.79E-09
HCCDB4 P=1.54E-56
HCCDB6 P=7.63E-17
HCCDB12 P=5.76E-05
HCCDB13 P=3.18E-29
HCCDB15 P=1.84E-28
HCCDB16 P=1.67E-08
Scaled expression
(D)
TNM stages
The relative expression of WDR46
≤ II
> II
(E)
Portal vein invasion
No
Yes
(F)
os
Percent survival
Low
High
P<0.001
Months
(G)
ARCHS4 database Predicted pathways (KEGG)
Z score
Viral carcinogenesis
Hepatitis B
(H)
Gao cohort
Adjacent tissues (n=159)
HBV-HCC tissues (n=159)
(I)
GSE94660
Adjacent tissues (n=21)
HBV-HCC tissues (n=21)
(J)
Age
≤ 50
> 50
(K)
AFP
≤ 20 ng/mL
> 20 ng/mL
(L)
OS
Low
High
P<0.05
Months
(M)
RFS
Low
High
P<0.05
Months
(N)
ICGC
Adjacent tissues
NBNC-HCC
HBV-HCC
HCV-HCC
(O)
HepG2
Huh7
SK-Hep1
PLC/PRF/5
Mock
HBV
(P)
HepG2
Huh7
SK-Hep1
PLC/PRF/5
WDR46
HBsAg
β-Tubulin
(Q)
Mock
HBC
HBsAg
HBX
HBe
HBp
preS1
preS2
WDR46
GAPDH
HepG2
Huh7
(R)
HepG2
Huh7
SK-Hep1
PLC/PRF/5
Mock
HBC
(S)
HepG2
Huh7
SK-Hep1
PLC/PRF/5
WDR46
Flag-HBC
GAPDH
(T)
HepG2
Huh7
IP: Flag-HBC
Mock-IgG
HBC-IgG
Mock-IP
HBC-IP
IB: Flag-HBC
IB: WDR46
Input
WDR46
Flag-HBC
IP: WDR46
IB: WDR46
IB: Flag-HBC
(U)
HepG2
Huh7
WDR46
HBC
DAPI
Merge
Mock
HBC
10 um

FIGURE 1 The expression of WDR46 is increased in HCC, and it can be modulated by HBC. (A) The expression of WDR46 in HCC and adjacent tissues that detected by IHC. Scale bar, 100 μm. (B) The expression of WDR46 in the ICGC cohort. (C) WDR46 expression in 7 HCC cohorts from the HCCDB database. (D) The expression of WDR46 in HCC tissues in patients with TNM stage ≤ II or > II. (E) WDR46 expression in HCC tissues in patients with or without portal vein invasion. (F) The association of WDR46 with the OS of HCC patients in the ICGC database. (G) The relationship of WDR46 with significant pathways in the ARCHS[4] database. (H) The expression of WDR46 in HBV-associated HCC tissues and adjacent tissues in the Gao cohort. (I) The expression of WDR46 in HBV-associated HCC tissues and adjacent tissues in the GSE94660. (J) The expression of WDR46 in HBV-associated HCC patients with age ≤ 50 or > 50. (K) The expression of WDR46 in HBV-associated HCC patients with AFP ≤20 ng/mL or > 20 ng/mL. (L) The association of WDR46 with the OS of HBV-associated HCC patients in the Gao cohort. (M) The association of WDR46 with the RFS of HBV-associated HCC patients in the Gao cohort. (N) The expression of WDR46 in HBV-positive HCC, HCV-positive HCC, and non-HBV/non-HCV HCC in the ICGC cohort. (O) The expression of the WDR46 gene in HCC cells induced by HBV. (P) The expression of WDR46 protein in HCC cells induced by HBV. (Q) The expression of WDR46 protein in HCC cells is induced by different HBV-encoded proteins. (R) The expression of the WDR46 gene in HCC cells induced by HBC. (S) The expression of WDR46 protein in HCC cells induced by HBC. (T) The interaction between WDR46 with HBC was detected by Co-IP assay. (U) The colocation of WDR46 with HBC in HCC cells that detected by immunofluorescence experiment. Scale bar, 10 μm; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Abbreviations: AFP, alpha-fetoprotein; Co-IP, co-immunoprecipitation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HBC, HBV core protein; IHC, immunohistochemistry; NBNC, non-HBV/non-HCV; OS, overall survival; RFS, recurrence-free survival; WDR46, WD repeat-containing 46.

expression levels of WDR46 in HBV, HCV, and NBNC HCC tissues were notably higher, implying that multiple factors, including HBV, can upregulate WDR46 in HCC.

Subsequently, we estimated the effect of HBV on WDR46 expression using 4 different hepatoma cell lines. Our observation indicated that HBV can upregulate WDR46 at both the mRNA and protein levels (Figures 1O, P). Next, the viral proteins that contribute to the upregulation of WDR46 induced by HBV were examined. The results revealed that HBC and HBsAg can enhance the expression of WDR46 in hepatoma cells (Figure 1Q). Since HBC is essential for the development of HCC,[4,5] and based on Co-IP with mass spectrometry (MS) analysis, the interaction between HBC and WDR46 has been previously identified.[7] Here, we are interestingly in estimating the impact of WDR46 on hepatocarcinogenesis induced by HBC. The effects of HBC on WDR46 protein and gene expression were determined in 4 HCC cell lines. We found that HBC only upregulated WDR46 protein expression (Figures 1R, S). In addition, the interaction and colocalization of HBC with WDR46 in hepatoma cells were validated by Co-IP and immunofluorescence (IF) assays (Figures 1T, U).

WDR46 contributes to the growth and migration of HCC cells caused by HBC

The effects of WDR46 on HBC-induced cell proliferation and migration were also investigated. We synthesized a specific shRNA targeting WDR6 for a loss-of-function study and validated its silencing effect on WDR46 expression (Figure 2A). In agreement with our previous studies,[5,7] the proliferation and colony formation capacities of hepatoma cells were markedly enhanced by HBC, based on the results of CCK-8, EdU, and cell colony-forming experiments (Figures 2B–D). However, WDR46 silencing inhibited these biological functions of HBC-positive HCC cells. Using a subcutaneous xenograft model in nude mice, we investigated the effect of WDR46 on malignant tumorigenesis induced by HBC in vivo. The results demonstrate that HBC promotes HCC cell growth in vivo. After WDR46 knockdown, significant suppression of cell growth mediated by HBC was revealed, based on observations of tumor weights, and volumes, accompanied by the expression of the proliferation marker KI67 in HCC tissues from different experimental groups (Figures 2E–G).

The effects of WDR46 on HBC-mediated cell migration were evaluated in vitro. These results indicated that HBC enhanced HCC cell migration. However, after treatment with shRNA against WDR46, the migration capacity of HCC cells induced by the viral protein was reduced (Figures 2H, I). Consistently, in experimental lung metastasis models in nude mice established by tail vein injection, HBC-related HCC cells formed more pulmonary nodules than the HCC cells in the control group in vivo. However, based on the observation using H&E staining, silencing of WDR46 in HBC-related cells markedly reduced the number of nodules in mouse lungs (Figure 2J).

HBC increases the stabilization of WDR46 protein by disrupting the interaction between WDR46 and TRIM25

Next, the mechanisms that benefit the overexpression of the WDR46 protein induced by HBC were explored. Because the expression of most cellular proteins is controlled by the ubiquitin–proteasome degradation pathway or autophagy–lysosome degradation pathway,[32] we assessed the influence of these 2 degradation pathways on WDR46 expression in HCC cells. Interestingly, based on GSEA analysis, the pathways of protein ubiquitination, as well as E3-ubiquitin ligases ubiquitinate target proteins, were found to be relevant to WDR46 in HCC (Figure 3A). Furthermore, WDR46 expression was promoted when HCC cells were treated with MG132 (a proteasome inhibitor), but not with bafilomycin A1 (BafA1) (an autolysosome inhibitor) (Figure 3B), indicating that the

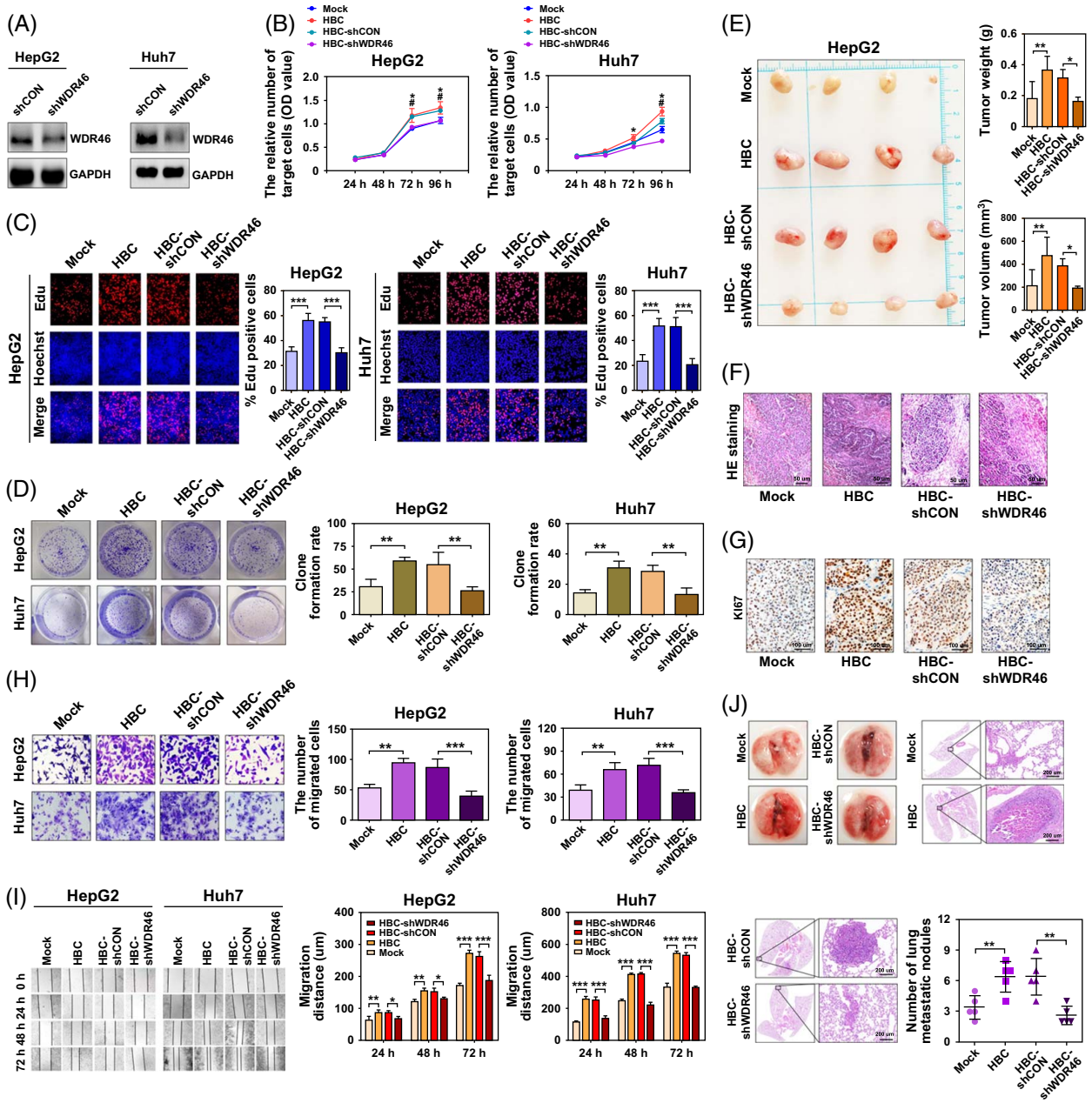
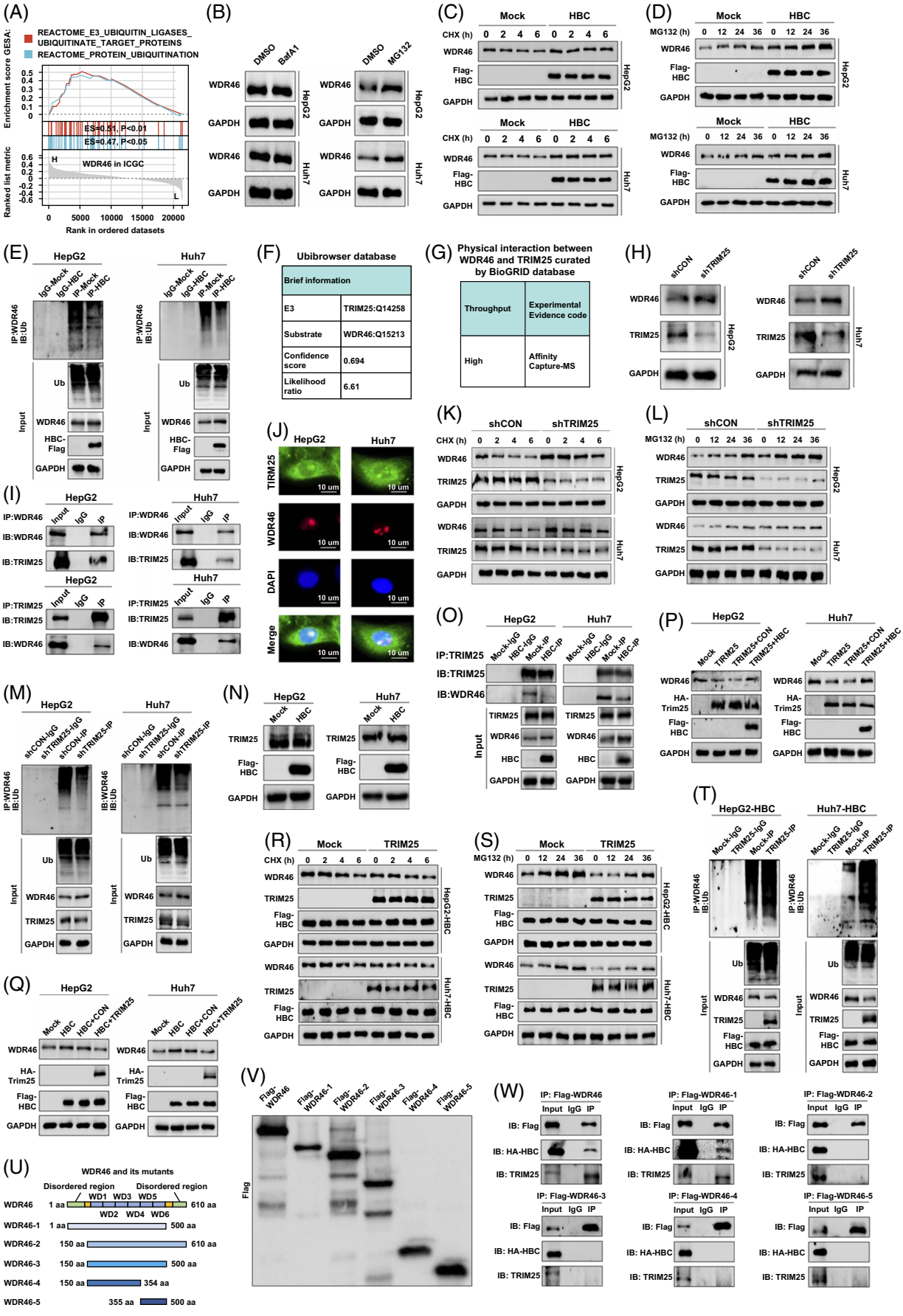


FIGURE 2 The effect of WDR46 on cell proliferation and migration mediated by HBC. (A) The inhibition of WDR46 protein expression is mediated by its specific shRNA. (B) The effect of WDR46 on cell viability mediated by HBC that detected by CCK-8. *$p < 0.05$, Mock group compares to HBC group, #$p < 0.05$, HBC-shCON group compares to HBC-shWDR46 group. (C) The effect of WDR46 on cell proliferation mediated by HBC that detected by EdU analysis. (D) The effect of WDR46 on colony formation mediated by HBC that detected by clone formation assay. (E) The effect of WDR46 on HCC cell proliferation mediated by HBC in nude mice. (F) H&E staining on the HCC tissues from nude mice in different groups. Scale bar, 50 μm. (G) KI67 expression was examined by IHC in tissues from nude mice in different groups. Scale bar, 100 μm. (H) The effect of WDR46 on cell migration mediated by HBC that detected by transwell assay. (I) The effect of WDR46 on cell migration mediated by HBC that detected by wound healing assay. (J) The effect of WDR46 on lung metastasis in HCC cells mediated by HBC in nude mice detected by H&E staining. Scale bar, 200 μm; *$p < 0.05$ and **$p < 0.01$. Abbreviations: CCK-8, cell counting kit-8; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H&E, hematoxylin–eosin; HBC, HBV core protein; IHC, immunohistochemistry; shRNA, short-hairpin RNA; WDR46, WD repeat-containing 46.

degradation of WDR46 mainly relies on the ubiquitin–proteasome pathway.

It has been demonstrated that HBC can control the stabilization of its target protein by disrupting the ubiquitination–proteasome degradation pathway.[7] We examined whether HBC affected the stabilization of WDR46 protein in this way. Consistent with these predictions, based on HCC cells treated with CHX (a protein synthesis inhibitor) and MG132, we found that HBC could elevate the half-life and protein stabilization of WDR46. Ubiquitination of WDR46 protein was suppressed by HBC (Figures 3C–E).


(A)
REACTOME_E3_UBIQUITIN_LIGASES_UBIQUITINATE_TARGET_PROTEINS
REACTOME_PROTEIN_UBIQUITINATION
Enrichment score GESA:
ES=0.51, P<0.01
ES=0.47, P<0.05
Ranked list metric
WDR46 in ICGC
Rank in ordered datasets
(B)
DMSO
BafA1
MG132
WDR46
GAPDH
HepG2
Huh7
(C)
Mock
HBC
CHX (h)
Flag-HBC
(D)
MG132 (h)
(E)
HepG2
Huh7
IgG-Mock
IgG-HBC
IP-Mock
IP-HBC
IP:WDR46 IB:Ub
Input
Ub
HBC-Flag
(F)
Ubibrowser database
Brief information
E3
TRIM25:Q14258
Substrate
WDR46:Q15213
Confidence score
0.694
Likelihood ratio
6.61
(G)
Physical interaction between WDR46 and TRIM25 curated by BioGRID database
Throughput
Experimental Evidence code
High
Affinity Capture-MS
(H)
shCON
shTRIM25
TRIM25
(I)
Input
IgG
IP
IP:WDR46
IB:WDR46
IB:TRIM25
IP:TRIM25
(J)
TIRM25
WDR46
DAPI
Merge
10 um
(K)
shCON
shTRIM25
(L)
(M)
shCON-IgG
shTRIM25-IgG
shCON-IP
shTRIM25-IP
IP:WDR46 IB:Ub
IB:WDR46 IB:Ub
(N)
(O)
Mock-IgG
HBC-IgG
Mock-IP
HBC-IP
IP:TRIM25
IB:TRIM25
IB:WDR46
(P)
Mock
TRIM25
TRIM25+CON
TRIM25+HBC
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(Q)
HBC
HBC+CON
HBC+TRIM25
(R)
(S)
(T)
HepG2-HBC
Huh7-HBC
(U)
WDR46 and its mutants
Disordered region
WD1 WD3 WD5
WD2 WD4 WD6
WDR46 1 aa 610 aa
WDR46-1 1 aa 500 aa
WDR46-2 150 aa 610 aa
WDR46-3 150 aa 500 aa
WDR46-4 150 aa 354 aa
WDR46-5 355 aa 500 aa
(V)
Flag-WDR46
Flag-WDR46-1
Flag-WDR46-2
Flag-WDR46-3
Flag-WDR46-4
Flag-WDR46-5
Flag
(W)
IP: Flag-WDR46
IP: Flag-WDR46-1
IP: Flag-WDR46-2
IP: Flag-WDR46-3
IP: Flag-WDR46-4
IP: Flag-WDR46-5
IB: Flag
IB: HA-HBC
IB: TRIM25

FIGURE 3 HBC disrupts the interaction between WDR46 with TRIM25 to stabilize WDR46 protein. (A) The significant pathways predicted by GSEA analysis with WDR46 in the ICGC HCC cohort. (B) The effect of BafA1 (200 nM) and MG132 (100 nM) on WDR46 expression in HCC cells. (C) The effect of HBC on the half-life of WDR46 protein in HCC tissues, when cells were treated with CHX (60 μg/mL). (D) The effect of HBC on stabilization of WDR46 protein in HCC tissues, when cells were treated with MG132 (100 nM). (E) The role of HBC on WDR46 ubiquitination in HCC cells. (F) The information on TRIM25 as a E3 ligase of WDR46 in the Ubibrowser database. (G) The predicted interaction between WDR46 and TRIM25 in the BioGRID database. (H) The expression of WDR46 in HCC cells with TRIM25 knockdown. (I) The interaction between WDR46 and TRIM25 in HCC cells was detected by Co-IP assays. (J) The colocation of WDR46 with TRIM25 in hepatoma cells was detected by immunofluorescence assay. Scale bar, 10 μm. (K) The effect of TRIM25 on half-life of WDR46 protein in HCC tissues, when cells were treated with CHX (60 μg/mL). (L) The effect of TRIM25 on stabilization of WDR46 protein in HCC tissues, when cells were treated with MG132 (100 nM). (M) The role of TRIM25 on WDR46 ubiquitination in HCC cells. (N) The effect of HBC on TRIM25 protein expression in HCC cells. (O) The effect of HBC on the interaction between WDR46 and TRIM25. (P) The effect of HBC on the expression of WDR46 mediated by TRIM25. (Q) The effect of TRIM25 on the expression of WDR46 mediated by HBC. (R) The effect of TRIM25 on the half-life of WDR46 protein mediated by HBC in HCC cells, when cells were treated with CHX (60 μg/mL). (S) The effect of TRIM25 on stabilization of WDR46 protein mediated by HBC in HCC cells, when cells were treated with MG132 (100 nM). (T) The role of TRIM25 on WDR46 ubiquitination mediated by HBC in HCC cells. (U) Pattern diagram of WDR46 mutants. (V) The expression of WDR46 mutants in HEK293T cells. (W) The interaction of WDR46 mutants with TRIM25 or HBC in HEK293T cells. Abbreviations: BafA1, bafilomycin A1; Co-IP, co-immunoprecipitation; CHX, cycloheximide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSEA, gene set enrichment analysis; HBC, HBV core protein; Ub, ubiquitin; WDR46, WD repeat-containing 46.

The ubiquitination of cellular proteins is regulated by E3 ligases.[33] Nevertheless, E3 ligases that benefit WDR46 ubiquitination are still unknown. Using the online Ubibrowser server,[29] WDR46 potential E3 ligases were predicted, and TRIM25, a vital E3 ligase that participates in the progression of HBV-associated HCC,[23] was shown to have the potential to regulate WDR46 ubiquitination with a high score (Figure 3F). According to the BioGRID database,[34] WDR46 was further identified to interact with TRIM25 (Figure 3G). Therefore, we speculated that based on the interaction, TRIM25 might facilitate the ubiquitination of WDR46 to inhibit its stabilization. The effect of TRIM25 on WDR46 expression was also evaluated. Our observation showed that inhibiting TIRM25 by shRNA can upregulate the WDR46 protein in hepatoma cells (Figure 3H). Subsequently, the interaction and colocation between WDR46 and TRIM25 were validated by Co-IP and IF experiments (Figures 3I, J). The effect of TRIM25 on WDR46 half-life, stabilization, and ubiquitination was also examined, and we noticed that TRIM25 can reduce WDR46 half-life and stabilization by enhancing its ubiquitination (Figures 3K–M).

Next, we assessed whether HBC affected TRIM25, leading to the inhibition of WDR46 ubiquitination to enhance its stability. Unexpectedly, no significant effect of HBC on TRIM25 expression was observed (Figure 3N). However, we found that the interaction between WDR46 and TRIM25 was restricted by HBC (Figure 3O). After transfection of the HBC gene into TRIM25-overexpressing HCC cells, the inhibition of WDR46 mediated by TRIM25 was restored (Figure 3P). Conversely, when TRIM25 was transfected into HBC-positive cells, the HBC-induced upregulation of WDR46 was inhibited (Figure 3Q). These results indicate that HBC can compete with TRIM25 to regulate WDR46 expression. To determine whether the role of HBC on WDR46 half-life, stabilization, and ubiquitination was strongly associated with TRIM25, we transfected ectopic TRIM25 into HBC-positive HCC cells and found that WDR46 half-life and stabilization mediated by HBC can be restricted by TRIM25. Meanwhile, TRIM25 overexpression enhanced WDR46 ubiquitination in HBC-positive HCC cells (Figures 3R–T).

Ultimately, we estimated that the regions of WDR46 interacted with HBC or TRIM25. As shown in Figures 3U, V, 5 WDR46 deletion mutants were constructed and their expression was confirmed. Based on the Co-IP assay, our findings demonstrated that the N-terminal region (1-149 aa) of WDR46 interacts with both HBC and TRIM25 (Figure 3W), suggesting that, to enhance WDR46 stabilization, HBC disrupts the binding of WDR46 to TRIM25 in N-terminal region of WDR46 to suppress WDR46 ubiquitination induced by TRIM25 in hepatoma cells.

Dependent on WDR46, HBC benefits NUSAP1 increase in HCC cells

Subsequently, we evaluated the underlying factors by which WDR46 enhanced HCC progression mediated by HBC. Based on RNA sequencing, DEGs regulated by WDR46 were identified (Figure 4A). Relying on GSEA analysis, we also examined the pathways significantly related to RNA-sequencing data. The investigation showed that the negative regulation of DNA replication was inversely associated with WDR46-associated DEGs (Figure 4B). GESA analysis was also performed on the ICGC HCC cohort, and the results showed that WDR46 was positively associated with DNA replication (Figure 4C). Collectively, these results suggested that molecules relevant to DNA replication are regulated by WDR46 in HCC cells. Next, we selected NUSAP1, a DEG shown in Figure 4A, for further analysis. NUSAP1 has been reported to participate in DNA replication.[35] Moreover, NUSAP1 is predicted to contribute to DNA replication, viral carcinogenesis, and hepatitis B, based on the ARCHS^4 database (Figure 4D), implying that NUSAP1 may have a vital role in the development of HBV-related HCC.

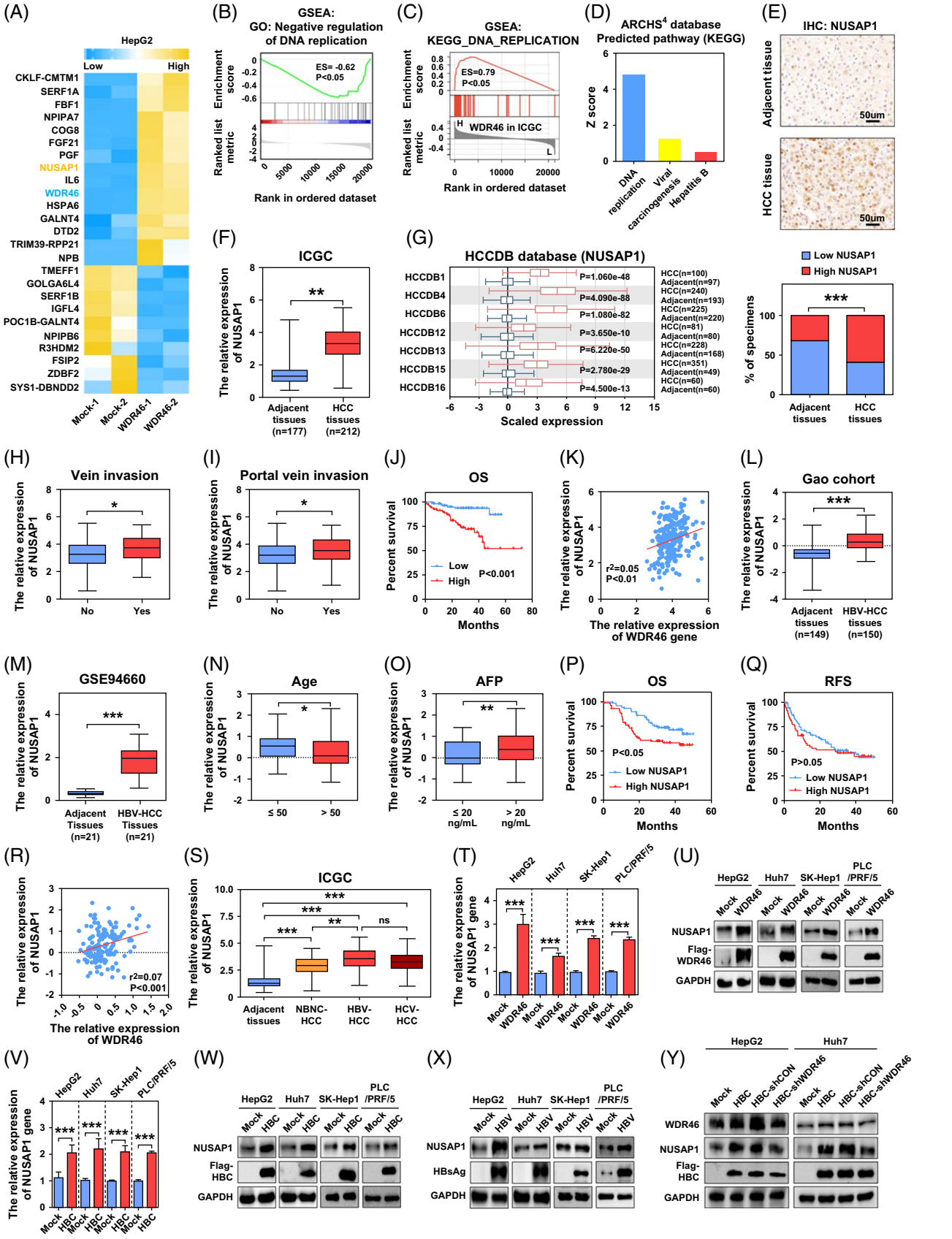

FIGURE 4 WDR46 promotes NUSAP1 transcription in HBC-positive HCC cells. (A) The heat map shows the effect of WDR46 on the expression of DEGs that were detected by RNA sequencing. (B) The pathway is enriched by GSEA analysis, based on the RNA-sequencing data. (C) The pathway is enriched by GSEA analysis, based on the ICGC HCC data. (D) The association of NUSAP1 with significant pathways in the

ARCHS[4] database. (E) The expression of NUSAP1 in HCC and adjacent tissues that detected by IHC. Scale bar, 50 μm. (F) The expression of NUSAP1 in the ICGC cohort. (G) NUSAP1 expression in 7 HCC cohorts in the HCCDB database. (H) The expression of NUSAP1 in HCC tissues in patients with or without vein invasion. (I) The expression of NUSAP1 in HCC tissues in patients with or without portal vein invasion. (J) The association of NUSAP1 with the OS of HCC patients in the ICGC database. (K) The association of WDR46 with NUSAP1 in HCC tissues in the ICGC cohort. (L) The expression of NUSAP1 in HBV-associated HCC tissues in the Gao cohort. (M) The expression of NUSAP1 in HBV-associated HCC tissues in GSE94660. (N) The expression of NUSAP1 in patients aged ≤ 50 years or > 50 years in Gao cohort. (O) The expression of NUSAP1 in HBV-related HCC patients with AFP ≤ 20 ng/mL or > 20 ng/mL in Gao cohort. (P) The association of NUSAP1 with the OS of HBV-related HCC patients in the Gao cohort. (Q) The association of NUSAP1 with the RFS of HBV-related HCC patients in the Gao cohort. (R) The association of WDR46 with NUSAP1 in HBV-associated HCC tissues in the ICGC cohort. (S) The expression of NUSAP1 in HBV-positive HCC, HCV-positive HCC, and non-HBV/non-HCV HCC in the ICGC cohort. (T) The effect of WDR46 on NUSAP1 gene expression in HCC cells. (U) The effect of WDR46 on NUSAP1 protein expression in HCC cells. (V) The expression of NUSAP1 gene in HCC cells induced by HBC. (W) The expression of NUSAP1 protein in HCC cells induced by HBC. (X) The expression of NUSAP1 protein in HCC cells induced by HBV. (Y) The effect of WDR46 on NUSAP1 protein expression in HCC cells mediated by HBC. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Abbreviations: DEGs, differentially expressed genes; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSEA, gene set enrichment analysis; HBC, HBV core protein; IHC, immunohistochemistry; NBNC, non-HBV/non-HCV; NUSAP1, Nucleolar spindle-associated protein 1; OS, overall survival; RFS, recurrence-free survival; WDR46, WD repeat-containing 46.

We subsequently evaluated the expression levels of NUSAP1 in our HCC cohort, the ICGC HCC cohort, and 7 HCC cohorts from the HCCDB database (Figures 4E–G). Similar to WDR46, elevated NUSAP1 expression was observed in these HCC cohorts. In the ICGC HCC cohort, higher NUSAP1 expression was observed in persons with vein invasion and portal vein invasion (Figures 4H, I). A significant association between NUSAP1 overexpression and poor OS in tumors was also identified (Figure 4J). Furthermore, a positive correlation between WDR46 and NUSAP1 expression was observed in the HCC tissues (Figure 4K). In addition, based on the Gao cohort and GSE94660, elevated expression of NUSAP1 was identified in HBV-positive HCC tissues (Figures 4L, M). Based on the Gao HBV-associated HCC cohort, elevated expression of NUSAP1 in persons aged ≤ 50 years and cases with AFP > 20 ng/mL was discovered (Figures 4N, O). In patients with HBV-associated tumors, a remarkable association between NUSAP1 overexpression and OS but not RFS was identified (Figures 4P, Q). A positive association between WDR46 and NUSAP1 expression was also observed in the HBV-positive HCC tissues (Figure 4R). Consistent with WDR46, the elevated expression of NUSAP1 in HBV-associated, HCV-associated, and NBNC-associated HCCs was validated, based on gene expression analysis from the ICGC cohort (Figure 4S).

Next, the effect of WDR46 on the upregulation of NUSAP1 was determined using 4 HCC cell lines (Figures 4T, U). We also assessed the role of HBC in NUSAP1 expression and found that HBC significantly enhanced NUSAP1 gene and protein expression (Figures 4V, W). HBV-mediated elevation of NUSAP1 expression in tumor cells was also confirmed (Figure 4X). In addition, when HBC-associated HCC cells were treated with WDR46 shRNA, HBC-mediated elevation of NUSAP1 was inhibited (Figure 4Y), suggesting that the role of HBC in upregulating NUSAP1 was dependent on WDR46.

NUSAP1 benefits WDR46-induced HCC cell proliferation and migration

We assessed the effects of NUSAP1 on WDR46-induced cell proliferation and migration. One specific NUSAP1 shRNA was constructed and its inhibitory role on NUSAP1 expression in HCC cells was confirmed (Figure 5A). Furthermore, NUSAP1 knockdown remarkably inhibited the growth of HCC cells mediated by WDR46 in vitro (Figures 5B–D). We also examined whether NUSAP1 affects tumorigenesis induced by WDR46 in vivo. As presented in Figures 5E–G, the tumor weight, volume, and expression of KI67 in the WDR46-overexpressing HCC group were higher than those in the control HCC group in the nude mice xenograft mode. However, NUSAP1 silencing efficiently repressed the growth of WDR46-associated HCC cells with reduced tumor weight, volume, and KI67 expression in tumor tissues.

Next, we determined the influence of NUSAP1 deficiency on HCC cell migration mediated by WDR46. These results suggest that inhibiting NUSAP1 could result in a marked decrease in HCC cell migration induced by WDR46 in vitro (Figures 5H, I). To validate the role of NUSAP1 in hepatoma cell metastasis induced by WDR46 in vivo, WDR46 overexpressing HCC cells with NUSAP1 knockdown were constructed and injected into nude mice via tail veins. Consistent with in vitro experimental results, a higher number of metastatic nodules were found in the lungs of the WDR46 over-expressing HCC group than in those of the control group. However, the number of metastatic nodules was lower in the WDR46 overexpressing HCC group with NUSAP1 knockdown than in the control group (Figure 5J).

WDR46 enhances NUSAP1 gene transcription via c-Myc in HBC-associated HCC cells

We further examined the mechanisms contributing to NUSAP1 upregulation mediated by WDR46. Dependent

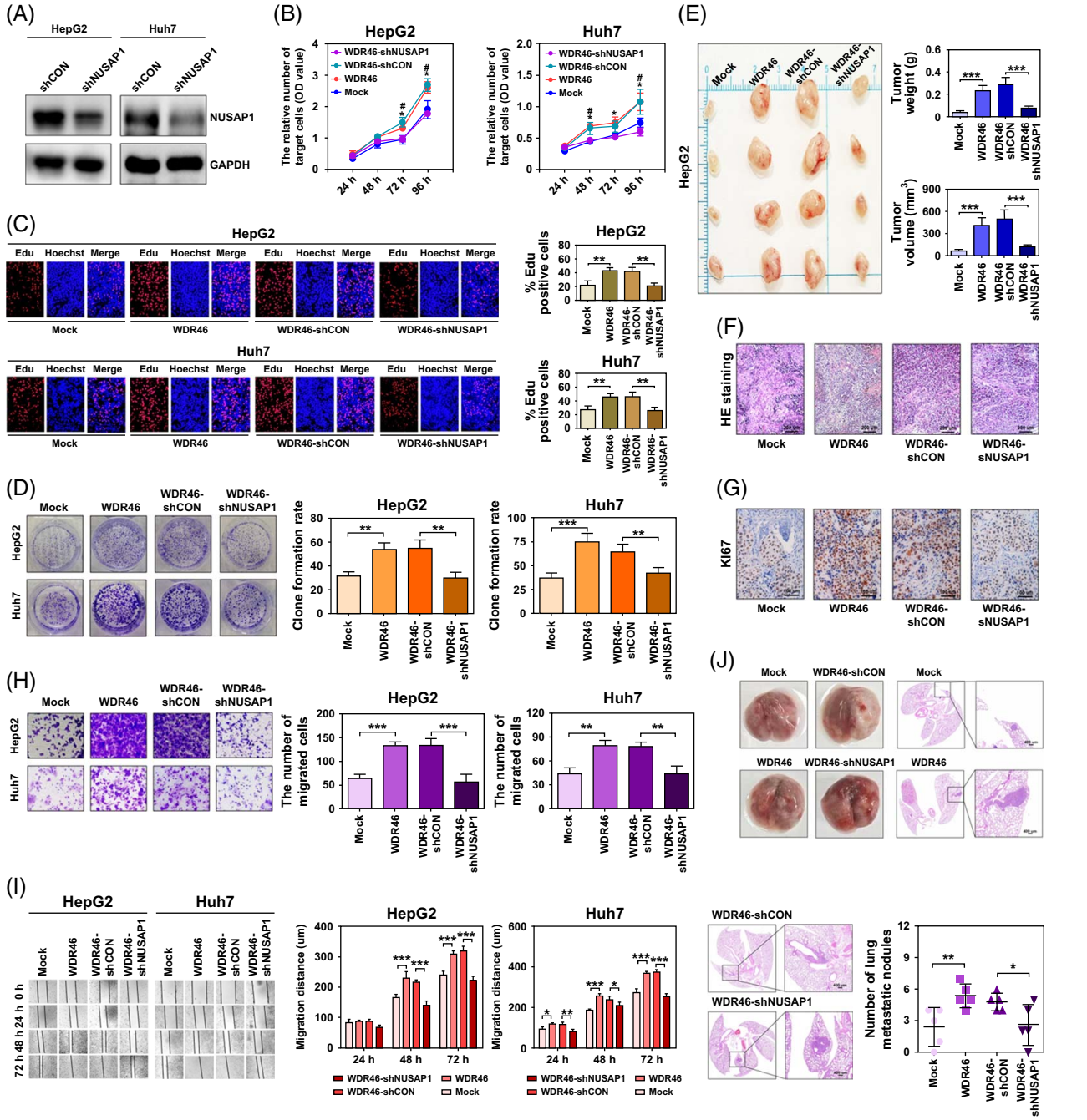

FIGURE 5 The effect of NUSAP1 on cell proliferation and migration is mediated by WDR46. (A) The inhibition of NUSAP1 protein expression is mediated by its specific shRNA. (B) The effect of NUSAP1 on cell viability mediated by WDR46 that detected by CCK-8; *$p < 0.05$, Mock group compares to WDR46 group, #$p < 0.05$, WDR46-shCON group compares to WDR46-shNUSAP1 group. (C) The effect of NUSAP1 on cell proliferation mediated by WDR46 that detected by EdU analysis. (D) The effect of NUSAP1 on colony formation mediated by WDR46 that detected by clone formation assay. (E) The effect of NUSAP1 on HCC cell proliferation mediated by WDR46 in nude mice. (F) H&E staining on the HCC tissues from nude mice in different groups. Scale bar, 200 μm. (G) KI67 expression was examined by IHC in tissues from nude mice in different groups. Scale bar, 100 μm. (H) The effect of NUSAP1 on cell migration mediated by WDR46 that detected by transwell assay. (I) The effect of NUSAP1 on cell migration mediated by WDR46 that detected by wound healing assay. (J) The effect of NUSAP1 on lung metastasis in HCC cells mediated by WDR46 in nude mice that were detected by H&E staining. Scale bar, 400μm; *$p < 0.05$ and **$p < 0.01$. Abbreviations: CCK-8, cell counting kit-8; H&E, hematoxylin–eosin; IHC, immunohistochemistry; NUSAP1, nucleolar spindle-associated protein 1; shRNA, short-hairpin RNA; WDR46, WD repeat-containing 46.

on the GSEA analysis, we observed that, in HCC tissues, WDR46 was significantly relevant to MYC pathways (Figure 6A). Furthermore, based on the Cistrome DB database[36], the transcription factor c-Myc was found to interact with the NUSAP1 promoter in hepatoma cells (Figure 6B), implying that WDR46 may enhance NUSAP1 transcription via c-Myc. Next, the influence of c-Myc on NUSAP1 expression was examined, and it was

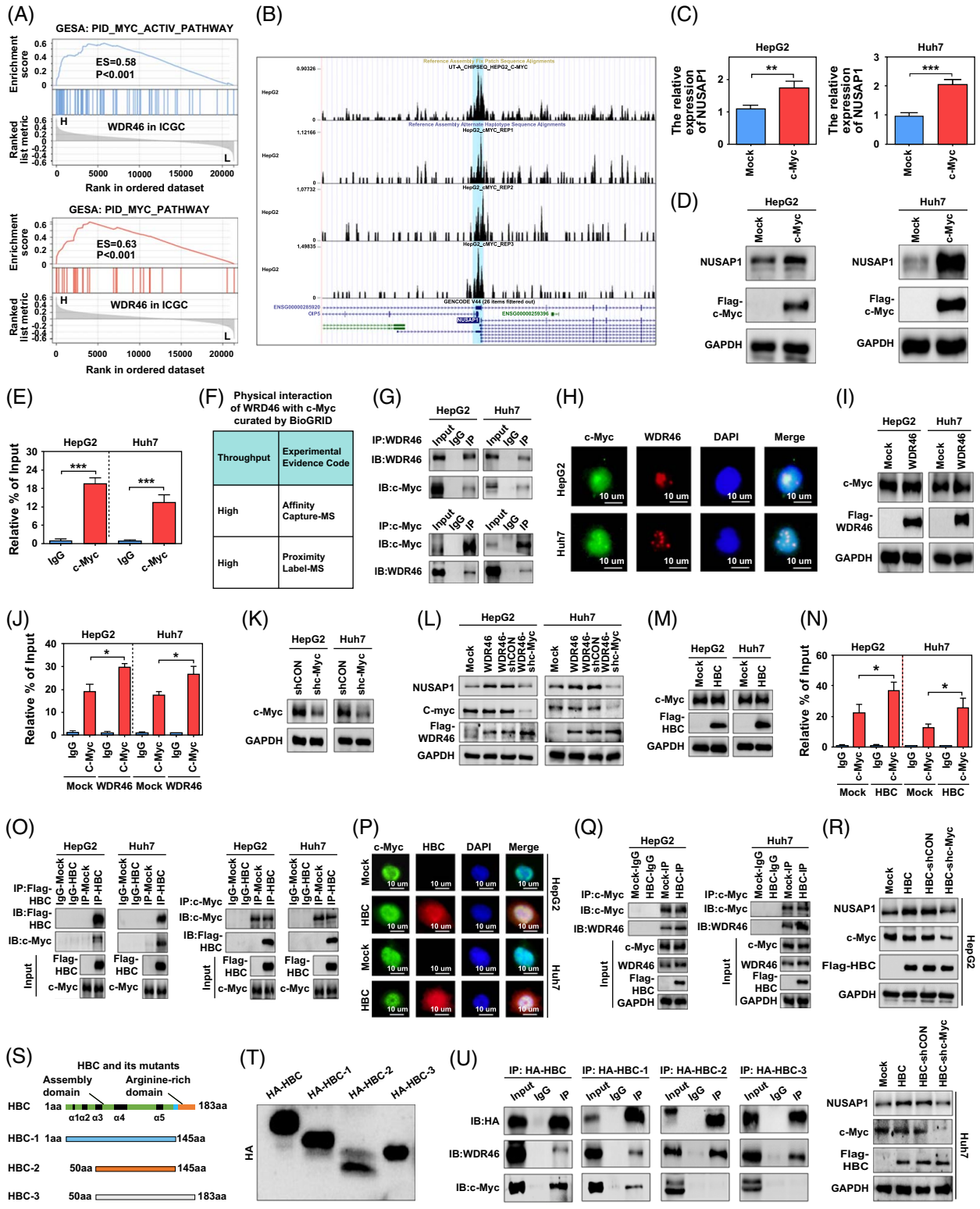

FIGURE 6 The effect of c-Myc on the expression of NUSAP1 mediated by WDR46 and HBC. (A) The pathways associated with WDR46 in the ICGC HCC cohort that predicted by GSEA analysis. (B) The information of ChIP sequencing on the interaction of c-Myc with NUSAP1 promoter in HepG2 cells in the Cistrome Data Browser database. (C) The effect of ectopic c-Myc on the expression of NUSAP1 gene in HCC cells. (D) The effect of ectopic c-Myc on the expression of NUSAP1 protein in HCC cells. (E) The binding of c-Myc to NUSAP1 promoter in HCC cells that detected by ChIP assay. (F) The interaction between WDR46 with c-Myc in the BioGRID database. (G) The interaction between WDR46 and c-Myc detected by Co-IP assay. (H) The colocation of WDR46 with c-Myc in HCC cells detected by immunofluorescence assay. Scale bar, 10 μm. (I) The effect of WDR46 on c-Myc expression. (J) The effect of WDR46 on the binding of c-Myc to NUSAP1 promoter in HCC cells that detected by ChIP assay. (K) The inhibition of c-Myc mediated by its shRNA. (L) The effect of c-Myc on expression of NUSAP1 mediated by WDR46. (M) The effect of HBC on c-Myc expression. (N) The effect of HBC on the binding of c-Myc to NUSAP1 promoter in HCC cells that detected by ChIP assay.

(O) The interaction between HBC and c-Myc detected by Co-IP assay. (P) The colocation of HBC with c-Myc in HCC cells detected by immunofluorescence assay. Scale bar, 10 μm. (Q) The effect of HBC on the interaction between WDR46 and c-Myc. (R) The effect of c-Myc on expression of NUSAP1 mediated by HBC. (S) Pattern diagram of HBC mutants. (T) The expression of HBC mutants in HEK293T cells. (U) The interaction of HBC mutants with c-Myc or WDR46 in HEK293T cells; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. Abbreviations: ChIP, chromatin immunoprecipitation; Co-IP, co-immunoprecipitation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSEA, gene set enrichment analysis; HBC, HBV core protein; NUSAP1, nucleolar spindle-associated protein 1; WDR46, WD repeat-containing 46.

revealed that c-Myc promoted NUSAP1 expression (Figures 6C, D). The location of c-Myc in the NUSAP1 promoter was also confirmed by a ChIP assay (Figure 6E).

Based on the BioGRID database, an interaction between WDR46 and c-Myc was identified (Figure 6F). WDR46 interaction and colocalization with c-Myc in hepatoma cells were subsequently validated by Co-IP and IF assays (Figures 6G, H). We also evaluated the role of WDR46 in the expression of c-Myc. However, no significant effects were observed (Figure 6I). However, the ChIP assay showed that WDR46 improved c-Myc recruitment to the NUSAP1 promoter (Figure 6J). To determine the role of c-Myc on NUSAP1 expression mediated by WDR46. The shRNA targeting c-Myc was used (Figure 6K), and the results suggested that after silencing c-Myc, NUSAP1 upregulation controlled by WDR46 could be inhibited (Figure 6L).

We then assessed the effect of HBC on c-Myc expression. Although no obvious effect on c-Myc expression mediated by HBC was observed (Figure 6M), recruitment of c-Myc to the NUSAP1 promoter was enhanced by the viral protein (Figure 6N). In addition, we discovered that HBC interacted with c-Myc, and the colocation between these 2 molecules in HCC cells was also observed (Figures 6O, P). Furthermore, the Co-IP experiment indicated that HBC enhanced the interaction between WDR46 and c-Myc (Figure 6Q). After inhibition of c-Myc by shRNA, HBC-mediated increase in NUSAP1 expression was suppressed (Figure 6R). As our findings indicate that HBC could interact with both WDR46 and c-Myc, we examined the regions of the viral protein that can interact with these 2 molecules. C-terminal, double-terminal, and N-terminal deletions of the HBC mutants were established (Figures 6S, T). Interestingly, dependent on the Co-IP assay, we discovered that HBC could interact with WDR46 and c-Myc in different regions (Figure 6U), suggesting that HBC could serve as an adaptor to improve the binding between WDR46 and c-Myc to increase NUSAP1 transcription.

NUSAP1 elevates the growth and migration efficiency of HCC cells induced by HBC

Finally, we investigated whether HBC affects cell growth in a NUSAP1-dependent manner. Indeed, NUSAP1 deficiency dampened the proliferation of HBC-positive HCC cells in vitro (Figures 7A–C). Additionally, in vivo studies indicated that depletion of NUSAP1 reversed the effect of HBC on tumor growth in nude mice subcutaneously injected with HBC-positive HCC cells (Figures 7D–F).

In addition to cell proliferation, we examined whether NUSAP1 participated in HBC-mediated hepatoma cell migration. The findings indicated that the migratory ability of HBC-positive HCC cells was repressed by NUSAP1 deficiency (Figures 7G, H). Moreover, fewer lung metastatic nodules were observed in nude mice after the injection of HBC-associated HCC cells with the suppression of NUSAP1 (Figure 7I). In summary, the above experiments demonstrated that NUSAP1 was responsible for the proliferation and migration of HBC-positive HCC cells.

DISCUSSION

Until now, although the treatment options, including surgery and immunotherapy[37] could be chosen for HCC caused by HBV, the 5-year survival rate has not shown significant improvement. Accumulating evidence indicates that HBC contributes to the malignant progression of HCC.[5,7] Therefore, targeting HBC and associated host factors could provide a novel strategy for treating HBV-associated HCC. Here, we noticed that WDR46 is upregulated in HCC, especially in HBV-related HCC and that its protein can be stabilized by HBC, which disrupts the interaction between WDR46 and TRIM25 to reduce WDR46 ubiquitination, subsequently promoting cell growth and migration (Figure 8). The DNA replication-related protein NUSAP1, which participates in the modulation of HCC development,[20] is identified as a vital downstream factor of WDR46. To facilitate NUSAP1 transcription, WDR46 interacts with c-Myc to enhance its recruitment to NUSAP1 promoter. Importantly, HBC served as a scaffold protein to strengthen the interaction between WDR46 and c-Myc.

WDR46 is a vital member of the WDR protein family, which is associated with the progression of various cancers.[9,10] Although the association of WDR46 with gastric carcinoma[14] and colorectal cancer[11] has been studied, little information on the biological effect of WDR46 in HCC was reported. Depending on various HCC cohorts, we observed an increase in WDR46 expression in HCC. Moreover, high WDR46 expression is an independent prognostic factor for HCC, and its overexpression is significantly correlated with worse tumor clinical progression. Although current investigations indicated that HCCs caused by different etiologic factors have distinct gene

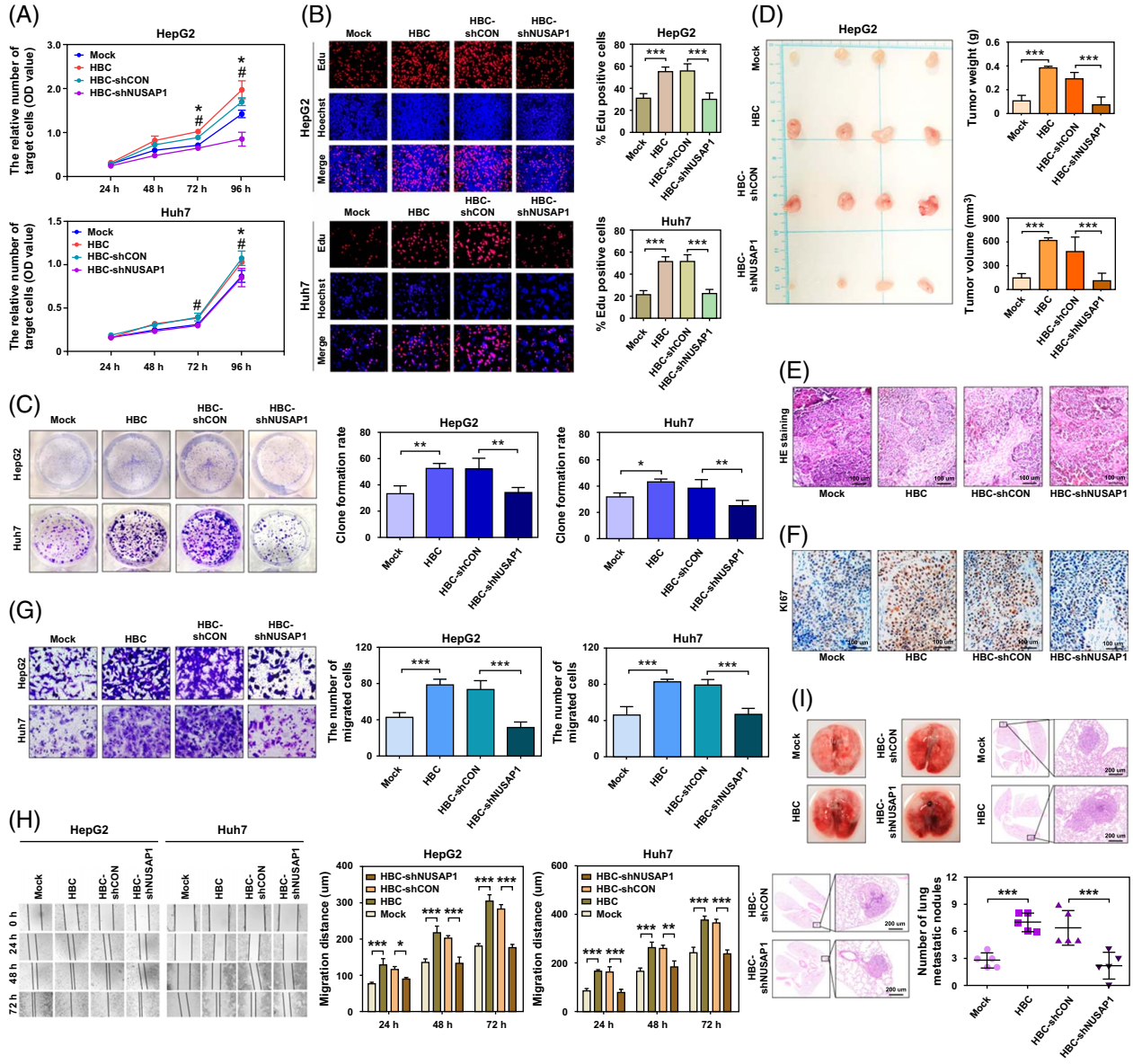

FIGURE 7 The role of NUSAP1 on cell growth and migration mediated by HBC. (A) The effect of NUSAP1 on cell viability mediated by HBC that detected by CCK-8; *$p < 0.05$, Mock group compares to HBC group, #$p < 0.05$, HBC-shCON group compares to HBC-shNUSAP1 group. (B) The effect of NUSAP1 on cell proliferation mediated by HBC that detected by EdU analysis. (C) The effect of NUSAP1 on colony formation mediated by HBC that detected by clone formation assay. (D) The effect of NUSAP1 on HCC cell proliferation mediated by HBC in nude mice. (E) H&E staining on the HCC tissues from nude mice in different groups. Scale bar, 100 μm. (F) KI67 expression examined by IHC in tissues from nude mice in different groups. Scale bar, 100 μm. (G) The effect of NUSAP1 on cell migration mediated by HBC that detected by transwell assay. (H) The effect of NUSAP1 on cell migration mediated by HBC that detected by wound healing assay. (I) The effect of NUSAP1 on the lung metastasis in HCC cells mediated by HBC in nude mice that detected by H&E staining. Scale bar, 200 μm; *$p < 0.05$ and **$p < 0.01$. Abbreviations: CCK-8, cell counting kit-8; HBC, HBV core protein; H&E, hematoxylin–eosin; IHC, immunohistochemistry; NUSAP1, Nucleolar spindle-associated protein 1.

expression patterns and pathogenic mechanisms,[38,39] the main etiologies of HCC also use common genes and pathways to induce hepatocarcinogenesis.[39,40] In this study, we found that HBV contributes to the upregulation of WDR46 in the tumor. However, no significant difference in WDR46 gene expression among HBV-associated, HCV-associated, and NBNC-associated HCC tissues was found, based on the ICGC cohort, implying that WDR46 may act as a common gene induced by different etiologic factors, to facilitate the occurrence and development of HCC. Besides these, only the expression of the WDR46 gene in HCC with different etiologic factors was evaluated in our study, whether WDR46 protein expression in various etiology-related HCC is similar or different is still unknown. Therefore, it is worth detecting the expression of WDR46 protein by using different methods to validate the influence of different etiologic factors on WDR46 in liver cancers. In particular, we discovered that HBV facilitates the upregulation of WDR46 in HCC, and that HBC is required for WDR46

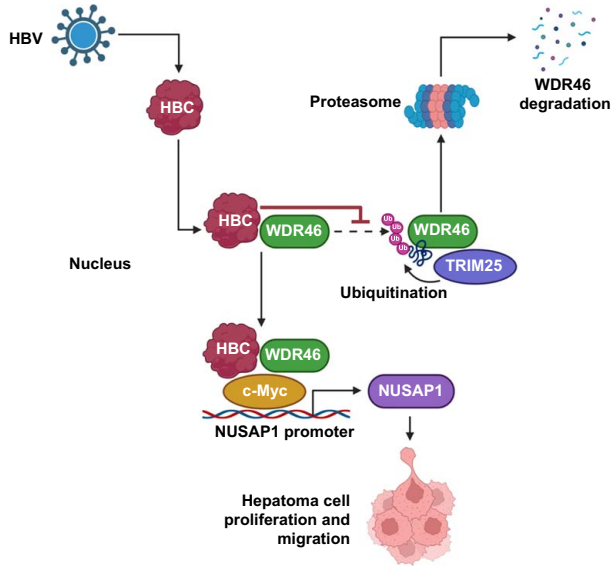

FIGURE 8 A schematic diagram showing the mechanisms regarding the increase of WDR46 induced by HBC to upregulate NUSAP1 and facilitate hepatocarcinogenesis. In HCC cells, HBC strengthens WDR46 stabilization by disrupting the interaction between WDR46 and TRIM25 to reduce ubiquitination-dependent degradation of WDR46. Subsequently, HBC promotes the interaction of WDR46 with c-Myc to benefit the recruitment of c-Myc to the NUSAP1 promoter, therefore increasing NUSAP1 gene transcription and enhancing cell growth and migration. Abbreviations: HBC, HBV core protein; NUSAP1, Nucleolar spindle-associated protein 1; WDR46, WD repeat-containing 46.

upregulation induced by the virus. Although we also discovered that WDR46 overexpression is also closely related to the poor prognosis of HBV-related HCC, whether WDR46 can act as an adverse prognostic indicator in both HBV-positive and HBV-negative HCC is still unknown, and needs to be explored in the future. Functionally, our results indicate that WDR46 supports hepatoma cell growth and metastasis mediated by HBC in vitro and in vivo, indicating that WDR46 plays a pivotal oncogenic role in HCC development caused by HBV infection.

Notably, HBC increased WDR46 protein expression. Accumulating evidence suggests that the expression of cellular proteins is mostly controlled by the ubiquitin–proteasome-associated degradation pathway and autophagy–lysosome-related degradation pathway.[41] Based on GESA analysis and associated experimental detection, we demonstrated that the ubiquitin–proteasome pathway is an important regulator of WDR46. Moreover, HBC effectively enhanced the stabilization of WDR46 by suppressing the pathway-induced degradation. TRIM25, an E3-ubiquitin ligase, is a critical regulatory factor in the development of HCC by controlling ubiquitination and stabilization of its substrate protein.[23,42] Relying on the uBibrowser and BioGRID databases, along with experimental verification, we identified that WDR46 can interact with TRIM25, and it is a new substrate protein of TRIM25 in HCC. However, it is noteworthy that HBC can stabilize WDR46 by disrupting the interaction of WDR46 with TRIM25. In addition, accumulating evidence indicates that HBsAg can promote hepatocarcinogenesis.[43–45] Similar to HBC, we found that HBsAg was responsible for enhancing HBV-mediated WDR46 protein expression in HCC (Figure 1Q). However, the mechanisms that contribute to the elevation of WDR46 induced by HBsAg remain unknown and need to be clarified in future studies.

NUSAP1 is a DNA replication regulation-associated protein.[35] To date, it has been demonstrated that NUSAP1 contributes to the development of multiple cancers,[17,35] including HCC,[20] especially HBV-related HCC.[21] Functionally, this molecule facilitates cell cycle transition,[46] regulates liver cancer cell stemness,[20] controls DNA damage,[46] and enhances HCC cell proliferation and migration.[22,47] Although E2F8[46] and miR-193a-5p[48] have been shown to participate in modulating NUSAP1 in HCC, the factors in controlling NUSAP1 expression are far from being well elucidated. Based on RNA-sequencing, NUSAP1 was identified as a vital downstream molecule of WDR46. Consistent with previous reports, the upregulation of NUSAP1 in HCC was also identified in our study. Moreover, a positive correlation between WDR46 and NUSAP1 in HCC tissues and HBV-related HCC tissues was identified. However, the Pearson *r* values indicated that the association of WDR46 with NUSAP1 in the tumor was weak. To better validate the clinical correlation between WDR46 and NUSAP1, future measurements based on a larger number of HCC tissues, including HBV-related HCC tissues, are required. Besides these, based on the ICGC cohort, we discovered that, compared to adjacent tissues, the expression levels of NUSAP1 were significantly elevated in NBNC-related, HBV-related, and HCV-related HCC. Functionally, NUSAP1 promoted the proliferation and metastasis of HCC cells mediated by WDR46 and HBC. In addition to NUSAP1, many other downstream genes of WDR46 were also identified in our study (Figure 4A), and the regulation and associated mechanisms of WDR46 on these molecules need to be explored in future studies.

It has been demonstrated that WDR family proteins, including WDR5 and WDR48,[49,50] can bind to the oncoprotein transcription factor c-Myc to promote tumorigenesis. Similar to WDR5 and WDR48, based on predictions from the GESA and BioGRID database, as well as detection by different assays, we demonstrated that WDR46 interacts with c-Myc, to benefit the recruitment of c-Myc to NUSAP1 promoter and facilitate NUSAP1 gene transcription. More importantly, HBC binds to WDR46 and c-Myc in different regions to form protein complexes, leading to an increase in the interaction between WDR46 and c-Myc. Thus, the disruption of the interaction between WDR46 and c-Myc mediated by HBC has the potential to enhance the anti-HBV–associated HCC effect.

CONCLUSIONS

In summary, this study indicates that overexpression of WDR46 and its downstream molecule NUSAP1 in HCC are closely related to unfavorable prognosis. The roles of WDR46 and NUSAP1 in the proliferation and migration of HCC cells were investigated using various models in vitro and in vivo. Furthermore, in HBV-associated HCC, based on HBC, the virus can increase WDR46 expression to upregulate NUSAP1, thereby regulating the biological functions of HCC cells. Therefore, targeting the WDR46 and its downstream molecule NUSAP1 may be a compelling strategy to treat HBV-induced HCC. This study had certain limitations. Our findings were mainly dependent on HCC cell models, subcutaneous xenograft models, and lung metastasis models. In the future, to further validate the effects and mechanisms of the WDR46 and NUSAP1 in HBC-induced hepatocarcinogenesis, as well as potential drugs targeting WDR46 or NUSAP1 for cancer treatment, orthotopic implantation models, transgenic models, and gene knockout mouse models are needed.

DATA AVAILABILITY STATEMENT

Data will be made available on request.

AUTHOR CONTRIBUTIONS

Fanyun Kong and Ensi Bao equally to the study. Fanyun Kong, Hongjuan You, and Ensi Bao checked the data and wrote and revised the manuscript. Yujie Zhong, Yuxin Wang, Huanyang Zhang, Lu Yang, Rong Jiang, Xuanke Liu, Chen Li, and Xiangye Liu performed laboratory work. Ruyu Liu and Xiucheng Pan contributed to clinical tissue collection, associated experiments, and statistical analysis. Fanyun Kong, Renxian Tang, Kuiyang Zheng, and Hongjuan You designed the study and revised the manuscript. All the authors have discussed and approved the final version of the manuscript.

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CONFLICTS OF INTEREST

The authors have no conflicts to report.

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