

N4BP3 promotes angiogenesis in hepatocellular carcinoma by binding with KAT2B

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Abstract

Although angiogenesis is a critical event in hepatocellular carcinoma (HCC), and this process provides the tumor with sufficient oxygen and nutrients, the precise molecular mechanism by which it occurs is not fully understood. NEDD4 binding protein 3 (N4BP3) was identified in this study as a novel pro-angiogenic factor in HCC cell lines and tissues. We discovered that N4BP3 was significantly expressed in HCC and that its level of expression was positively correlated with the density of tumor microvessels in HCC tissues. Cell biology experiments have shown that N4BP3 knock-down in HCC cells significantly inhibits the formation of complete tubular structures by HUVECs in vitro and HCC angiogenesis in vivo. In HCC cells, overexpression of N4BP3 has the opposite effects. Further cell and molecular biology experiments have revealed that N4BP3 interacts with KAT2B (lysine acetyltransferase 2B), increasing signal transducer and activator of transcription 3 (STAT3) expression by regulating the distribution of acetyl-histone H3 (Lys27) (H3K27ac) in its promoter region. This, in addition, regulates the activity of the STAT3 signaling pathway, which promotes the proliferation of microvessels in HCC and accelerates the malignant process of the tumor. In vivo experiments in nude mice have confirmed our findings, and also suggested that N4BP3 could be a potential target for the treatment of HCC in combination with sorafenib.

KEYWORDS

angiogenesis, hepatocellular carcinoma, metastasis, N4BP3, STAT3

1 | INTRODUCTION

Hepatocellular carcinoma, as one of the most common malignant tumors of the digestive system, ranks sixth in the number of new cases and is the second leading cause of cancer-related deaths

globally, with a high incidence rate and a high degree of malignancy.¹ Tumors in HCC are frequently associated with an abundance of new blood vessels, which play a critical role in tumor growth, invasion, and metastasis.² As a result, the majority of patients lose the opportunity for radical surgery when diagnosed.³ Therefore, targeting

Abbreviations: Co-IP, co-immunoprecipitation; HCC, hepatocellular carcinoma; HIF-1a, hypoxia inducible factor-1alpha; IHC, immunohistochemistry; KAT2B, lysine acetyltransferase 2B; MVD, microvascular density; N4BP3, NEDD4 binding protein 3; qPCR, quantitative PCR; STAT3, signal transducer and activator of transcription 3; VEGFA, vascular endothelial growth factor A.

Hexu Han and Wei Zhu are co-first authors of this paper.

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pro-angiogenic factors and their associated signaling pathways has become a growing area of research in targeted therapy for HCC,⁴ and a class of targeted drugs such as sorafenib has emerged,⁵ but their high treatment costs, toxicity, and side-effects, and the extremely limited improvement in patients' survival, have limited their use.⁶ All of these factors contribute significantly to patients' experience and psychological distress, as well as significant social and familial burdens.⁷ This necessitates additional research and exploration of new factors that contribute to angiogenesis in HCC to advance our understanding of angiogenesis and establish a new theoretical basis for the therapeutic targets.

STAT3 is an important gene involved in the regulation of cell physiology and pathology, and the STAT3 signaling pathway can mediate a variety of biological functions, including promoting angiogenesis.⁸ The primary activation pathway for this signaling pathway is that, after being stimulated by the extracellular environment,⁹ cytokines bind to its receptor to cause the dimerization and activation of receptor molecules (JAKs), which then results in phosphorylation of STAT3 at Tyr705.¹⁰ The phosphorylated form induces its dimerization and nuclear translocation, and then combines with the corresponding DNA to promote the transcription of downstream effector molecules, including VEGFA.¹¹ Studies have shown that STAT3 activation has been associated with a poor prognosis in patients with HCC.¹² More importantly, in HCC, increased STAT3 activity is closely related to the histological grade and the increase in intratumoral microvessel density.¹³ All of these findings suggest that blocking the STAT3 signaling pathway will significantly benefit HCC antivasculature targeted therapy.

NEDD4 binding protein 3 was first discovered as a NEDD4 binding protein; research on this molecule has thus far been mostly focused on the nervous system.¹⁴ In 2013, Schmeisser et al¹⁵ found that N4BP3 is required for the axon and dendritic branching of developing neurons, and this finding was confirmed in 2017 by Kiem et al.¹⁶ Although numerous bioinformatics research findings have indicated that N4BP3 might be involved in tumor distant metastasis and that its expression is negatively correlated with patient prognosis in recent years,¹⁷ the specific biological role of N4BP3 in tumors, particularly HCC, remains unknown.¹⁸

In this article, using the N4bp3 KO mouse model, we found that N4BP3 plays an important role in the development and progression of HCC through chemical induction. Additionally, we discovered

that N4BP3 is highly expressed in tumor tissues and has a negative correlation with the prognosis of HCC. Other studies have revealed that N4BP3 promotes angiogenesis by activating the STAT3 signaling pathway, a mechanism that is mediated through the interaction of the N4BP3 and KAT2B proteins. In vivo experiments in nude mice further verified the above findings, and combination therapy with sorafenib or bevacizumab also established a theoretical basis for N4BP3 as a therapeutic target for HCC.

2 | MATERIALS AND METHODS

2.1 | Cell culture

The cell lines and culture methods used in this research are shown in Appendix S1.

2.2 | Experiments on animals

Tumorigenesis assay in nude mice and N4bp3 KO mice were used in this paper, please refer to Appendix S1.

2.3 | Cellular experiments, molecular experiments, and statistical methods

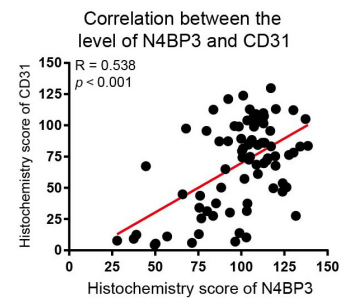
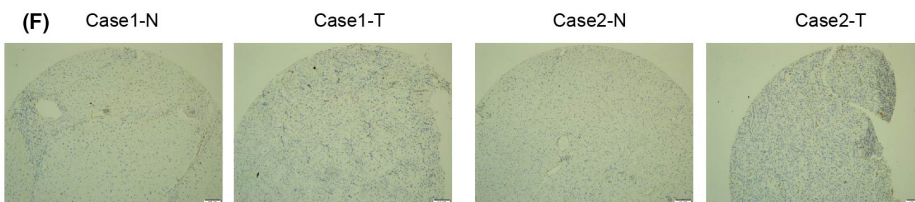
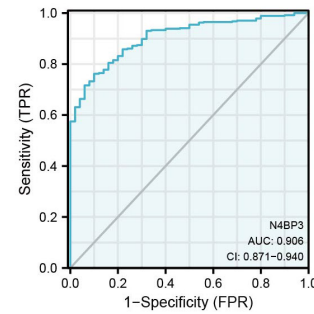
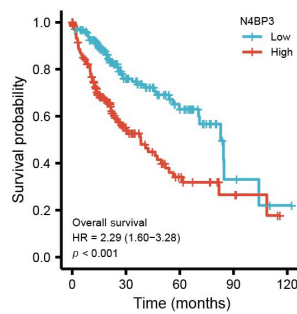
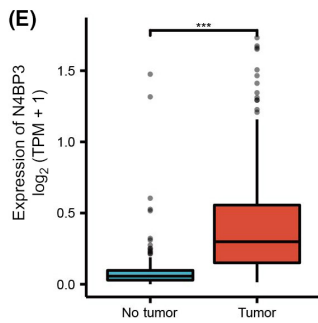
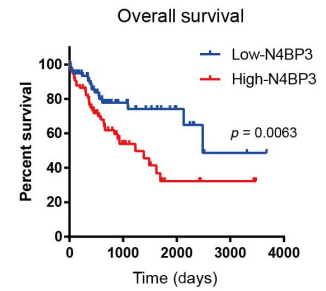
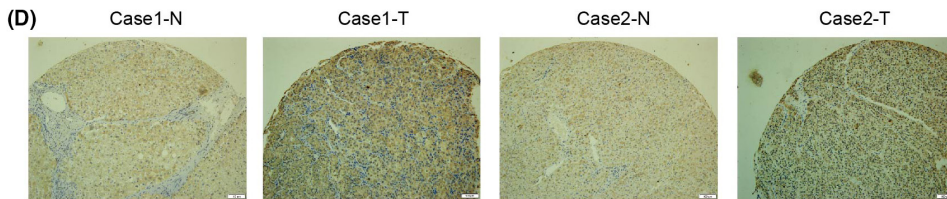
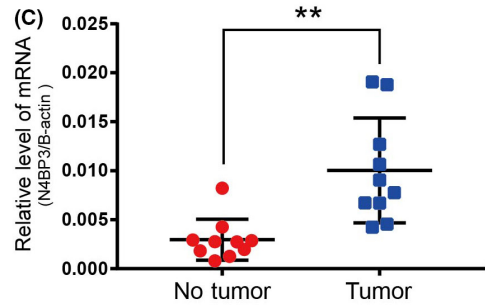
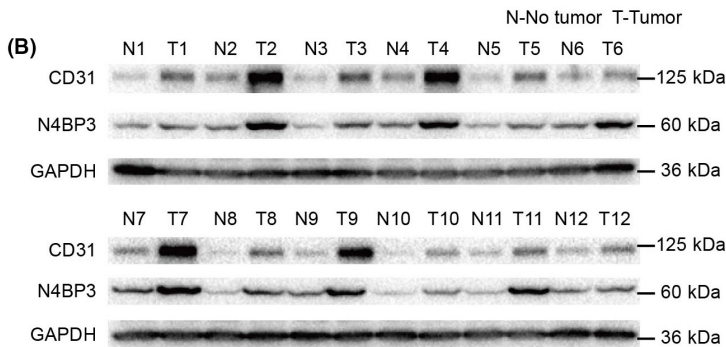
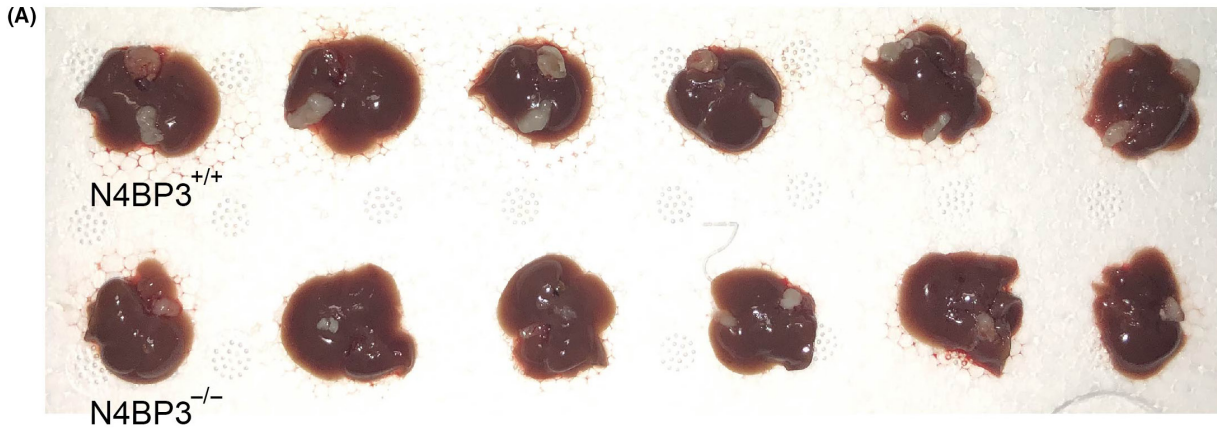
Please refer to Appendix S1 for various experimental methods and statistical methods.

3 | RESULTS

3.1 | Expression levels of N4BP3 are positively correlated with MVD in HCC tissues

To explore whether N4BP3 plays an important role in the occurrence and development of HCC, we used N-nitrosodiethylamine and CCl4 to model WT mice and N4bp3 KO mice to induce spontaneous liver cancer models in mice. The results showed that in the group of N4bp3 KO mice, the number of tumors was smaller and the size

FIGURE 1 Expression levels of NEDD4 binding protein 3 (N4BP3) were positively correlated with microvessel density in hepatocellular carcinoma (HCC) tissues. (A) CCL4 and N-nitrosodiethylamine were used for modeling. After 16 weeks, liver tissues of WT mice and N4bp3 KO mice were obtained and assessed. The number and volume of tumor tissues in the KO group were significantly reduced. (B) Western blot analysis of N4BP3 levels in paired tumor tissues (T) and matched normal tissues (N); GAPDH was used as the loading control. (C) Quantitative PCR assays of N4BP3 levels in paired tumor tissues and matched normal tissues; β -actin was used as the control. (D) Immunohistochemical (IHC) staining revealed that, compared to adjacent nontumor tissues, N4BP3 levels in HCC were significantly increased (left panels) and that they were negatively correlated with the prognosis of patients (right panel). (E) By analyzing the data of HCC patients in The Cancer Genome Atlas database, it was established that, compared to paracancer tissues, N4BP3 was highly expressed in tumor tissues (left panel), and its high expressions were negatively correlated with the prognosis of patients (middle panel). Receiver operating characteristic curve (area under the curve [AUC] > 0.9) reflected the high accuracy of N4BP3 in predicting patient prognosis (right panel). CI, confidence interval; FPR, false positive rate; TPR, true positive rate. (F) IHC staining showed elevated CD31 levels in cancer samples versus corresponding normal tissues, the chip was from the same group as above, and the same location was selected (left panels). CD31 levels gradually increased with increasing N4BP3 expression, implying a correlation between N4BP3 and angiogenesis (right panel)



was reduced, which indicated that N4BP3 plays an important role in HCC (Figure 1A and Figure S1A). To further explore the biological role of N4BP3 in HCC, we analyzed the expression level of N4BP3 and analyzed its expression difference with the corresponding adjacent tissues by western blotting assays. Results showed that the protein levels of N4BP3 were significantly higher in most tumor samples when compared with the paired adjacent tissues (Figure 1B and Figure S1B). In another cohort of samples, quantitative RT-PCR assays also showed significantly higher N4BP3 expression in tumor tissues compared to matched nontumor tissues (Figure 1C). These experimental results in the human tissue specimens not only verified the results in the abovementioned animal models but also showed that N4BP3 might indeed participate in the process of HCC. The IHC staining results in another queue further verified the results of the above experiment. When we further analyzed the staining results, the corresponding results showed that the high expression of N4BP3 in HCC tissues often accompanies the poor prognosis of patients (Figure 1D). By analyzing the HCC information in The Cancer Genome Atlas database, we obtained similar results with the abovementioned collected tissues; that is, compared with the adjacent tissues, N4BP3 is highly expressed in tumor tissues, and the highly expressed N4BP3 is negatively correlated with the prognosis of patients. Moreover, the receiver operating characteristic curve reflects that the molecular prediction is true with high accuracy (Figure 1E), which not only verified the data of our center, but at the same time, it further proved that N4BP3 could play an important role in the occurrence and development of HCC. Previous research has established that microvessel proliferation plays a critical role in the progression of HCC. Using the same group of tissue microchips to stain CD31, a vascular marker, was found to have an obvious positive correlation between N4BP3 expression and tumor MVD (Figure 1F), indicating that N4BP3 might promote tumor development by promoting angiogenesis in HCC.

3.2 | NEDD4 binding protein 3 promotes tumor angiogenesis both in vivo and in vitro

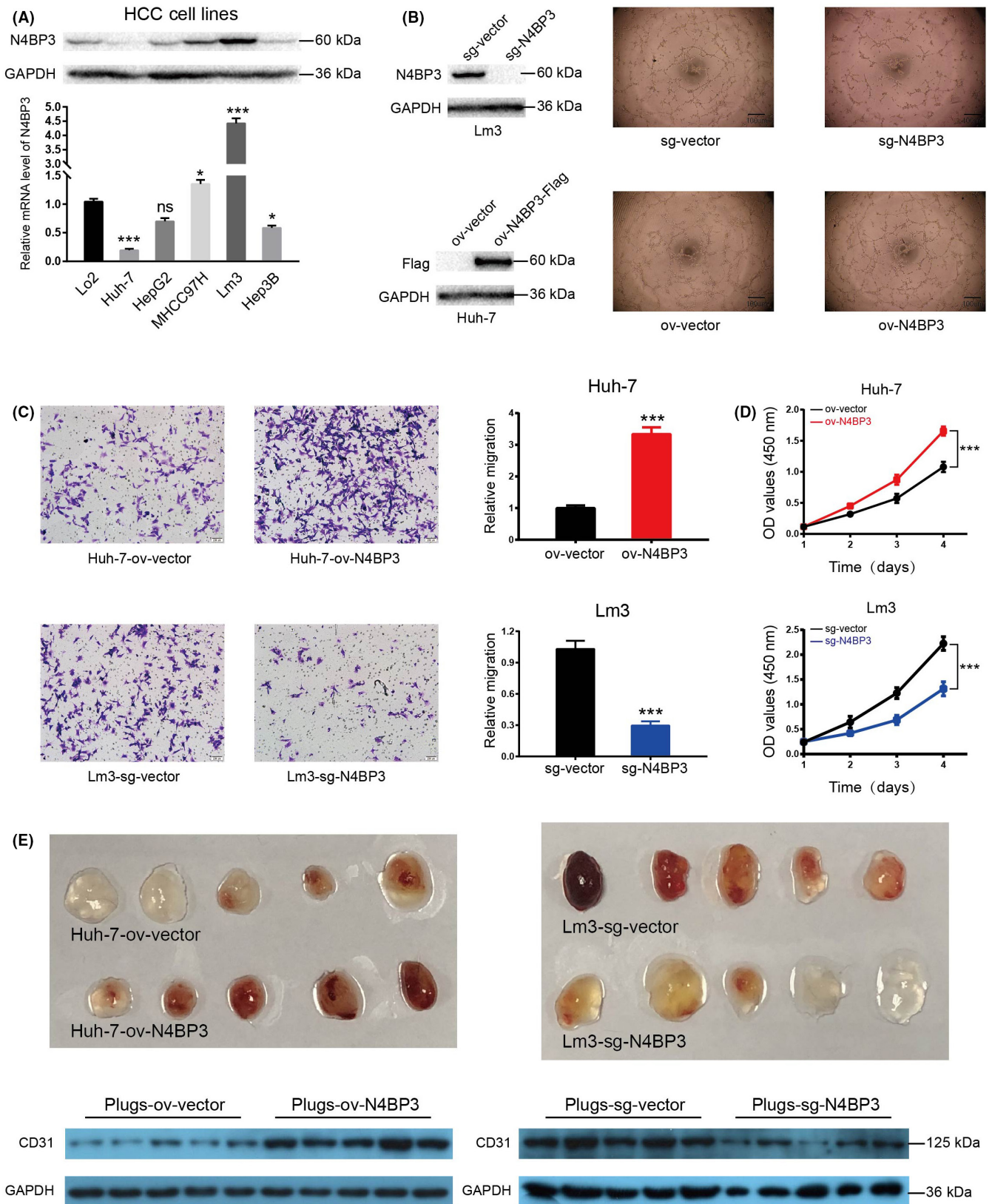
To confirm that N4BP3 can promote angiogenesis in HCC and to elucidate the precise mechanism, we first determined the level of expression in each cell line (Figure 2A). We knocked out N4BP3 in Lm3 cells and overexpressed it in Huh-7 cells. The average rate at which complete tubular structures were generated by HUVECs

was significantly greater in cells receiving conditioned medium from Huh-7-ov-N4BP3 cells and significantly lower in cells incubated with medium from Lm3-sg-N4BP3 cells than in the control cells (Figure 2B and Figure S2A,B). Additionally, the proliferation and migratory abilities of endothelial cells are critical for angiogenesis. Further Transwell migration assays and CCK-8 proliferation assays showed that, compared with the control group, the proliferation and migration ability of HUVECs was significantly enhanced by using supernatant from the overexpressed-N4BP3 group. In contrast, when N4BP3 was knocked, the stimulation and chemotaxis of HUVECs in the supernatant group decreased significantly, indicating that N4BP3 does indeed promote angiogenesis in HCC (Figure 2C,D). Using in vivo experiments, we then combined cell supernatants from Huh-7-ov-N4BP3/Lm3-sg-N4BP3 cells and corresponding controls with Matrigel and subcutaneously injected the mixture into mice. We extracted Matrigel plugs 7 days after injection for a more complete study of blood vessel formation and perfusion within the Matrigel plugs. We used western blot assays for quantitative analysis after grinding the emboli (Figure 2E), and further IHC analysis of the infiltrated MVD (indicated by CD31) confirmed the above findings (Figure S2C,D).

3.3 | NEDD4 binding protein 3 promotes angiogenesis through VEGFA

The above results indicated that high expression of N4BP3 in HCC can indeed promote angiogenesis. To further explore the molecular mechanism of N4BP3 in promoting angiogenesis, we collected the supernatants of Huh7-ov-vector and Huh7-ov-N4BP3 cells and analyzed them with a Human Angiogenesis Array Kit (ARY007, R&D Systems). The results showed that, compared with the control group, the content of VEGFA in the supernatant of the Huh-7-ov-N4BP3 group was significantly increased (Figure 3A), further qPCR and ELISA assays in Huh-7 and Lm3 cells also confirmed this finding (Figure 3B,C), which suggested that N4BP3 might promote angiogenesis by enhancing the expression and secretion of VEGFA. At the same time, we also verified the expression levels of other cytokines by qPCR and ELISA assays that can promote angiogenesis, such as fibroblast growth factor, Angiopoietin2, and hepatocyte growth factor, which showed no significant change (Figure S3A,B). To confirm the role of VEGFA in N4BP3-regulated HCC cell angiogenesis, we carried out VEGFA rescue and VEGFA

FIGURE 2 NEDD4 binding protein 3 (N4BP3) promoted tumor angiogenesis in vivo and in vitro. (A) We evaluated N4BP3 levels in hepatocellular carcinoma (HCC) cells using western blot and quantitative PCR assays. (B) Based on the above experimental results, N4BP3 was knocked out in Lm3 cells and overexpressed in Huh-7 cells. At the same time, supernatants for each group of cells were collected and tube formation assays carried out to verify that N4BP3 promotes angiogenesis. (C) Transwell migration assays revealed that the supernatant from Huh-7-ov-N4BP3 cells strongly promoted HUVECs migration in vitro. The opposite effect was observed in Lm3 cells when N4BP3 was knocked out. (D) CCK-8 proliferation assays revealed that the supernatant from Huh-7-ov-N4BP3 cells promoted HUVEC growth, whereas the stimulating effects of the supernatant from Lm3-sg-N4BP3 cells on HUVEC growth were inconspicuous. (E) Images of retrieved Matrigel plugs. Compared to the control group, the Matrigel from the supernatant of the overexpressed N4BP3 group show more vascular infiltration, and vice versa (upper panels). Western blot assays were used to assess blood vessel contents of the Matrigel plugs (indicated by CD31⁺ cells) (lower panels).



blocking assays. We found that the inhibited tubular structure formations in the conditioned medium from N4BP3-deficient Lm3 cells can be rescued by the addition of recombinant VEGFA; in contrast, the application of sufficient VEGFA Ab abrogated the

elevated tube formation in conditioned medium from N4BP3-overexpressed Huh-7 cells (Figure 3D and Figure S3C,D). These results strongly suggested that N4BP3 promotes angiogenesis in HCC by promoting the expression and secretion of VEGFA. In

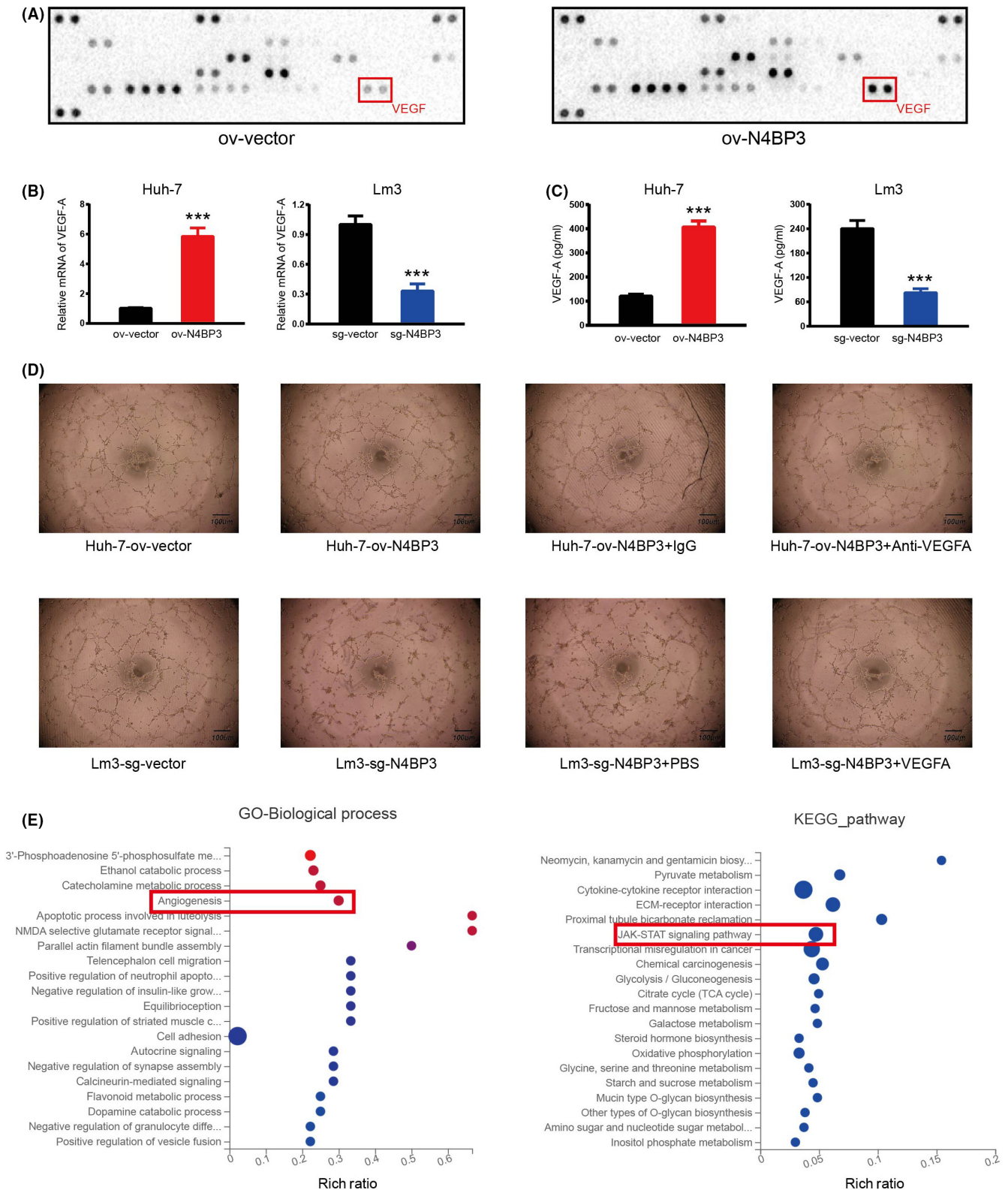


FIGURE 3 NEDD4 binding protein 3 (N4BP3) promoted angiogenesis through vascular endothelial growth factor A (VEGFA). (A) Angiogenesis-related factors were screened from the medium collected from Huh-7-ov-N4BP3 cells and corresponding control cells by proteomic Ab array. Among the angiogenesis-promoting cytokines, only VEGFA levels were significantly increased after N4BP3 overexpression. (B) Quantitative PCR assays were used to assess VEGFA mRNA levels in cells of each group. (C) ELISA assays were undertaken to evaluate VEGFA levels in cell supernatants of each group. (D) Through tube formation assays, it was established that the ability to promote the formation of complete tubular structures of HUVECs decreased significantly when anti-VEGFA Abs were added to supernatants of cells in the Huh-7-ov-N4BP3 group. In contrast, when the purified protein (VEGFA) was added to the supernatant of cells in the Lm3-sg-N4BP3 group, the ability of HUVECs to form complete tubular structures was significantly improved. (E) Through transcriptome sequencing and analysis of corresponding results, we found that N4BP3 might promote hepatocellular carcinoma angiogenesis through the STAT3 signaling pathway. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes

addition, transcriptome sequencing was undertaken to not only comprehensively explore the function of N4BP3 in HCC, but also the signaling pathway associated with N4BP3 mediating angiogenesis in HCC. The results suggest that N4BP3 overexpression in HCC appears to promote angiogenesis by regulating the JAK-STAT signaling pathway (Figure 3E).

3.4 | NEDD4 binding protein 3 promotes the expression of VEGFA by enhancing the STAT3 signaling pathway

Based on the sequencing results above, to further investigate the specific mechanism by which N4BP3 elevates the expression of VEGFA to enhance angiogenesis, we examined several key molecules in the related signaling pathways that promote tumor angiogenesis. Western blot assays revealed that STAT3 and its phosphorylated form (p-STAT3) showed significant changes, which was consistent with the results of the high-throughput results above (cells of each group were cultured in a hypoxic environment with 1% oxygen for 16 h and then the expression of HIF-1 α was detected) (Figure 4A,C). Previous studies have established the STAT3 signaling pathway as an important pathway affecting angiogenesis in tumors, and the dual-luciferase reporter gene assays further verified the above results of western blots, that is, after overexpression of N4BP3 in Huh-7 cells, the fluorescence signal intensity of STAT3 was significantly increased, and vice versa in Lm3 cells (Figure 4B). Additionally, because N4BP3 affected the expression of STAT3 molecules, but not the content of other proteins in this pathway, our findings suggested that N4BP3, which is highly expressed in HCC, might promote angiogenesis through the STAT3 signaling pathway (Figure 4C). To further verify this finding, we undertook rescue experiments in different cell types. In Lm3 cells, we introduced the WT STAT3 plasmid into Lm3-sg-N4BP3 cells, as well as the corresponding phosphomimetic mutant form (STAT3 Y705D) and dominant-negative mutant form (STAT3 Y705F). The results showed that VEGFA expression would rebound only when the WT and phosphomimetic mutant form STAT3 plasmids were transferred, but not in the Lm3-sg-N4BP3 cells overexpressing the STAT3 Y705F plasmid. The expression of VEGFA did not change significantly compared with the control group (Figure S4A). However, in Huh-7 cells with increased STAT3 expression due to the overexpression of N4BP3, when we knocked down the elevated expression of STAT3 or treated Huh-7-ov-N4BP3 cells with STAT3 inhibitors, we found that the expression level of VEGFA could fall back to varying degrees at this time, which was confirmed by qPCR and ELISA assays (Figure 4D). Further tube formation assays also verified these experimental results (Figure 4E and Figure S4B). Corresponding further studies found that the N4BP3-STAT3 regulatory axis also exists in HUVECs, which indicates that the regulation model seems to be universal (Figure S4C). Taken together, these results revealed that the highly expressed N4BP3 in HCC promotes the malignant course of the tumor through the STAT3 signaling pathway.

3.5 | NEDD4 binding protein 3 promotes the expression of STAT3 by interacting with KAT2B

To further explore the specific mechanism of N4BP3 promoting the STAT3 signaling pathway, we used Co-IP to analyze the proteins interacting with N4BP3 followed by Coomassie bright blue staining assays. The results showed that there was a distinct band near 95 kDa, which was identified as KAT2B by mass spectrometry (Figure S5A). Further Co-IP experiments also verified the mutual binding between N4BP3 and KAT2B in Huh-7 cells, and the immunofluorescence assay showed that the heterodimer of N4BP3-KAT2B was colocalized in the nucleus (Figure 5A). The interaction is direct because GST or His pull-down experiments showed that purified His-tagged KAT2B protein interacted with purified GST-N4BP3, but not GST alone (Figure 5B). KAT2B is a transcription adapter protein and histone acetyltransferase, which is the catalytic subunit of the transcription coactivator protein complex. Therefore, we speculated that N4BP3 activates the signaling pathway to promote angiogenesis by promoting the expression of STAT3 by interacting with KAT2B. To further verify the role of KAT2B in N4BP3 in promoting angiogenesis, we used shRNA and molecular inhibitors targeting KAT2B. The results showed that after knocking down or inhibiting the function of KAT2B in Huh-7-ov-N4BP3 cells, the expression of STAT3 and the content of p-STAT3 obviously fall back (Figure 5C). Both ELISA and tube formation assays were also used to confirm that blocking KAT2B or interfering with KAT2B could rescue the increase in VEGFA secretion and average rate of complete tubular structures caused by N4BP3 overexpression in Huh-7 cells (Figure 5D,E and Figure S5B). The abovementioned various experimental results fully show that N4BP3 does rely on the interaction with KAT2B to perform its role in promoting neovascularization in HCC. It is worth noting that KAT2B is a histone acetylation modification enzyme, and its main modification site in cells is H3K27ac, which can be used as a marker for transcriptional activation. We hypothesized that the N4BP3-KAT2B heterodimer affects STAT3 gene expression by regulating intracellular H3K27ac distribution. We confirmed this hypothesis using ChIP and found that H3K27ac enrichment in the STAT3 promoter region increased after N4BP3 overexpression in Huh-7 cells. In contrast, H3K27ac enrichment in the STAT3 promoter region was significantly reduced after N4BP3 KO in Lm3 cells (Figure 5F). In summary, these experimental results show that N4BP3 affects the distribution of H3K27ac in the STAT3 promoter region by interacting with KAT2B, thereby regulating its transcriptional activity.

3.6 | C-terminal of N4BP3 is essential for its function

To further understand the working mechanism of the N4BP3-KAT2B heterodimer and map the specific N4BP3-interacting region in KAT2B, we constructed several expression plasmids with myc-tagged truncated KAT2B (K-JD1-myc, K-JD2-myc, K-JD3-myc, and

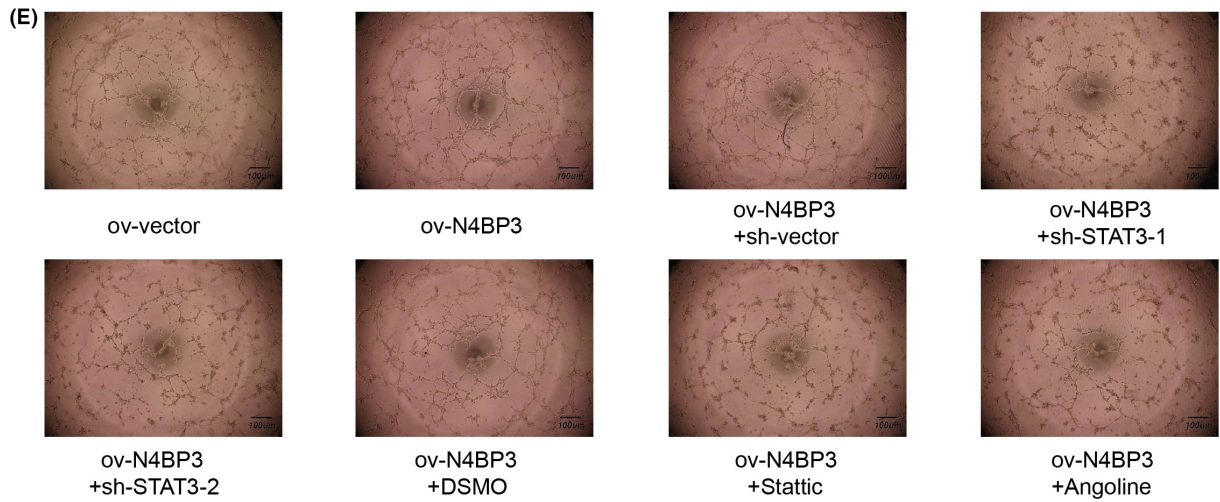
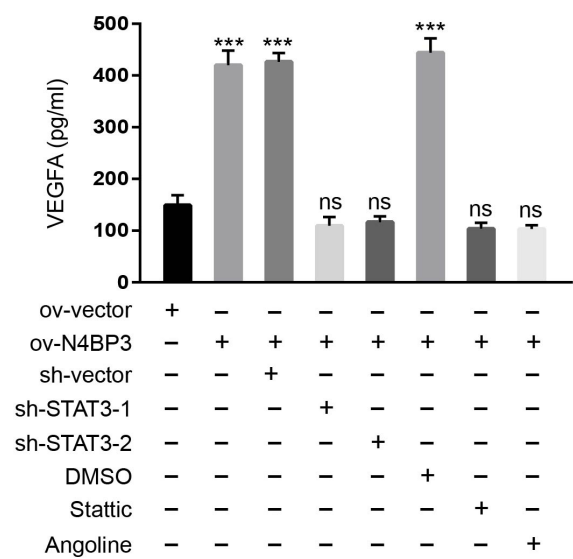
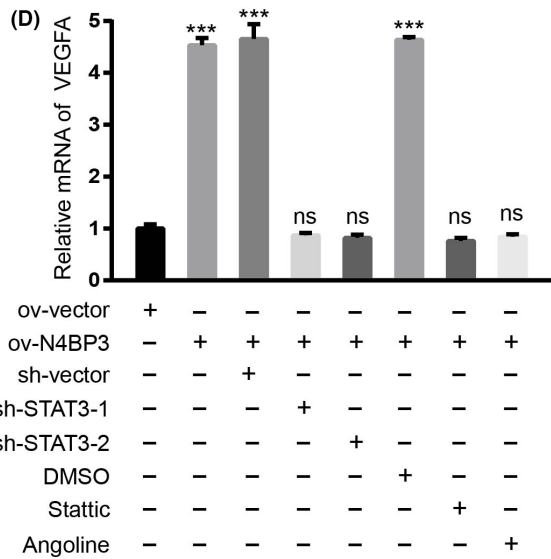
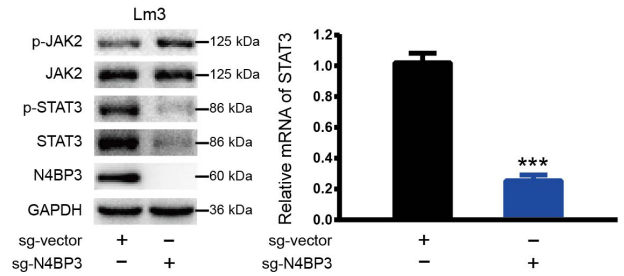
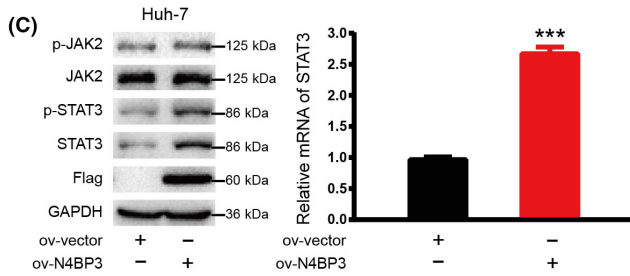
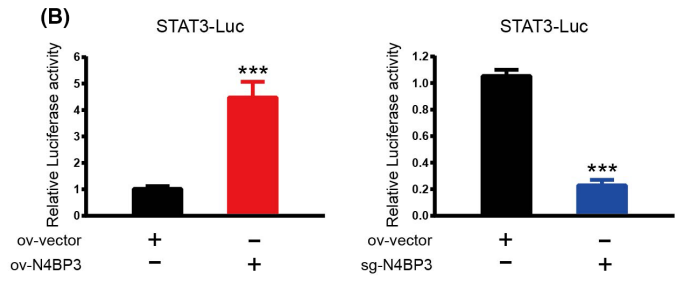
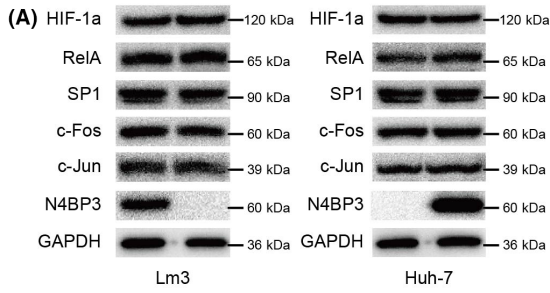


FIGURE 4 NEDD4 binding protein 3 (N4BP3) promoted vascular endothelial growth factor A (VEGFA) expression by enhancing the signal transducer and activator of transcription 3 (STAT3) signaling pathway. (A) Western blot analysis of the expressions of key executive molecules in various pro-angiogenesis signaling pathways. Consistent with the above sequencing results, other key molecules showed no significant changes. (B) Through dual-luciferase reporter gene assays, fluorescence intensity of the STAT3 signaling pathway was significantly increased after N4BP3 was overexpressed in Huh-7 cells, whereas the fluorescence intensity of STAT3 signaling pathway was significantly reduced when N4BP3 was knocked out in Lm3 cells. (C) Western blot and quantitative PCR (qPCR) assays showed that N4BP3 only affected STAT3 expressions and p-STAT3 levels in the JAK2/STAT3 signaling pathway, but did not affect the other molecules involved in this signaling pathway. (D) qPCR and ELISA assays were undertaken to confirm that blocking the STAT3 signaling pathway or interfering with STAT3 could rescue the increased secretion of VEGFA caused by N4BP3 overexpression in Huh-7 cells. (E) Tube formation assays were carried out to confirm that blocking STAT3 signaling or interfering with STAT3 could rescue the increased number of complete tubular structures following treatment with supernatants from Huh-7-ov-N4BP3 cells

K-JD4-myc), and each was cotransfected into Huh-7 cells with the N4BP3-Flag expression plasmid. Co-immunoprecipitation results revealed that the region spanning residues 696–832 aa of KAT2B (BROMO domain) was required for its interaction with N4BP3 (Figure 6A). Similarly, three FLAG-tagged truncated forms of N4BP3 (N-Delta-Flag, M-Delta-Flag, and C-Delta-Flag) were constructed and coexpressed with KAT2B-myc in Huh-7 cells, followed by Co-IP. The results suggested that the C-terminal of N4BP3 (361–544 aa) was involved in the interaction with KAT2B (Figure 6B). Therefore, N4BP3 was found to bind to the BROMO domain of KAT2B through its C-terminus. To further verify the role of the C-terminal of N4BP3 in promoting angiogenesis, we transferred N4BP3-Flag, N-Delta-Flag, M-Delta-Flag, and C-Delta-Flag into Huh-7 cells. Compared with other groups, when N4BP3 lacks its C-terminal domain, the results of western blot assays showed that the expression of STAT3 and the content of p-STAT3 did not increase significantly (Figure 6C). The ELISA experiments also showed that, compared with other groups, when only N-Delta-Flag plasmid was transferred, the content of VEGFA in the supernatant of this group did not change significantly compared with the control group (Figure 6D). The results of the above series of experiments proved the important role of the C-terminal of N4BP3 in promoting angiogenesis.

Thus far, we have fully explored the potential mechanism of N4BP3 promoting angiogenesis in HCC, but the related reasons for the high expression of N4BP3 in HCC remain unclear. Previous studies have shown that hypoxia is an important feature in malignant tumor tissues, and some studies have shown that severe hypoxia also exists in HCC. Therefore, further exploration was carried out, combined with the analysis of the expression of N4BP3 in multiple HCC cells in Figure 2. We selected two cell lines (HepG2 and Hep3B) with moderate expression for subsequent experiments. After hypoxia treatment on HepG2 and Hep3B cells, qPCR assays and western blot assays were used to detect that the expression level of N4BP3 in cells was significantly increased (Figure S6A), indicating that hypoxia could indeed promote the expression of N4BP3. To this end, we analyzed the N4BP3 promoter region and found that there was indeed a transcription binding site for HIF-1 α protein in this region. Using the dual-luciferase reporter gene assays, we found that this potential binding site played an important role in the transcription of N4BP3 in the hypoxic environment (Figure S6B).

To further verify the above experimental findings, the *HIF1 α* gene was overexpressed in HepG2 and Hep3B cells. The experimental

results showed that overexpression of HIF1 α protein could indeed further enhance the transcription of the *N4BP3* gene in the hypoxic environment (Figure S6C). The corresponding ChIP assays confirmed that HIF1 α could directly bind to the potential binding site of the *N4BP3* promoter region (Figure S6D). In summary, we found that hypoxia is an important factor in inducing *N4BP3* gene expression.

3.7 | NEDD4 binding protein 3 could be used as a target for the treatment of HCC

Based on the above experimental findings, using IHC staining methods, we undertook the corresponding analysis in spontaneous liver cancer tissues of induced mice. We found that, compared with WT mice, the content of p-STAT3 was significantly reduced in the chemically induced liver cancer tissues in N4BP3 KO mice, which is consistent with the experimental findings at the cell level (Figure 7A). To further verify the role of N4BP3 in the progression of HCC in vivo, stable Huh-7-ov-N4BP3 or corresponding vector control cells were injected subcutaneously into nude mice. After the subcutaneous implant tumors were removed, accelerated tumor growth was reported in the N4BP3 overexpression group. At this time, after knocking down KAT2B in Huh-7-ov-N4BP3 cells, we also found that the effect of N4BP3 on tumor promotion could be significantly reduced, indicating that the promotion effect of N4BP3 on HCC depends on KAT2B (Figure 7B). Additionally, IHC revealed that N4BP3-overexpressing tumors showed higher MVD (indicated by CD31⁺ cells) than control tumors. Furthermore, IHC staining (Ki-67) results confirmed tumor proliferation, and the above-described effects could be rescued by interfering with KAT2B (Figure 7C). Correspondingly, in vivo, the proliferation rate of Lm3-sg-N4BP3 decreased significantly compared with the control group and the IHC results also verified this finding (Figure 7D). To further explore the role of N4BP3 in the treatment of HCC, sorafenib (20 mg/kg) was intragastrically administered to the Lm3-sg-vector/N4BP3 group. The results showed that the combination of sorafenib after N4BP3 KO can greatly enhance the therapeutic effect of sorafenib, which provided the theoretical basis for N4BP3 as a therapeutic target for HCC (Figure 7D). Further IHC staining results showed that, after N4BP3 KO, the proliferative potential of the tumor was greatly reduced, which was accompanied by a significant decrease in MVD in the tumor (Figure S7A,B).

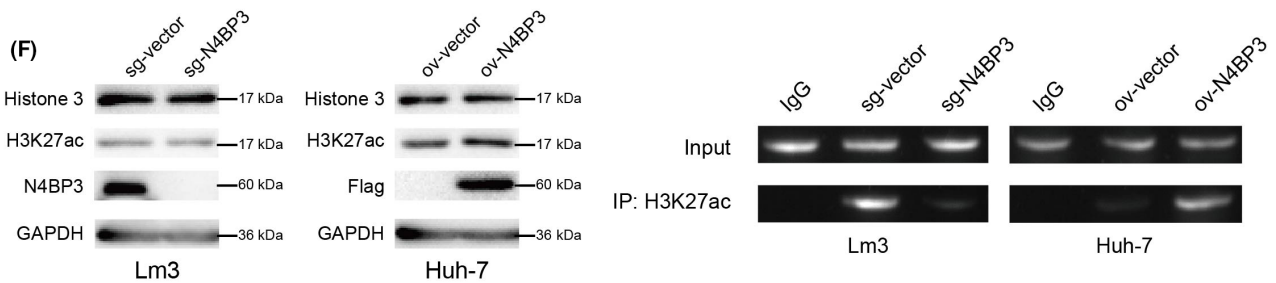
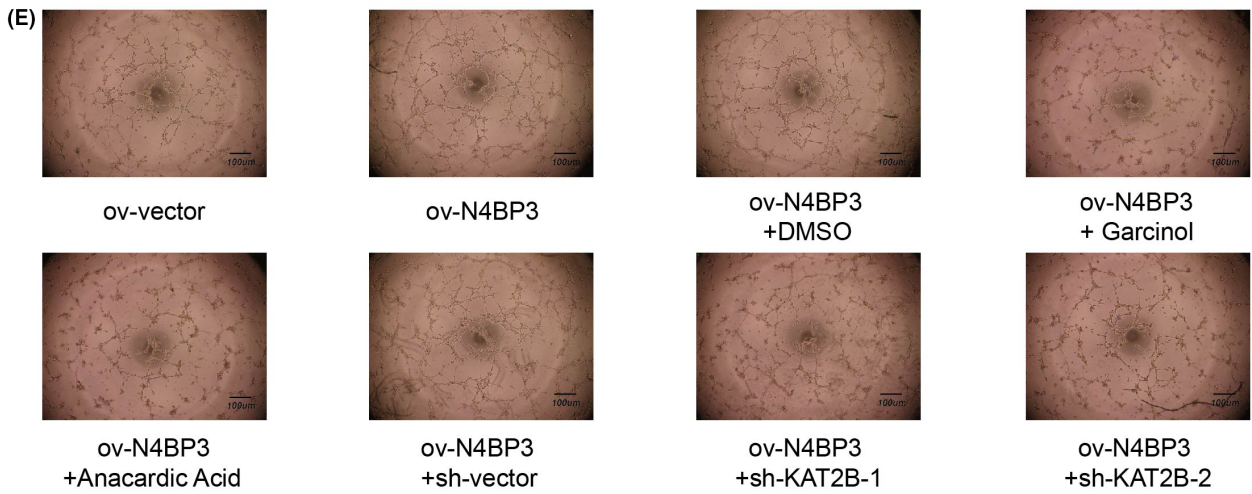
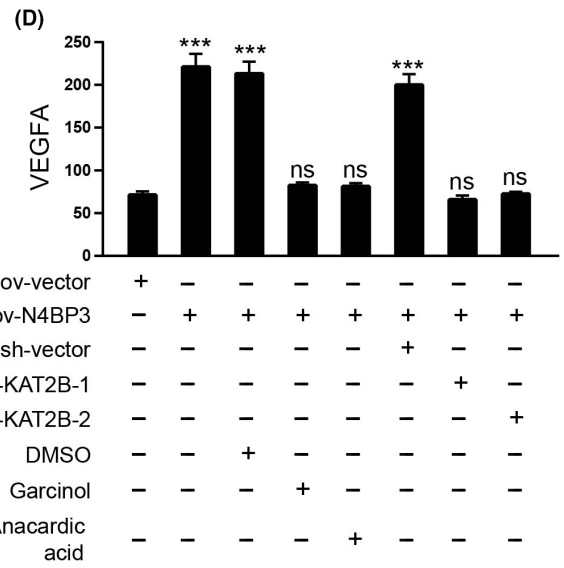
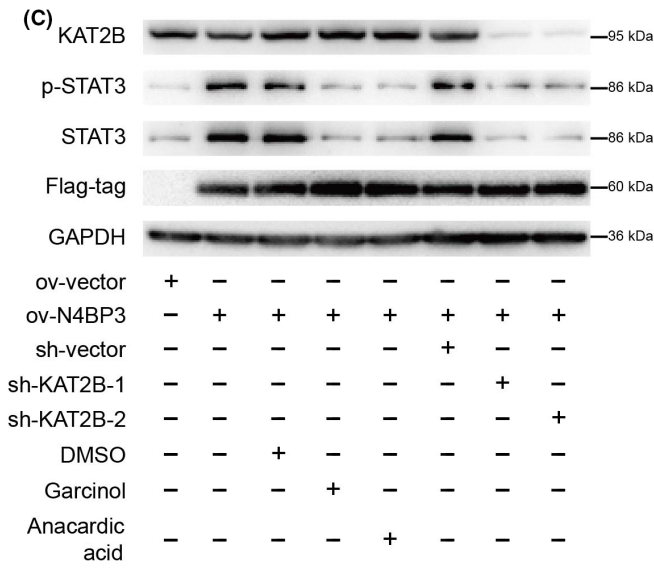
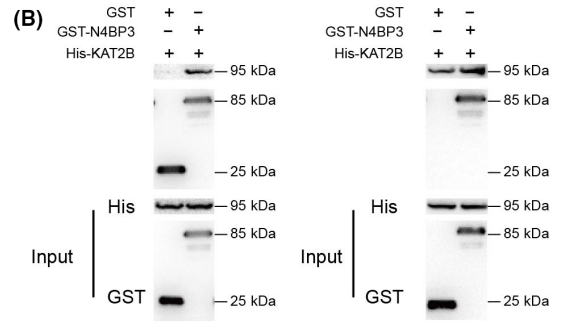
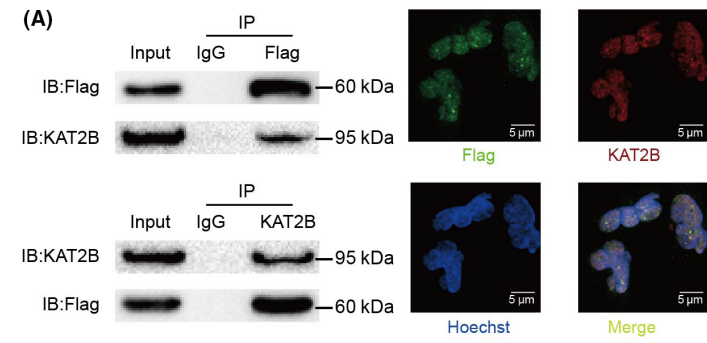


FIGURE 5 NEDD4 binding protein 3 (N4BP3) promoted the expressions of signal transducer and activator of transcription 3 (STAT3) by interacting with lysine acetyltransferase 2B (KAT2B). (A) In Huh-7 cells, interactions between N4BP3 and KAT2B were confirmed by co-immunoprecipitation assays (left). Further immunofluorescence experiments showed that N4BP3 and KAT2B were co-located in the nucleus (right). (B) Protein purifications and pull-down results confirmed direct interactions between N4BP3 and KAT2B. In the GST- and His-pull-down experiments, only GST-N4BP3 was shown to directly interact with His-KAT2B, instead of a separate GST protein. (C) Western blot assays showed that upregulation of STAT3 caused by overexpression of N4BP3 could be rescued by knocking down KAT2B or inhibiting its functions in Huh-7-ov-N4BP3 cells. (D) ELISA was carried out to confirm that knocking down KAT2B or inhibiting its functions could rescue the increased secretions of vascular endothelial growth factor A (VEGFA) caused by N4BP3 overexpression in Huh-7 cells. (E) Tube formation assays were used to confirm that knocking down KAT2B or inhibiting its functions could rescue the increased number of complete tubular structures following treatment with supernatants from Huh-7-ov-N4BP3 cells. (F) Western blot assays showed that regulation of N4BP3 expression in hepatocellular carcinoma did not affect intracellular acetyl-Histone H3 (Lys27) (H3K27ac) levels (left panels), while ChIP assays showed that N4BP3 promoted intracellular H3K27ac enrichment in the STAT3 promoter region by coactivating with KAT2B (right panels). *** $p < 0.001$; IB, immunoblot; IP, immunoprecipitation; ns, not significant

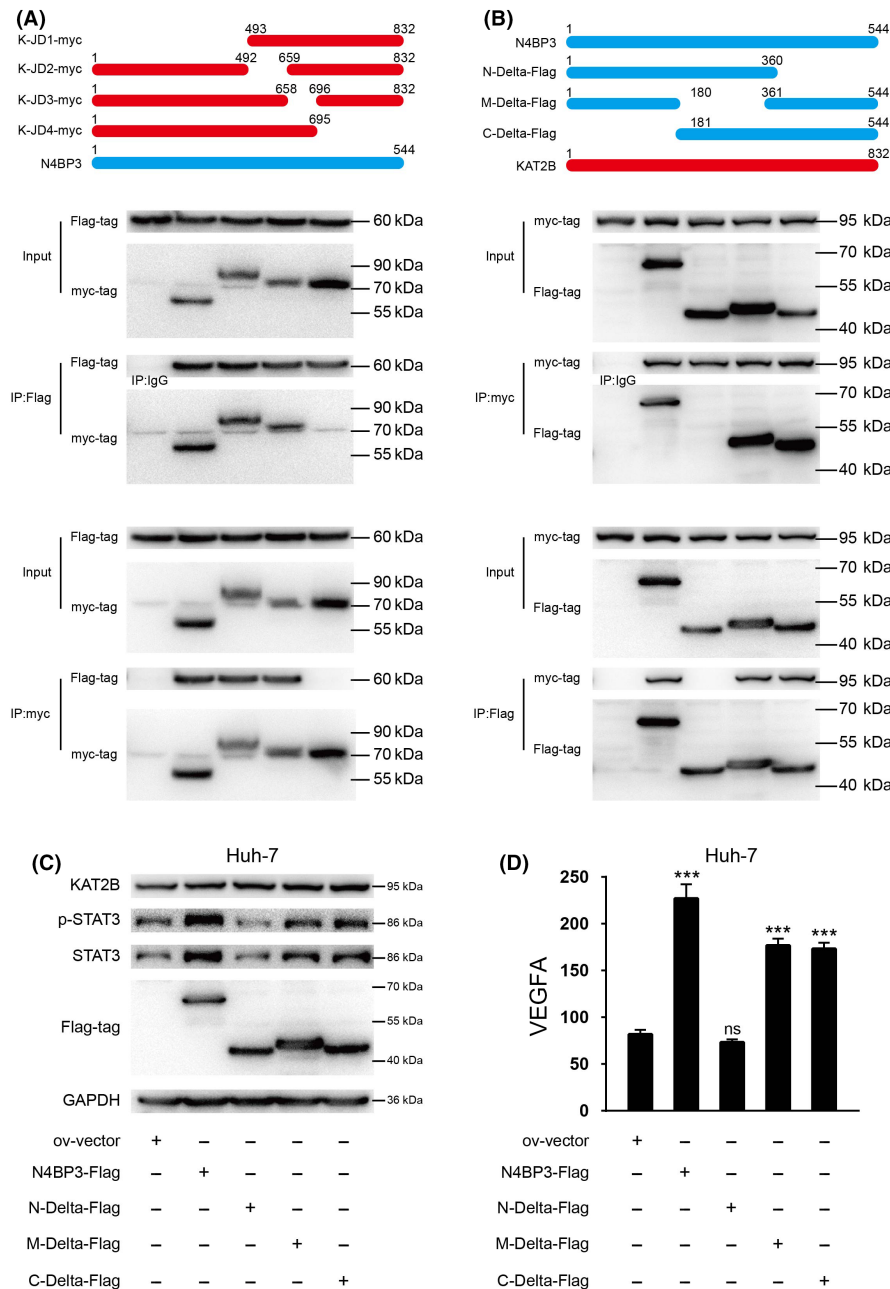


FIGURE 6 C-terminal of NEDD4 binding protein 3 (N4BP3) is essential for its functions. (A) Co-immunoprecipitation (Co-IP) assays of Huh-7 cells transfected with indicated FLAG-tagged-N4BP3 and Myc-tagged-lysine acetyltransferase 2B (KAT2B) truncations. Schematic presentations of KAT2B. Its truncated mutants are also shown (upper panel). (B) Co-IP assays of Huh-7 cells transfected with indicated Myc-tagged-KAT2B and FLAG-tagged-N4BP3 truncations. Schematic presentations of N4BP3. Its truncated mutants are also shown (upper panel). (C) Western blot assays confirmed that when N4BP3 lacks its C-terminal domain, its effect on promoting signal transducer and activator of transcription 3 (STAT3) expression disappears, indicating the importance of the C-terminal domain in the biological functions of N4BP3. (D) ELISA was used to determine that the C-terminal domain of N4BP3 plays an important role in its biological functions. IP, immunoprecipitation; VEGF, vascular endothelial growth factor

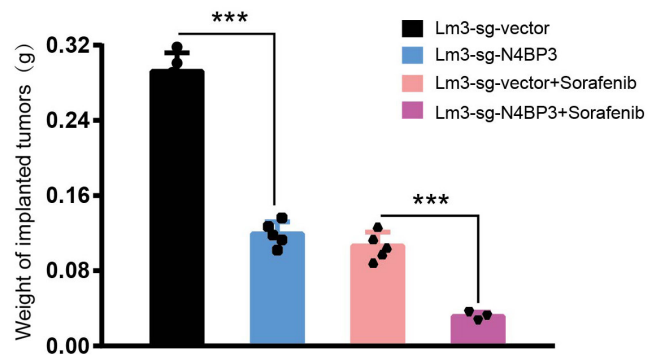
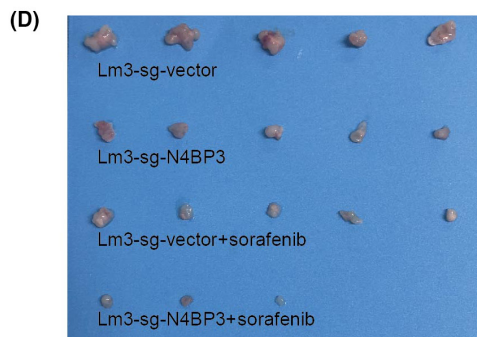
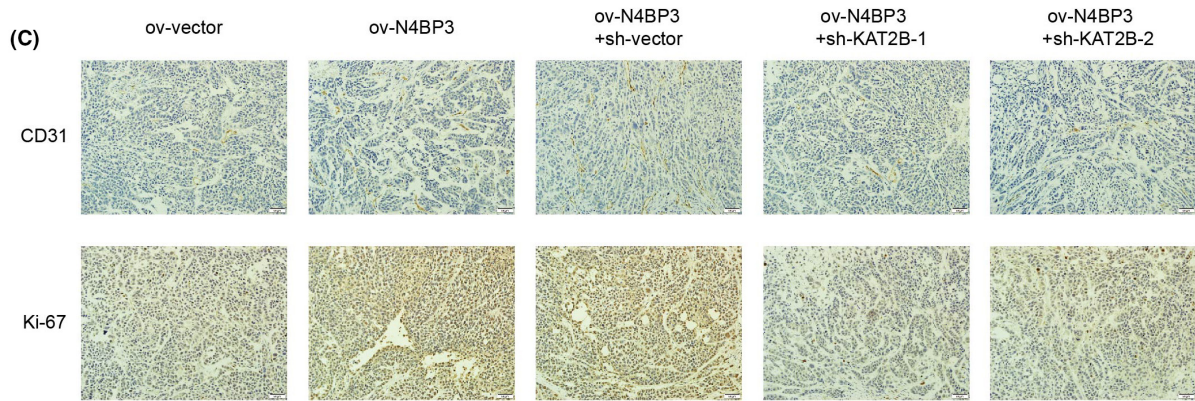
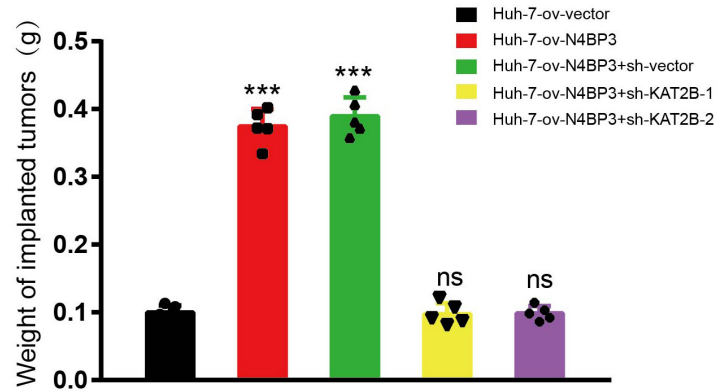
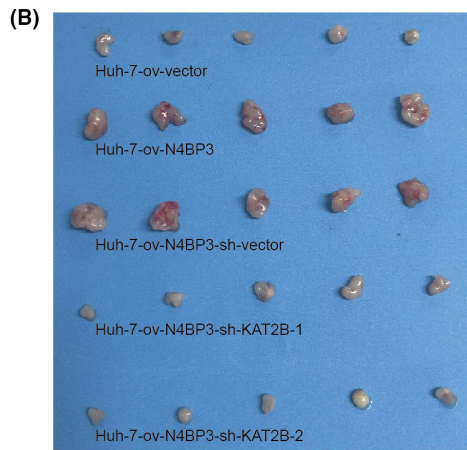
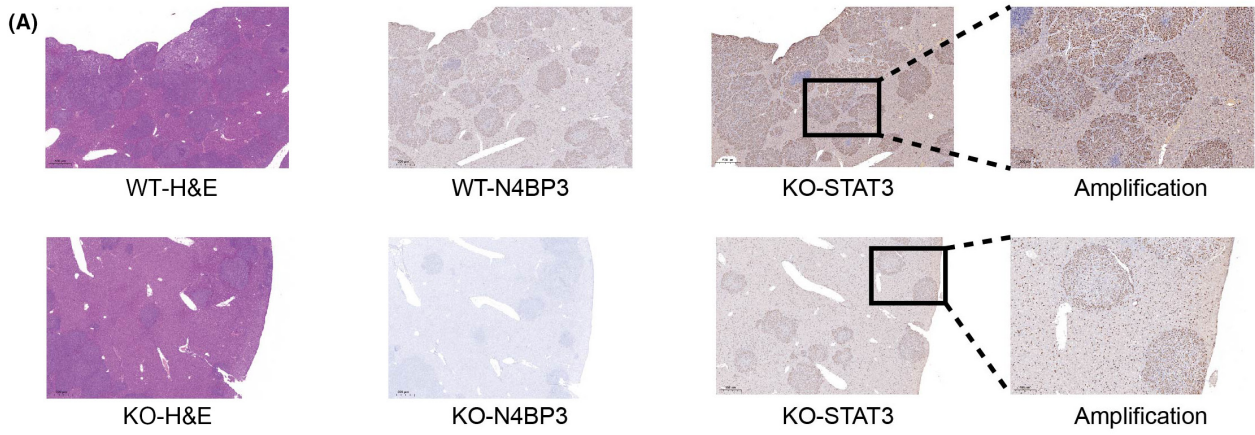


FIGURE 7 NEDD4 binding protein 3 (N4BP3) is a potential target for the treatment of hepatocellular carcinoma. (A) Using the tumor tissue induced in Figure 1, we verified that, compared to the sample from WT mice, phosphorylated signal transducer and activator of transcription 3 (p-STAT3) levels were significantly reduced in chemically induced liver cancer tissues from N4BP3 KO mice, as revealed by immunohistochemistry (IHC) and H&E staining. (B) Subcutaneous tumorigenesis experiments in nude mice showed that overexpression of N4BP3 promoted the growth of tumor xenografts in nude mice, whereas KAT2B knockdown inhibited this effect (left panel). The corresponding statistical results supported our experimental results (right panel). (C) IHC staining of tumor microvessel density (indicated by CD31⁺ cells) and related proliferation indices (Ki-67) in different groups. (D) Compared to the corresponding control cells, N4BP3 KO in Lm3 cells significantly reduced their proliferation rate in nude mice. When sorafenib was added, cells in the Lm3-sg-N4BP3 group showed higher drug sensitivities compared to the control group (left panel). The corresponding statistical results supported our experimental results (right panel)

As a vascularly rich solid tumor, HCC has been clinically applied with a variety of VEGF inhibitors, including bevacizumab, for antivasular therapy. Therefore, we undertook a preliminary comparative study between N4BP3 KO and bevacizumab treatment. Relevant experimental results showed that, compared with the control group, N4BP3 KO can indeed inhibit the malignant proliferation of HCC *in vivo*, and can further enhance the therapeutic effect of bevacizumab. However, the therapeutic effect of N4BP3 KO is still slightly inferior to that of drug therapy, and further IHC staining results and statistical analyses also confirmed the findings of *in vivo* experiments in nude mice (Figure S7C–E).

The results of these two experiments in nude mice showed that N4BP3 could reduce the malignant proliferation of HCC *in vivo* and enhance the efficacy of related anticancer drugs, thus providing a theoretical basis for N4BP3 as a potential therapeutic target for HCC. In summary, we can conclude that N4BP3, which is highly expressed in HCC, interacts with the BROMO domain of KAT2B through its C-terminus to promote STAT3 expression, activating the STAT3 signaling pathway, and thus increasing VEGFA expression and secretion to promote angiogenesis in HCC.

4 | DISCUSSION

In the ranking of human malignant tumors, HCC has long occupied an important position with its high incidence and high mortality.¹⁹ As a tumor with rich blood supply, the recently developed antivasular molecular targeted therapy is a potential treatment strategy for patients who have lost the chance for radical surgery.²⁰ As the representative drug, sorafenib is associated with various side-effects, including diarrhea, increased blood pressure, and skin lesions.²¹ Moreover, cost implications and the extremely limited improvement in survival time greatly limits its clinical application prospects.²² To this end, studies have aimed at developing new antivasular treatment strategies, with improvements in new drugs such as lenvatinib and regorafenib.²³ Efforts have also aimed at improving the sensitivity of these drugs while reducing resistance. To achieve optimal outcomes, there is a need to elucidate the mechanisms involved in angiogenesis in HCC and to further identify the targets that can improve the sensitivity of drug therapy. In this study, through multilayer experiments in cells, animals, and tissues, we analyzed the specific mechanisms through which N4BP3 promotes angiogenesis in HCC. It was established that, following N4BP3 KO, the efficacy of

sorafenib was significantly enhanced; similarly, the efficacy of bevacizumab was also significantly improved after N4BP3 KO.

The JAK2/STAT3 signaling pathway mediates several biological mechanisms in various physiological and pathological processes.²⁴ Its main conduction processes are as follows. The binding of the cytokine to the receptor causes dimerization of the receptor molecule, which brings the receptor-coupled JAK2 close to each other and activated through reciprocal tyrosine phosphorylation.²⁵ Activated JAK2 catalyzes tyrosine phosphorylation of the receptor and forms the corresponding STAT3 docking site.²⁶ STAT3 binds the receptor through the SH2 domain and phosphorylates and activates it under the actions of JAK2.²⁷ Then STAT3 forms a dimer and is incorporated into the nucleus, from where it binds the corresponding target gene promoter to activate the corresponding gene transcription and expression.²⁸ Various regulation modes of this signaling pathway have been reported. These modes involve negative regulators of the JAK2/STAT3 signaling pathway, including SOCS3, PIAS3, and SHP2.²⁹ In addition to typical tyrosine phosphorylation, covalent modification of STAT also involves serine phosphorylation, acetylation, glycosylation, and ubiquitination, among others.³⁰ Jointly, these regulatory forms regulate the transcriptional activities of the intracellular JAK2/STAT3 signaling pathway.³¹ In this study, we found that N4BP3 regulates the distribution of H3K27ac, an important histone modification, in the promoter region of STAT3 by interacting with KAT2B, thereby realizing the effective regulation of the latter's transcriptional activity.

We identified N4BP3, a protein that has never been systematically studied before, to be an oncogene that can promote angiogenesis in HCC. We found that through its C-terminal domain, it can interact with KAT2B, thereby promoting the expression of STAT3 to increase the activity of the STAT3 signaling pathway. However, Figure 6 shows that, although its C-segment still exists, when N4BP3 lacks the M-segment or N-segment domain, it can still interact with KAT2B, but the promotion of STAT3 expression is not as good as its full length, indicating that M-segment and N-segment domains have corresponding biological functions, which should be further explored. Through transcriptome sequencing, it was revealed that N4BP3 might also be involved in various biological behaviors, and some of these biological events are dependent, or partly dependent, on the STAT3 signaling pathway, but some are independent of this signaling pathway. This indicates that N4BP3 could have a biological function that is independent of the STAT3 signaling pathway. Therefore, there is a need to assess

the specific roles of N4BP3 in HCC. Figure 7 shows that N4BP3 KO in Lm3 cells in combination with sorafenib in nude mice significantly enhanced the therapeutic effects of the combined drugs, when compared to those of the single drug group. These findings provide a theoretical basis for N4BP3 as a therapeutic target for HCC. We also postulated that deletion of N4BP3, in addition to its own existence of reducing angiogenesis to enhance the efficacy of sorafenib, whether there are other unknown mechanisms that N4BP3 may increase the sensitivity of sorafenib. Based on these findings, we will explore the role of N4BP3 in the mediation of other diverse biological functions in HCC.

In summary, N4BP3, a proto-oncogene, is involved in the promotion of angiogenesis in HCC, which elucidates the malignant processes of HCC and also provides a new perspective for us to understand STAT3 signaling pathway regulation. Moreover, our findings provide a theoretical basis for the selection of therapeutic targets for HCC.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

DATA AVAILABILITY STATEMENT

Sequencing data have been deposited in the SRA database under accession number PRJNA804643.

ETHICS STATEMENT

Approval of the research protocol by an institutional review board: Institutional Research Ethics Committee of Taizhou People's Hospital.

Animal Studies: All animal experiments were approved by the Institutional Animal Care and Use Committee of Nantong University.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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