HMBOX1 inhibits hepatocellular carcinoma progression via PTPN1 mediated AKT1 phosphorylation

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Abstract. Hepatocellular carcinoma (HCC) represents the most common form of primary liver cancer and is characterized by a significant rate of recurrence. However, there is still a lack of effective therapeutic methods. Accumulating evidence has highlighted the importance of homeobox containing 1 (HMBOX1) in tumorigenesis. However, the relationship between HMBOX1 expression and HCC remains unclear. In the present study, through the analysis of public databases and staining analysis of tissue microarrays, it was found that compared with normal tissues, HMBOX1 was significantly downregulated in tumor tissues. Furthermore, through analyses such as Cell Counting Kit-8 assay, wound healing assay and colony formation, it was found that overexpression of HMBOX1 could inhibit cell proliferation and migration, while silencing of HMBOX1 promoted tumor biological characteristics in HCC cell lines. The molecular biological mechanism was explored by using proteomics combined with bioinformatics analysis and western blotting. Mechanistically, AKT1 was identified as a downstream effector of HMBOX1, and protein tyrosine phosphatase non-receptor type 1 (PTPN1) signaling might mediate the regulation of AKT1 by HMBOX1. *In vivo* tumor-bearing experiments also verified the function of the HMBOX1/PTPN1/AKT1 pathway in HCC development. Taken together, the present findings revealed a new HMBOX1/PTPN1/AKT1 axis that inhibits tumor progression and provides new candidate therapy targets for HCC.

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Introduction

Hepatocellular carcinoma (HCC) is the second most common cause of cancer-related deaths worldwide and is the most prevalent type of primary liver cancer, accounting for ~85% of all malignant liver tumors. Clinically, surgical resection and ablation are commonly used for treatment, but the recurrence rate within five years remains high at 40-70% (1). The advent of targeted immunotherapies has largely mitigated disease progression in patients with HCC (2). However, medication alone does not function as well (3). By contrast, immunotherapy in combination with targeted inhibitors offers greater clinical benefits for patients with HCC and represents a new direction for future HCC treatment (4,5). Therefore, it is important to study the pathogenesis of HCC and explore new therapeutic targets.

The transcriptional repressor homeobox containing 1 (HMBOX1) was first isolated from a human pancreatic cDNA library and is widely expressed in various human organ tissues. As a novel transcriptional repressor, there is relatively little research on the biological activity of HMBOX1, mainly involving studies on cell differentiation and development, immune regulation, cellular autophagy and aging, and tumor pathology (6). As previously reported, HMBOX1 exerts distinct regulatory effects on different types of tumors. For instance, in osteosarcoma, cervical cancer and ovarian cancer, HMBOX1 impedes tumor cell immune evasion and malignant proliferation (7,8). Conversely, in gastric cancer, it can facilitate tumor growth and metastasis, underscoring the intricate regulatory mechanisms of HMBOX1 in tumorigenesis (9). Preliminary research found that the expression level of HMBOX1 in liver cancer tissue is significantly decreased (10), but the specific regulatory mechanism and its effect on tumor growth are still unclear.

Protein Tyrosine Phosphatase Non-receptor type 1 (PTPN1), also known as PTP1B, is a member of the protein tyrosine phosphatase (PTP) family. It plays an important role in different physiological processes, especially insulin and leptin sensitivity (11). Therefore, PTPN1 may be an effective target for the treatment of type 2 diabetes. As the research continues, PTPN1 has also been shown to play an important role in tumor-related studies. However, the specific role of PTPN1 activation in promoting or suppressing tumors is not consistent across different tumors (12-15). In HCC, PTPN1 has

also been reported to have both a tumor-promoting effect (16) and a possible tumor-inhibiting effect (17). However, the specific regulatory effects and mechanisms of PTPN1 in tumors, especially HCC, remain unclear, and its regulatory relationship with HMBOX1 has not yet been reported.

In the present study, it was identified, for the first time to the best of the authors' knowledge, the important role of HMBOX1-mediated activation of the PTPN1/AKT signaling pathway in HCC malignant biological behaviors, such as proliferation and migration. Importantly, these molecular events account for the protective effects of HMBOX1 on HCC development, thereby providing a new potential target for the clinical diagnosis and treatment of HCC.

Materials and methods

Data sources. GEPIA2 (18) (http://gepia2.cancer-pku.cn/) was used to compare the expression level of genes in tumor and normal tissues and to perform correlation analysis of the expression of two genes. Kaplan-Meier plotter (https://kmplot.com/analysis/) was used to compare differences in the survival of patients with different gene expression levels (19).

Metascape database (https://metascape.org/) was used for Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of differentially expressed genes screened by proteomics (20). STRING database (https://cn.string-db.org/) is used to analyze protein-protein interactions (21). cBioPortal database (http://www.cbioportal.org/) was used to analyze the effect of clinical interventions on the expression of HMBOX1 and PTPN1 in tumor tissues of patients with HCC (22). JASPAR (https://jaspar.elixir.no/) was used to predict potential HMBOX1-binding sites within the 2-kb region upstream of the transcription start site of PTPN1 (23). The use of patient sample data from the public databases was approved by the Ethics Committee of Shandong First Medical University (approval no. R202403040155; Jinan, China).

Cell lines and culture. Mouse hepatoma cell line Hepa1-6 (cat. no. CL-0105; Procell Life Science & Technology Co., Ltd.) and human hepatoma cell line Huh-7 (cat. no. CL-0120; Procell Life Science & Technology Co., Ltd.) were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM; cat. no. PM150210; Procell Life Science & Technology Co., Ltd.) with 10% fetal bovine (cat. no. 11011-8611; Zhejiang Tianhang Biotechnology Co., Ltd.) and 1% penicillin/streptomycin (cat. no. PB180120; Procell Life Science & Technology Co., Ltd.) at 37˚C and 5% CO_2 in a cell culture incubator. In some experiments, the Akt activator SC79 (cat. no. HY-18749; MedChemExpress) at a concentration of 4 μM was used to treat Hepa1-6 cells for different times. All cell experiments were completed within two months after transfection.

Vector constructs, lentivirus production and cell transduction. Lentiviral particles were produced by transfecting 293T cells with the transfer plasmid (pLV3-CMV-MCS-3flag-EF1-Zs-Green-T2A-PURO, Research Cloud Biology) containing the HMBOX1 transcrip, along with the packaging plasmid (psPAX2) and the envelope plasmid (pMD2.G), using PEI at a mass ratio of 4:3:1 at 37˚C and 5% CO_2 in a cell culture incubator. Supernatants containing viral particles were collected at 48 h post-transfection, filtered, and concentrated. To generate stable HMBOX1-overexpressing cell lines, target HCC cells were then transduced with the virus in the presence of 8 μg/ml Polybrene (cat. no. C0351; Beyotime Institute of Biotechnology) at 37˚C and 5% CO_2 in a cell culture incubator. The multiplicity of infection values used to transduce Hepa1-6 and Huh-7 cells were 5 and 10, respectively. After transduction for 24 h, the culture medium containing the lentivirus was washed, and changed to fresh complete medium to continue culture for 48 h. Finally, cell lines were selected using complete medium containing the appropriate concentration of puromycin for seven days (Hepa1-6 2.2 μg/ml and Huh-7 1.0 μg/ml).

Cell proliferation assay. Cells were seeded into 96-well plates at a density of 1,000 cells/well. Cell viability was determined using a Cell Counting Kit-8 (CCK-8; cat. no. BS350B; Biosharp Life Sciences) following the instructions. At 6, 24, 48 and 72 h respectively, 10 μl/well CCK-8 reagent was added in each well and incubated at 37˚C for 4 h. Absorbance was measured at 450 nm using a multimode reader (SpectraMax i3x) to assess cell viability.

Colony formation assay. Cells were seeded into 6-well plates at a density of 1,000 cells/well. 2 ml of DMEM was added to each well, and the medium was changed every two days. After 10 days, the cells were fixed with 4% paraformaldehyde at 25˚C for 15 min and stained with 0.1% crystal violet solution at 25˚C for 7 min. The number of colonies (minimum number of cells forming a colony equals to 50) formed was counted using ImageJ software (Version 1.54f; National Institutes of Health).

Wound healing assay. The cells were seeded into a 6-well plate overnight and allowed to proliferate to 90% density. To minimize the influence of cell proliferation on wound closure, cells were serum-starved by incubating in serum-free DMEM for 12 h prior to creating the scratch. Then, a uniform scratch wound was then created in the center of each well using a 10-μl sterile pipette tip. The plate was washed three times with PBS to remove scraped cells, and 2 ml of 2% serum DMEM was added per well. At 0, 48 and 72 h, images were captured under bright-field illumination usingan inverted phase-contrast microscope (IX73; Olympus Corporation). The width of the wound was assessed utilizing ImageJ software in order to determine the relative rate of healing. The relative healing rate was calculated using the following formula: $(S_{0h}-S_{ah})/S_{0h}$ x100% (where ah is the scratch area time).

Proteomic analysis. The protein expression of the two groups (Vector and Over-HMBOX1) of Hepa1-6 cells were detected by liquid chromatography-tandem mass spectrometry (Thermo Fisher Scientific, Inc.). The sample processing and analysis methods have been described in a recent study by the authors (24). In brief, Hepa1-6 cells were lysed with RIPA protein lysate containing 1% PMSF (cat. no. P0013B; Beyotime Institute of Biotechnology) to extract total cell protein, according to the manufacturer's instructions. The protein concentration was determined using a BCA protein

concentration detection reagent kit (cat. no. ZJ101; Epizyme, Inc.). The protein samples are pre-treated and then then further tests be conducted using a mass spectrometry instrument. Mass spectra were searched against the mouse UniProt database (organism_id_10090_2022_07_15.fasta; https://www.uniprot.org/) using Proteome Discoverer version 2.4.

Western blotting. Cells were lysed with RIPA (cat. no. P0013B; Beyotime Institute of Biotechnology) containing 1% PMSF to extract total cell protein. The protein concentration was determined by BCA kit. The detection quantity for each sample was 25 micrograms of protein. β-actin was used as the loading control. Protein samples were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane (MilliporeSigma). Membranes were blocked and incubated with primary antibodies overnight at 4˚C. The following antibodies were used: anti-HMBOX1 (1:1,000; cat. no. 16123-1-AP; Proteintech Group, Inc.), anti-PTPN1 (1:2,000; cat. no. 11334-1-AP; Proteintech Group, Inc.), anti-AKT1 (1:2,000; cat. no. A17909; ABclonal Biotech Co., Ltd.), anti-phospho-AKT1-S473 (1:1,000; cat. no. AP0637; ABclonal Biotech Co., Ltd.), and anti-β-actin (1:10,0000; cat. no. AC026; ABclonal Biotech Co., Ltd.). Incubation with the horseradish peroxidase-labeled secondary antibody (1:1,000; cat. no. A0208; Beyotime Institute of Biotechnology) at 37˚C for 1 h was performed to test the binding of the antibodies. Finally, the proteins were visualized on a chemiluminescence instrument (5200Multi; Tanon Science and Technology Co., Ltd.) using an ultrasensitive ECL chemiluminescence kit (cat. no. P0018AS; Beyotime Institute of Biotechnology). ImageJ software (Version 1.54f; National Institutes of Health) was used for densitometric analysis.

Identification of HMBOX1 binding molecules by Co-IP and mass spectrometry. Protein samples from Hepa1-6 cells overexpressing HMBOX1 were carried out using the Immunoprecipitation Kit with Protein A+G Magnetic Beads kit (cat. no. P2179; Beyotime Institute of Biotechnology) in accordance with the manufacturer's instructions. Briefly, following thorough cell lysis with lysis buffer, the lysate was centrifuged at 12,000 x g for 5 min at 4˚C. Then the protein concentration was detected using the BCA kit. The HMBOX1 antibody (cat. no. 16123-1-AP; Proteintech Group, Inc.) was incubated overnight with the protein sample at 4˚C at 0.5-4.0 ug for 1.0-3.0 mg of total protein lysate. The Protein A+G Magnetic Beads were co-incubated with the protein-antibody complex at room temperature for 2 h. After incubation was completed, the mixture was placed on a Magnetic Separation Rack (cat. no. FMS004; Beyotime Institute of Biotechnology) for 10 sec and the supernatant was removed. The magnetic beads were washed three times with the lysis buffer containing the inhibitor. The protein samples were eluted using SDS-PAGE loading buffer elution. Subsequently, the samples were tested using western blotting. The SDS-PAGE gel was stained with Coomassie Blue Staining Solution (cat. no. P0003S; Beyotime Institute of Biotechnology), and gels were collected for mass spectrometry analysis to identify the protein molecules interacting with HMBOX1.

Animal models. Animal studies were approved by the Institutional Animal Care Committee of Central Hospital Affiliated to Shandong First Medical University (approval no. W202403040279; Jinan, China). The experimental procedures were in accordance with the ARRIVE guidelines. Wild type male C57BL/6j mice (6-week-old; weight, 20±2g) were purchased from Beijing HFK Bioscience Co. Ltd. All mice were housed in the Laboratory Animal Center Central Hospital Affiliated to Shandong First Medical University in a specific pathogen-free (SPF) conditions. The temperature of the raising environment was controlled at 22±2˚C, and the humidity was 50%±10%. A 12/12-h light/dark cycle was adopted. Animals could freely consume standard experimental feed and drink sterile water. To evaluate the antitumor effect of HMBOX1 overexpression *in vivo*, Hepa1-6 cells overexpressing HMBOX1 (Over-HMBOX1) or transfected with an empty vector (Vector, as Ctrl group) were used to construct a mouse subcutaneous tumor-bearing model. A total of 12 mice were involved in the experiment and randomly divided into 2 groups using completely random design, with 6 mice in each group, every mouse is an experimental unit. Hepa1-6 cells ($5x10^6$, Over-HMBOX1 or Vector) were mixed with Matrigel (cat. no. 256234; Corning, Inc.) in a total volume of 100 μl. Cells were subcutaneously injected into the right subaxillary region of every C57BL/6 mouse. Tumor sizes of every mouse were measured every three days using a Vernier caliper, and tumor volume (mm^3)=a x $b^2/2$ (where a is the largest and b is the smallest tumor diameter). At the endpoint, all mice were euthanized by carbon dioxide overdose inhalation which was carried out in accordance with the Guidelines for the Euthanasia of Animals made by American Veterinary Medical Association. A gradual fill method was employed, where the chamber was initially filled to 30% CO_2, followed by a maintained flow rate of 50% of the chamber volume per min until respiratory arrest and confirmed death. Tumors from individual mice were isolated to compare tumor weights.

Chromatin immunoprecipitation (ChIP assay). ChIP experiments were performed using a ChIP Assay Kit (cat. no. P2078; Beyotime Institute of Biotechnology). The cells were collected after cross-linking the protein and genomic DNA using DMEM containing 1% formaldehyde. DNA was cleaved into 200-1,000 kb fragments using sonication after cell lysis. Anti-HMBOX1 (cat. no. 16123-1-AP; Proteintech Group, Inc.) was added and incubated overnight at 4˚C, followed by Protein A+G Agarose/Salmon Sperm DNA slowly rotated at 4˚C for 60 min to precipitate the protein DNA complex recognized by the primary antibody. After removing the cross-links between the protein and DNA and PCR amplification of the PTPN1 promoter sequence, the binding of HMBOX1 to the PTPN1 promoter was verified through 2% agarose gel electrophoresis. The gel was stained with Goldview Nucleic acid dye (cat. no. BS357A; Biosharp Life Sciences) and visualized under UV light using a gel documentation system (5200Multi; Tanon Science and Technology Co., Ltd.). Primers were designed based on possible binding sites predicted by JASPAR. The primer sequences were as follows: PTPN1-1 forward, 5'-CTCTTCCAGGTTTTCAAACCTC-3' and reverse, 5'-GAT GAGACCCGGAATCTGCAT-3'; and PTPN1-2 forward,

5'-CCTCAAAATAGCACAAGTGTCC-3' and reverse, 5'-TGATTTCCCAAGCAGACCGTT-3'.

Immunohistochemistry (IHC). HCC tissue chips (cat. no. D097Lv01; Bioaitech®; https://www.bioaitech.com/) containing tumor and matched adjacent non-tumor tissues from 48 patients with HCC were used to evaluate the expression levels of HMBOX1 and PTPN1. The use of the tissue chips was approved by the Ethics Committee of Shandong First Medical University (approval no. R202403040155; Jinan, China). For immunohistochemical staining, the tissue chips were incubated at 60˚C for 30-60 min and then subjected to deparaffinization. Briefly, the slides were incubated in xylene twice for 10 min each to completely dissolve the paraffin. Subsequently, the slides were rehydrated through a graded ethanol series: 100% ethanol twice for 5 min each, followed by 95% ethanol for 5 min, 80% ethanol for 5 min and finally 70% ethanol for 5 min. After rehydration, the slides were rinsed thoroughly in water for 5 min. Repaired the antigen with citric acid and then blocked the endogenous peroxidase activity with 3% H_2O_2-methanol. After incubation with anti-HMBOX1 (1:200; cat. no. 16123-1-AP) or anti-PTPN1 (1:200; cat. no. 11334-1-AP; both from Proteintech Group, Inc.) at 4˚C for 16 h and secondary antibodies (1:50; cat. no. A0208; Beyotime Institute of Biotechnology), streptavidin-peroxidase was added dropwise. Images were captured under bright-field illumination using an upright microscope (BX53; Olympus Corporation) to analyze the positivity rate, and the expression of HMBOX1 and PTPN1 was compared between the patient's liver cancer tissue and adjacent tissues.

Statistical analysis. All experiments were repeated at least three times. All experimental data were presented as the mean ± standard. ANOVA was used for comparisons among multi-group and if a statistically significant difference was found, Tukey's post hoc test was applied for multiple comparisons between all groups. Paired t-test was used for comparisons between two groups in IHC assay results and unpaired t-test was used for comparisons between two groups in other results. $P<0.05$ was considered to indicate a statistically significant difference. GraphPad Prism 8 (Dotmatics) was used for all statistical analyses.

Results

HMBOX1 expression is downregulated in tumor tissues and correlates with poor prognosis in patients with HCC. Analysis of clinical data from the TCGA database revealed that HMBOX1 expression was significantly downregulated in HCC tumor tissues (Fig. 1A). Survival analysis conducted using the Kaplan-Meier Plotter database also suggested a negative correlation between HMBOX1 levels and the survival rate of patients with HCC (Fig. 1B). To further confirm the expression pattern of HMBOX1 in HCC tumor tissues, IHC analysis was performed using tissue microarray from patients with HCC. The results showed that the expression of HMBOX1 in tumor tissues of was significantly downregulated compared with that in non-tumor tissues (Fig. 1C). These results suggested that HMBOX1 is downregulated in HCC, which is associated with a poor prognosis. In addition, the expression of HMBOX1 in tumor tissues of patients at different stages was further analyzed through the GEPIA2 database. Although there was no statistical difference, it could be observed that the expression of HMBOX1 in tumor tissues of patients at stage IV was significantly downregulated compared with patients at stage I-III (Fig. S1A and B). The expression of HMBOX1 was not significantly correlated with that of the HCC marker molecule AFP (Fig. S1C and D). Moreover, there was no significant difference in the expression of HMBOX1 in tumor tissues between patients who received treatment and those who did not (Fig. S1E-H).

HMBOX1 inhibits HCC cell proliferation, migration and colony formation in vitro. The potential role of HMBOX1 in HCC progression was explored. Lentiviral infection was used to overexpress HMBOX1 in Hepa1-6 and Huh-7 cells and the overexpression effect was verified using western blotting (Fig. S2A and B). The CCK-8 assay was utilized for the assessment of cellular proliferation. The growth curves indicated that HMBOX1 overexpression impeded the proliferation of HCC cells (Fig. 2A and B). Wound healing assay was used to explore the effect of HMBOX1 on the migration ability of HCC cells. It was found that HMBOX1 overexpression significantly inhibited the migration ability of Hepa1-6 cells (Fig. 2C and D). The colony formation assays showed that exogenous HMBOX1 consistently inhibited colony formation in HCC cells (Fig. 2E). To further validate these results, small interfering RNA (siRNA) silencing of HMBOX1 was performed in Huh-7 cells (Fig. S2C) and it was found that downregulation of HMBOX1 expression significantly enhanced the proliferation and migration capabilities of Huh-7 cells (Fig. 2F and G).

Inhibitory effect of HMBOX1 on HCC is dependent on the blocking of AKT1 phosphorylation. To explore the potential mechanisms by which HMBOX1 inhibits the malignant behavior of HCC cells, a proteomic analysis of Hepa1-6 cells overexpressing HMBOX1 and control cells was performed. The results revealed that, compared with the Vector (Ctrl) group, there were 1,152 upregulated and 243 downregulated protein molecules in the over-HMBOX1 group (Fig. 3A and B). GO and KEGG enrichment analysis of the differential proteins was conducted using the Metascape database (Fig. S3). Among protein molecules with significant variations, it was found that the expression of AKT1 was significantly downregulated in the cells of the overexpression group. AKT1, as one of the effective targets for HCC therapy, has been shown in numerous studies to inhibit tumor growth both directly and indirectly through its inhibitors (25,26). Among the phosphorylation sites of AKT1, phosphorylation at Ser473 is critical for its functional activity (27). However, whether HMBOX1 regulates tumor progression by modulating AKT1 activation has not been reported. A preliminary study was conducted on the relationship between these two molecules. First, the expression levels of AKT1 and its phosphorylated proteins were examined in Hepa1-6 cells overexpressing HMBOX1 through western blotting. The phosphorylation level of AKT1 at Ser473 was markedly downregulated in Hepa1-6 and Huh-7 overexpressing HMBOX1 compared with control cells infected with the empty vector (Fig. 3C and D). To

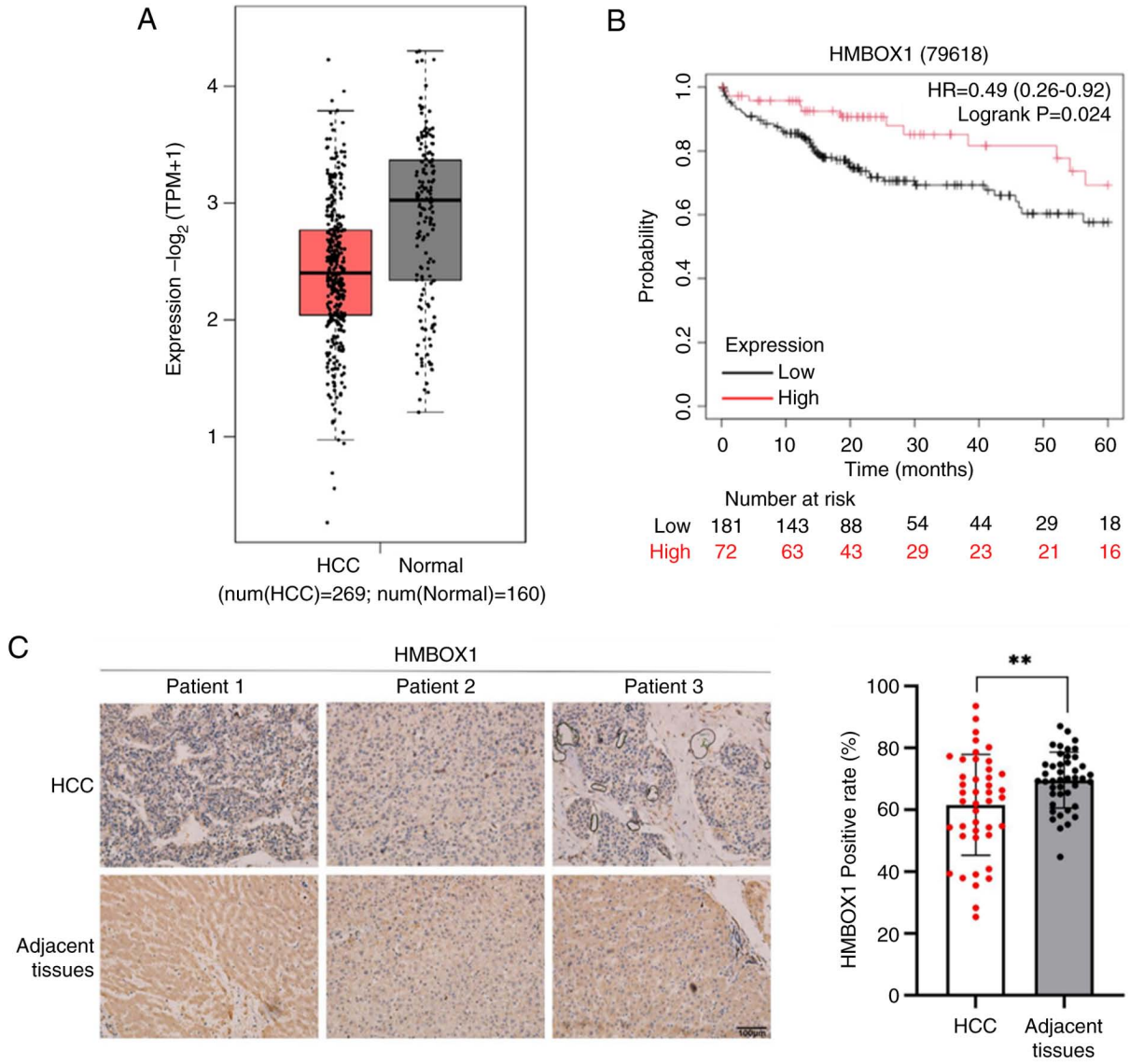

Figure 1. HMBOX1 expression is downregulated in tumor tissues and correlates with poor prognosis in patients with HCC. (A) The expression of HMBOX1 in HCC tumor tissues was analyzed using the GEPIA2 database. (B) The relationship between HMBOX1 expression and survival was analyzed via Kaplan-Meier plotter database. (C) IHC analysis of HMBOX1 expression in HCC tumor tissues and adjacent non-tumor tissues from 48 patients with HCC using TMA. **P<0.01. HMBOX1, homeobox containing 1; HCC, hepatocellular carcinoma.

further confirm the impact of HMBOX1 on HCC through the AKT pathway, HMBOX1-overexpressed Hepa1-6 cells were treated with the AKT agonist SC79. The results demonstrated that the inhibition of HMBOX1-overexpressed HCC cells on phosphorylated (p-)AKT1 (Ser473) was reversed when these cells were treated with SC79 (Fig. 3C and D). Moreover, SC79 treatment restored the inhibitory effects on HCC cell proliferation, migration, and clone formation induced by HMBOX1 overexpression (Fig. 3E-I). These results suggested that the inhibitory effect of HMBOX1 overexpression on the malignant biological behavior of HCC occurs through inhibition of AKT signaling.

PTPN1 may be a key regulator downstream of HMBOX1. PTPN1 has been documented to play a significant role in the modulation of AKT activation across various types of tumors (28,29). PTPN1 is abnormally expressed in liver cancer. Therefore, it was hypothesized that HMBOX1 may affect AKT1 phosphorylation by regulating PTPN1 expression. Using the GEPIA2 database, the correlation between HMBOX1 and PTPN1 expression levels was analyzed in HCC tissues, and it was found that the expression levels of the two molecules are positively correlated (Fig. 4A). In addition, it could be observed that the expression of PTPN1 in tumor tissues of patients at stage IV was significantly downregulated compared with patients at stage I-III (Fig. S1B). However, there is no significant correlation between the expression of PTPN1 and that of AFP in tumor tissues (Fig. S1D). There was no significant difference in the expression of PTPN1 in tumor tissues between patients who received treatment and those who did not (Fig. S1F and H). The TMA of patients with HCC was further used for IHC staining analysis. It was found that PTPN1 expression in tumor was downregulated compared with that in adjacent non-tumor tissues (Fig. 4B). Combined with the expression analysis of HMBOX1 in HCC shown in Fig. 1C, the correlation between the expression of HMBOX1

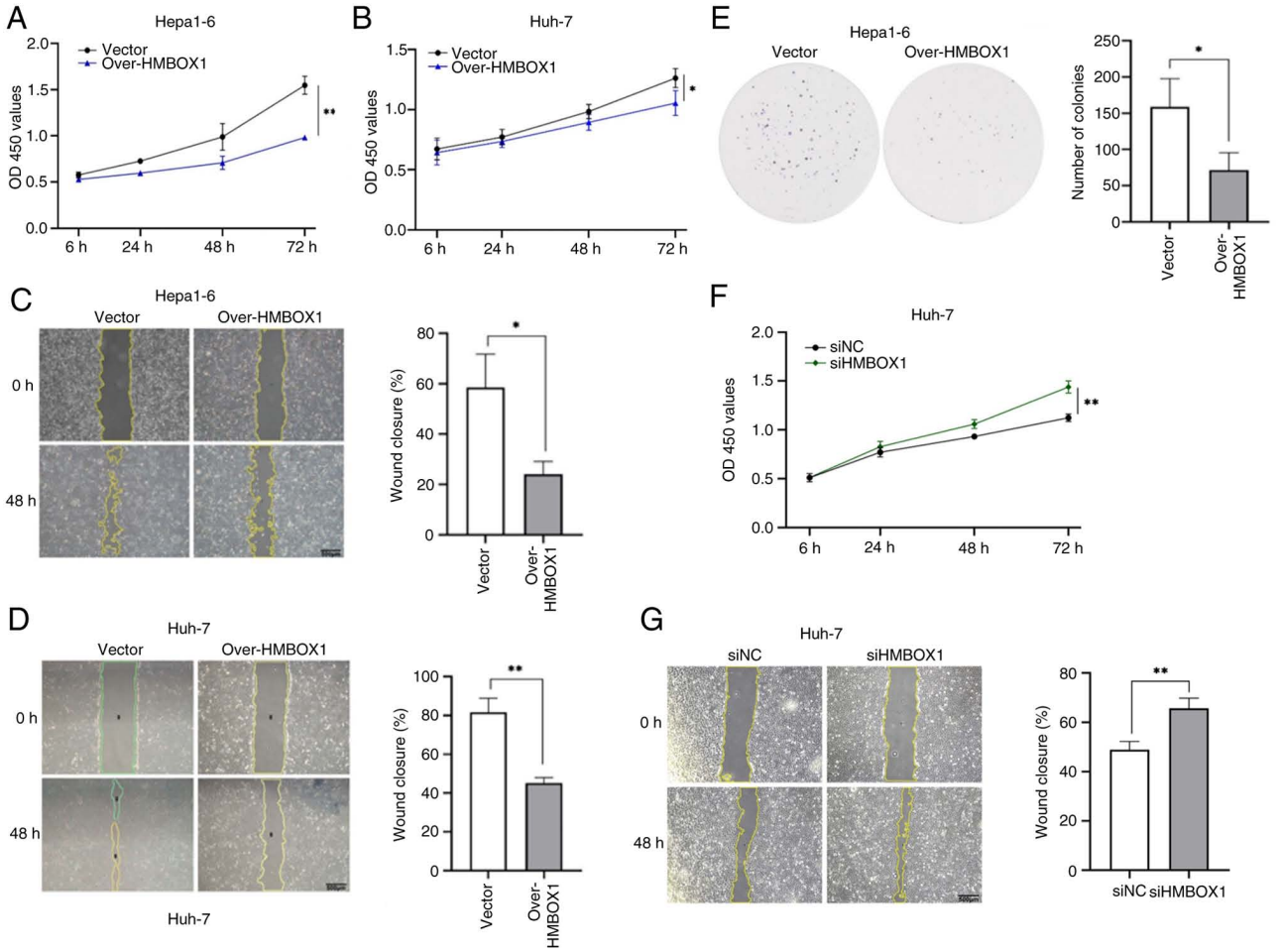

Figure 2. HMBOX1 inhibits HCC cell proliferation, migration and colony formation *in vitro*. (A and B) The effect of HMBOX1 overexpression in HCC cells proliferation was assessed through a CCK-8 assay. (C and D) The effect of HMBOX1 overexpression in HCC cells migration capacity was assessed through the wound healing assay. (E) The effect of HMBOX1 overexpression in Huh-7 cell proliferation was assessed through the colony formation assay. (F) The effect of HMBOX1 knockdown in Huh-7 cells proliferation was assessed through the CCK-8 assay. (G) The effect of HMBOX1 knockdown in Huh-7 cells migration capacity was assessed through the wound healing assay. *P<0.05 and **P<0.01. HMBOX1, homeobox containing 1; HCC, hepatocellular carcinoma; CCK-8, Cell Counting Kit-8; si-, small interfering; NC, negative control.

and PTPN1 was analyzed in the tumor tissues of patients with HCC, and it was found that the expression of these two proteins was positively correlated (Fig. 4C). HMBOX1 overexpression significantly upregulated the expression level of PTPN1 in Hepa1-6 and Huh-7cells (Fig. 4D and E). These results suggested that PTPN1 may act as a downstream target molecule of HMBOX1.

HMBOX1 inhibits HCC proliferation by regulating PTPN1/AKT1 signaling. Building on previous findings that HMBOX1 inhibits AKT1 phosphorylation and that PTPN1 may act as a downstream regulator of HMBOX1, it was hypothesized that HMBOX1 may further regulate the phosphorylation of AKT1 by modulating the expression of PTPN1, which may contribute to the oncogenic suppression activity of HMBOX1. To confirm the regulatory relationship between PTPN1 and AKT1, PTPN1 was silenced in HMBOX1-overexpressing HCC cells using siRNA targeting PTPN1 (Fig. S2D and E) and it was found that silencing of PTPN1 reversed the downregulation of AKT1 phosphorylation caused by the overexpression of HMBOX1 (Fig. 5A and B). Further functional studies showed that silencing PTPN1 significantly restored the effect of HMBOX1 overexpression on tumor cell proliferation and migration (Fig. 5C-F).

To further demonstrate the antitumor effect of HMBOX1 *in vivo*, a subcutaneous tumor model was established using HMBOX1-overexpressing (Over-HMBOX1) and control (Vector) Hepa1-6 cells in mice of the same strain. It was found that HMBOX1 overexpression significantly inhibited the growth of subcutaneous tumors in tumor-bearing mice (Fig. 6A and B). The expression of related markers in tumor tissues was analyzed, and it was found that HMBOX1 overexpression promoted PTPN1 expression and inhibited AKT1 phosphorylation (Fig. 6C). Taken together, these results indicated that HMBOX1 plays a vital role in the antitumor effects of HCC by regulating the PTPN1/AKT pathway.

These results suggested that PTPN1 may be a regulatory target of HMBOX1. To further clarify the relationship between the two, a ChIP assay was performed. However, the results showed that the regulation of PTPN1 by HMBOX1 may occur through an indirect mode of action (Fig. S4A-C). The CO-IP experiment was further used to analyze the protein molecules that might interact with HMBOX1 and 69 protein molecules interacting with HMBOX1 were further identified through

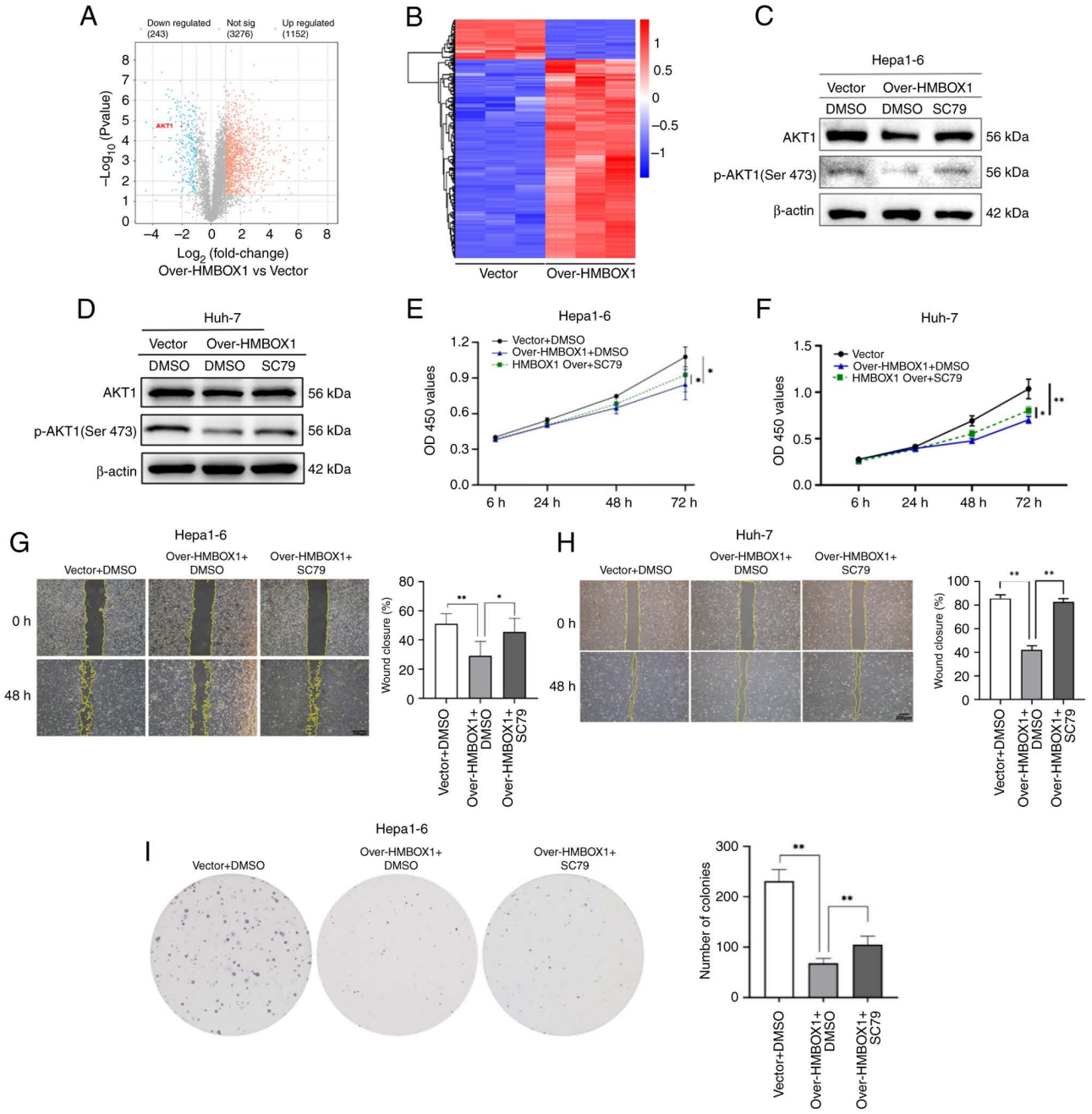

Figure 3. Inhibitory effect of HMBOX1 on HCC is dependent on the blocking of AKT1 phosphorylation. (A and B) The difference in protein expression between HMBOX1-overexpressing cells and control cells was analyzed through proteomics, and the results are shown in the form of volcano map (A) and heat map (B) respectively. (C and D) HCC cells were transfected with lentiviral vector to overexpress HMBOX1 and then treated with 4 μM SC79 for different times. The expression levels of AKT1 and p-AKT1 (Ser473) were determined through western blotting in Hepa1-6 and Huh-7 cells from different groups. (E-H) The proliferation and cell migration ability of Hepa1-6 and Huh-7 cells from each group were analyzed using a Cell Counting Kit-8 assay (E and F) and wound healing assay (G and H) (I) The proliferation ability of Huh-7 cells from different groups was assessed through the colony formation assay. *P<0.05 and **P<0.01. HMBOX1, homeobox containing 1; HCC, hepatocellular carcinoma.

mass spectrometry (Fig. S4D). GO enrichment analysis was conducted on PTPN1 and HMBOX1-interacting proteins. GO terms containing PTPN1 in the enrichment analysis results were selected to draw the Upset plots. It was found that SOD2 repeatedly appears in 9 GO terms (Fig. S4E). Furthermore, the STRING database was used to construct the interaction network between SOD2 and PTPN1. It was observed that SOD2 is directly associated with PTPN1 through JAK2, APP and GHR and the remaining nodes form a broader indirect interaction network (Fig. S4F). These results also suggested that HMBOX1 may regulate the expression of PTPN1 through an indirect regulatory approach, but the specific regulatory mechanism still needs further exploration.

Discussion

In the present study, the low expression of HMBOX1 was confirmed in tumor tissues by searching a database and analyzing tissue chips from patients with HCC, containing both tumor and matched non-tumor liver tissues. Lentiviral

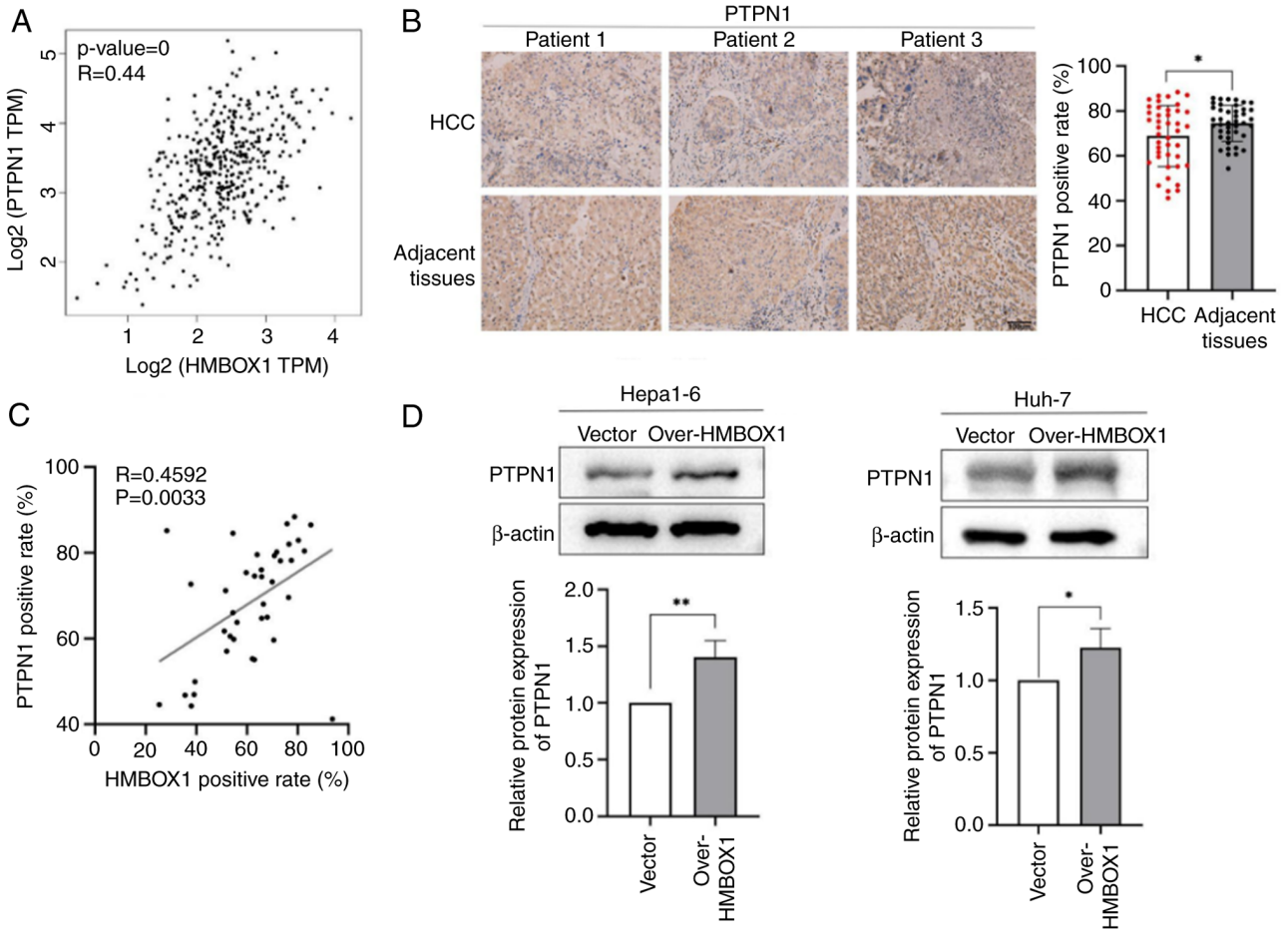

Figure 4. HMBOX1 overexpression promotes PTPN1 expression in HCC cells. (A) The correlation between the expression of HMBOX1 and PTPN1 in HCC tissues was analyzed via the GEPIA2 database. (B) IHC analysis of PTPN1 expression in HCC tumor tissues and adjacent non-tumor tissues from 48 patients with HCC. (C) PTPN1 expression level was positively correlated with HMBOX1 expression level in HCC tissues according to IHC analysis. (D) Effect of overexpression of HMBOX1 on PTPN1 expression was determined through western blotting. *P<0.05 and **P<0.01. HMBOX1, homeobox containing 1; PTPN1, protein tyrosine phosphatase non-receptor type 1; HCC, hepatocellular carcinoma; IHC, immunohistochemical.

infection was used to overexpress HMBOX1 in HCC cell lines and observed its effects on tumor progression through *in vitro* and *in vivo* experiments. HMBOX1 overexpression significantly inhibited the proliferation, migration and clonal formation of tumor cells, and significantly inhibited tumor growth in mice. Further proteomic analysis combined with experimental studies showed that HMBOX1 affected the phosphorylation of AKT1 by regulating the expression of downstream PTPN1 indirectly, thus playing a role in inhibiting HCC.

As a novel transcriptional suppressor, HMBOX1 is widely expressed in various tissues of the human body. Previously, in addition to the regulation of differentiation and development, its role in tumors has gradually attracted attention. However, the expression levels of HMBOX1 were not consistent among different tumors. Differences in expression levels also imply diverse functions (6,30). Our previous studies confirmed that the expression of HMBOX1 is significantly downregulated in tumor tissues and HCC cell lines, and that its expression level decreases with disease progression (10). In the present study, the LIHC dataset in the TCGA database and IHC staining of tumor tissues of patients with HCC were further analyzed, both of which confirmed that HMBOX1 had low expression in tumor tissues compared with non-tumor tissues. Proteomic analysis of Hepa1-6 cells overexpressing HMBOX1 (Over-HMBOX1) and the Vector (Ctrl) showed that AKT1 expression in the Over-HMBOX1 group was significantly downregulated compared with that in the Vector group. The activation of AKT1 is significantly implicated in the advancement of multiple types of malignant tumors, particularly HCC (25,31). Consequently, it was hypothesized that the overexpression of HMBOX1 could contribute to tumor suppression by downregulating the expression and activation of AKT1. The proteomic results were verified through western blotting, and it was found that the expression of total AKT1 protein was not significantly different between the two groups. However, the phosphorylation level of AKT1 (Ser473) was significantly inhibited in the HMBOX1 overexpression group. SC79 (AKT1 agonist) was used to activate p-AKT1 (Ser473) and it was found that the inhibitory effect of HMBOX1 on HCC could be reversed to a certain extent. These results indicated that HMBOX1 could inhibit the phosphorylation of AKT1. The downregulation of total AKT1 in proteomics conflicts with the western blotting experiments, probably because proteomic methods such as mass spectrometry are generally more sensitive than western blotting and

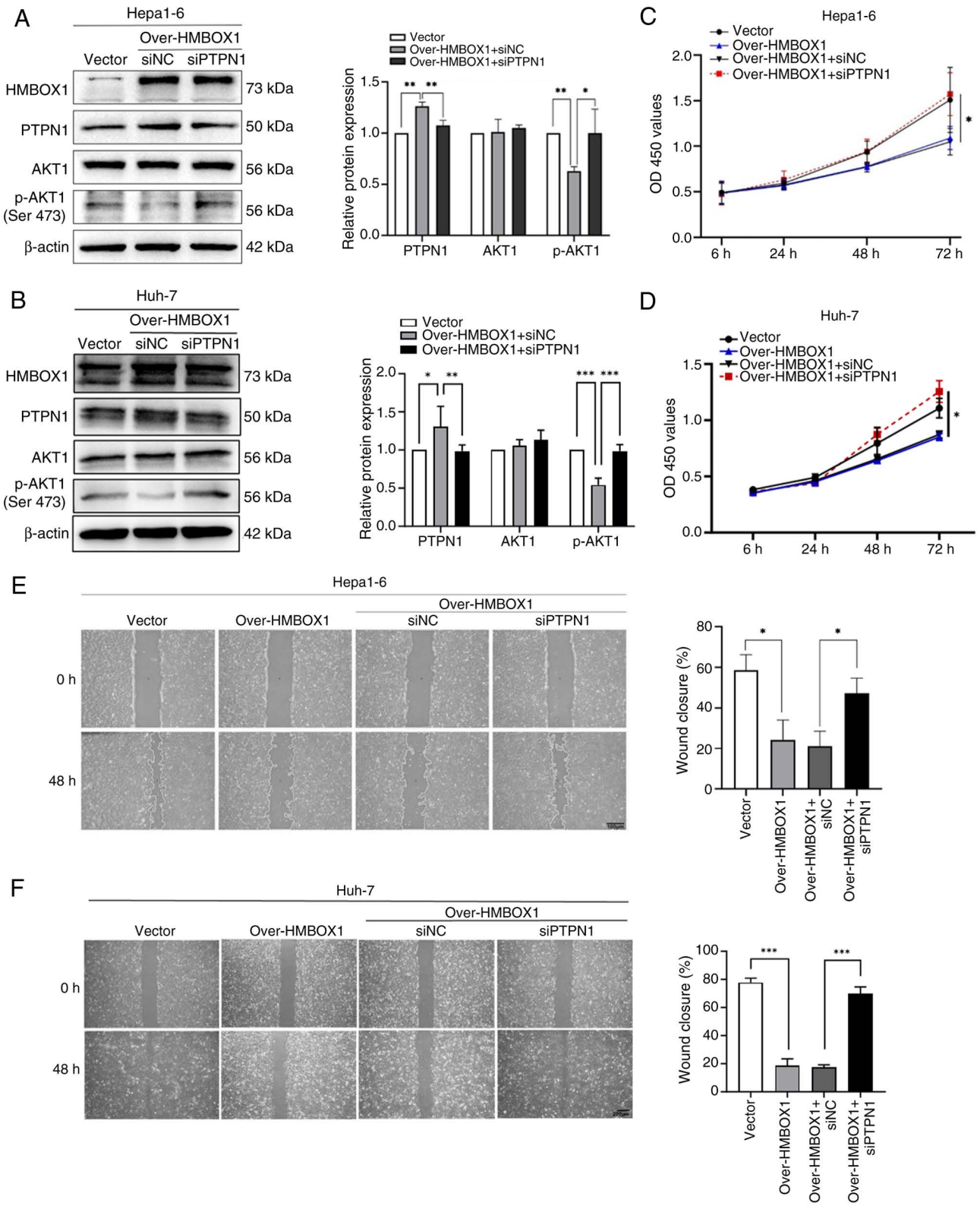

Figure 5. HMBOX1 inhibits HCC proliferation ability by regulating PTPN1/AKT1 signaling. (A and B) siRNA-targeted PTPN1 was used to silence PTPN1 in HCC cells which overexpressing HMBOX1, and western blotting was used to detect the expression of related molecules. (C and D) The proliferative ability of HCC cells from each group was analyzed using the Cell Counting Kit-8 assay. (E and F) Cell migration ability of HCC cells from each group was measured through the wound healing assay. *P<0.05, **P<0.01 and ***P<0.01. HMBOX1, homeobox containing 1; PTPN1, protein tyrosine phosphatase non-receptor type 1; HCC, hepatocellular carcinoma; si-, small interfering; NC, negative control.

different variant isoforms of AKT1 may affect the recognition of proteins by antibodies in western blot experiments. The antibody used in the present western blotting experiments is highly specific for the AKT1 isoform and recognizes a single, defined epitope. By contrast, bottom-up proteomics relies on the detection of tryptic peptides. It is possible that the specific

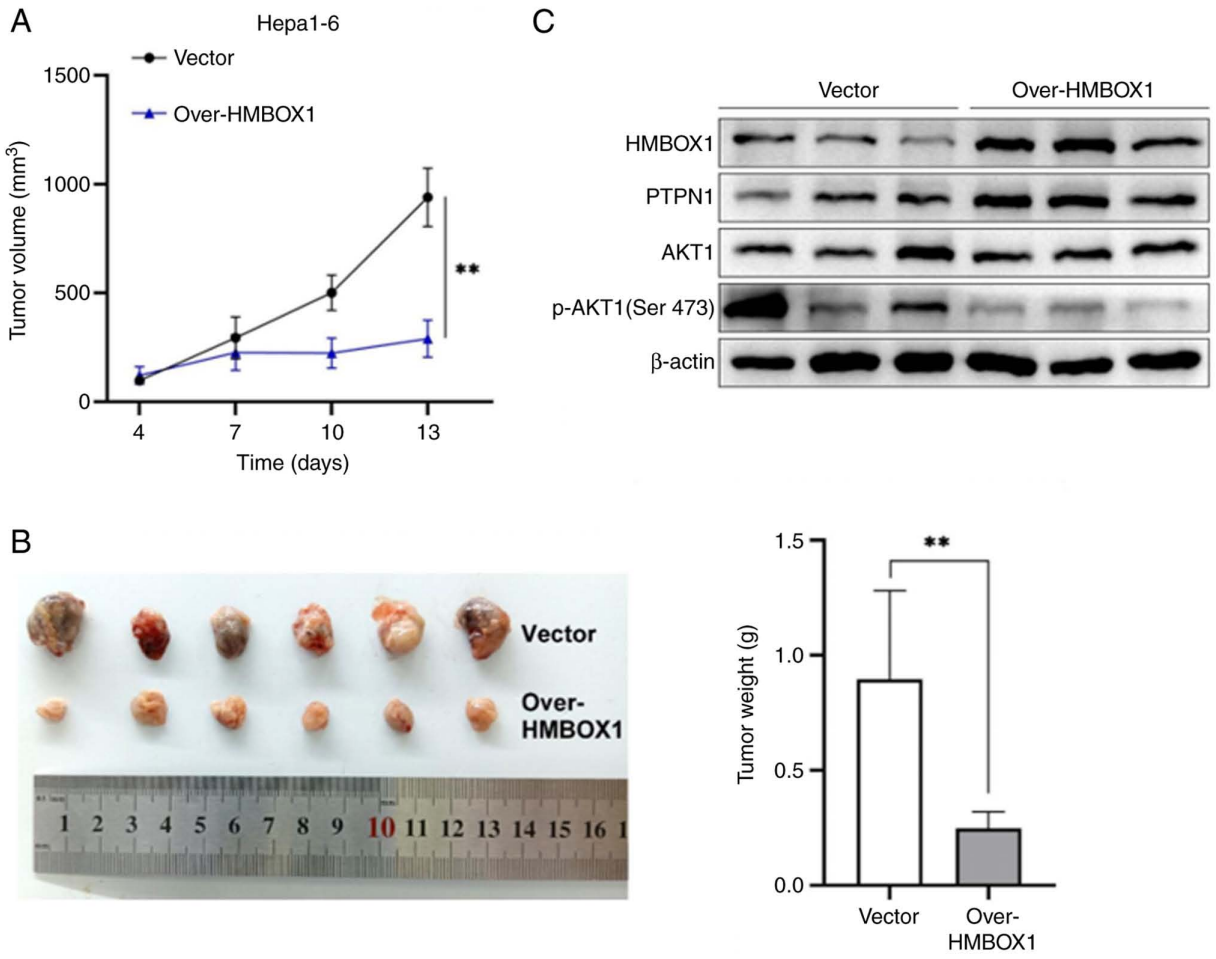

Figure 6. Overexpression of HMBOX1 can inhibit tumor growth in hepatocellular carcinoma bearing mice. A total of $5x10^6$ Hepa1-6 cells overexpressing HMBOX1 (Over-HMBOX1) or transfected with empty vector (Vector) were subcutaneously injected into the right subaxillary region of C57BL/6 mice (n=6). (A) Tumor growth curves were measured every three days. (B) Representative images and the tumor weight of the subcutaneous tumors from each group. (C) The expression levels of indicated molecules were determined through western blotting in different tumor tissues. **P<0.01. HMBOX1, homeobox containing 1; PTPN1, protein tyrosine phosphatase non-receptor type 1; p-, phosphorylated.

peptides quantified for 'AKT1' in the current proteomic experiment were derived from a region whose detectability was altered (for example, by a co-occurring post-translational modification not related to the phosphorylation studied, or by a sequence variant), leading to an apparent downregulation. The western blotting, assessing the intact protein, would be unaffected by such a localized change. However, the downregulation of p-AKT1 expression was detected in HMBOX1-overexpressing cells, suggesting that HMBOX1 inhibited the activation of the AKT1 pathway.

PTPN1, a key post-translational mechanism in the cellular processes of proliferation, migration, differentiation and apoptosis (32), has attracted attention for its abnormal expression in HCC tissues. Overexpression of HMBOX1 upregulated the expression of PTPN1, and the expression of PTPN1 in tumor tissues of patients with HCC was downregulated, which is consistent with and correlated with the changes in HMBOX1. In the present study, the rescue experiments were only verified by silencing PTPN1 with siRNA in HCC cells overexpressing HMBOX1; future gain-of-function studies will provide complementary validation. Moreover, there is a significant positive correlation between the expressions of HMBOX1 and PTPN1, that suggests that there might be a regulatory role between the two molecules. Further silencing of PTPN1 in HCC cells overexpressing HMBOX1 can reverse the inhibition of AKT1 phosphorylation by HMBOX1, which can to some extent explain the regulatory role among these three molecules. Whereas the precise temporal sequence and kinetics of these events remain to be fully elucidated by future time-course studies. In addition, whether other isoform of AKT (AKT2 and AKT3) are involved in signal regulation also remains to be further explored as the function of different isoforms of AKT in other tumor types is not redundant.

As a transcriptional suppressor, the positive regulation of HMBOX1 on PTPN1 expression may be indirect. Through the CHIP experiment, we also did not observe a direct interaction between HMBOX1 and PTPN1. This suggests that other molecules may play a regulatory role between HMBOX1 and PTPN1. The Co-IP experiment was used to pull down the proteins interacting with HMBOX1 and further identified 69 protein molecules interacting with HMBOX1 through mass spectrometry (Fig. S4D).

Using metascape database, GO enrichment analysis was conducted on PTPN1 and HMBOX1-interacting proteins. GO terms containing PTPN1 in the enrichment analysis results

were selected to draw the Upset plots. It was found that SOD2 repeatedly appears in 9 GO terms (not in GO:0080135, GO:0071732 and GO:1901699) (Fig. S4E). Further, by using the STRING database to construct the interaction network between SOD2 and PTPN1. It can be seen that SOD2 is directly associated with PTPN1 through JAK2, APP and GHR and the remaining nodes form a broader indirect interaction network (Fig. S4F). The proteomics identification results and bioinformatics analysis results also suggested that HMBOX1 may regulate the expression of PTPN1 through an indirect regulatory approach, but the specific regulatory mechanism still needs further exploration. The precise temporal sequence and kinetics of these events remain to be fully elucidated by future time-course studies.

The effect of PTPN1 on tumors is not consistent, and even in studies of the same tumor, the regulatory effect of PTPN1 is completely opposite in different research contexts (33,34). Lessard *et al* (35) reviewed the double-sided role of PTPN1 in tumors, demonstrating that PTPN1 has the potential to function as both a tumor suppressor and a tumor promoter, contingent upon the specific substrate and the surrounding cellular environment. Yuan *et al* (36) confirmed that in the highly metastatic HCC cell line MHCC97-H, OP-B could inhibit the activation of PI3K/AKT signaling by inhibiting the expression of PTPN1, subsequently playing a role in inhibiting HCC. This is inconsistent with the present findings that PTPN1 inhibits tumors by inhibiting AKT activation. This could be attributed to the utilization of various HCC cell lines. A previous study using liver cancer cell line HepG2 also confirmed that decreased PTPN1 protein expression can induce the activation of the PI3K/Akt pathway (29). These findings suggest that PTPN1 may play different regulatory roles in cells with different genetic and molecular characteristics, even within the same tumor. The fundamental function of a phosphatase is determined by its interacting partners and substrates in a specific cellular context. In the current HCC model, the expression and activity of PTPN1 are driven by the transcription factor HMBOX1. Although this driving effect is indirect, this unique upstream regulation might create a specific signaling complex. The outcome of PTPN1 activity is likely shaped by the specific signaling complex. Different binding partners in different cell types could recruit PTPN1 to distinct sets of substrates, leading to either activation or inhibition of oncogenic pathways. On the other hand, it is possible that PTPN1 participates in a negative feedback loop to dampen excessive AKT signaling, which is a common feature in cancers with hyperactive PI3K/AKT. In this scenario, in the specific context of the HCC model of the present study, the tumor-suppressive, AKT-dephosphorylating function of PTPN1 becomes dominant and unmasked. Whether PTPN1's regulatory role on tumors is related to the disease process remains to be further determined.

In the present study, the data suggested that HMBOX1 can inhibit HCC progression by upregulating the expression of downstream PTPN1 and inhibiting the phosphorylation at Ser473 of AKT1. These results confirmed the protective effect of HMBOX1 in HCC development, and providing a new potential target for the clinical diagnosis and treatment of HCC.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

HLZ conceptualized the study, reviewed and edited the manuscript. CNZ conducted data curation and investigation. CNZ, JHL, WYZ and JQ developed the methodology. YJ performed formal analysis. HLZ and YJ were acquired funding, conducted project administration and supervised the study. HLZ and QW validated data. YJ wrote the original draft of the manuscript. HLZ and CNZ confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Animal studies were approved by the Ethical Committee of Central Hospital Affiliated to Shandong First Medical University (approval no. W202403040279; Jinan, China). Animal experiments were performed in accordance with the ARRIVE guidelines. The use of HCC tissue chips and the patient information in the public database were approved by the Ethics Committee of Shandong First Medical University (approval no. R202403040155; Jinan, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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