

CPNE8 Suppresses Hepatocellular Carcinoma Progression Through Downregulation of ITGB1/CDC42/PAK1 Signaling and an Altered Immune-Marker Profile

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Background: Hepatocellular carcinoma (HCC) is among the most lethal malignancies, and its immunosuppressive microenvironment limits the efficacy of current therapies. The integrin $\beta 1$ (ITGB1)-CDC42-p21-activated kinase 1 (PAK1) axis is an established driver of HCC progression; however, the role of copine 8 (CPNE8), a calcium-dependent phospholipid-binding protein previously reported to have oncogenic effects in gastric cancer, has not been defined in HCC. The aim of this study was to investigate the expression, function, and mechanistic relevance of CPNE8 in HCC, as well as its association with the tumor immune microenvironment.

Methods: CPNE8 expression was assessed in the TCGA-LIHC cohort and HCC cell lines. Stable CPNE8-overexpression (Huh7) and -knockdown (Hep3B) cell lines were generated and evaluated using cell counting kit-8 (CCK-8) and Transwell migration assays. Colony-formation and Transwell invasion assays were additionally performed in the overexpression cell lines. ITGB1 and CDC42 protein levels and PAK1 phosphorylation (p-PAK1/total PAK1 ratio) were examined by Western blotting, ITGB1 mRNA expression was measured by quantitative real-time PCR (qRT-PCR), and an ITGB1 rescue experiment was performed. Cpne8-overexpressing Hepa1-6 cells were implanted subcutaneously into immunocompetent C57BL/6J mice, and immune markers were evaluated by immunohistochemistry.

Results: CPNE8 was consistently downregulated in HCC (TCGA-LIHC, 371 tumors vs. 50 adjacent normal tissues; Wilcoxon rank-sum test, $p < 0.0001$) and showed a weak inverse correlation with estimated regulatory T-cell infiltration (Spearman $r = -0.20$, $p = 1.03 \times 10^{-4}$). CPNE8 overexpression suppressed cell viability, colony formation, migration, and invasion (all $p < 0.001$), whereas knockdown increased cell viability and migration (both $p < 0.001$). CPNE8 overexpression reduced ITGB1 and CDC42 protein levels and the phospho-PAK1/total PAK1 ratio (all $p < 0.001$), while ITGB1 re-expression reversed these effects (all $p < 0.001$). *In vivo*, Cpne8 overexpression suppressed tumor growth, increased the CPNE8-positive area, and reduced the ITGB1-positive area and Ki67⁺ nuclear density (all $p < 0.001$). These changes were accompanied by higher densities of CD8⁺ and Granzyme B⁺ cells, lower FOXP3⁺ cell density, and a reduced PD-L1-positive area (all $p < 0.001$).

Conclusion: CPNE8 is downregulated in HCC and suppresses malignant phenotypes through reduced ITGB1 and CDC42 protein levels and decreased PAK1 phosphorylation. Its restoration is also associated with an altered immune-marker profile in a syngeneic model, although the present study design cannot distinguish a direct effect of CPNE8 on the tumor microenvironment from secondary changes resulting from reduced tumor burden. These findings identify CPNE8 as a candidate tumor suppressor in HCC and warrant further investigation.

Keywords: HCC; CPNE8; ITGB1; CDC42; tumor immune microenvironment; tumor suppressor

Introduction

Hepatocellular carcinoma (HCC), which accounts for approximately 75–85% of primary liver cancers, remains one of the most lethal human malignancies, with an estimated 684,659 new cases and 597,434 deaths attributed to the disease worldwide in 2022 alone [1,2]. Chronic hepatitis B or C virus infection underlies a substantial proportion of cases, although metabolic dysfunction- and alcohol-

associated liver disease are steadily reshaping its etiologic landscape [3,4,5]. Therapeutically, the introduction of multikinase inhibitors and, more recently, immune checkpoint-based regimens such as atezolizumab plus bevacizumab has improved clinical outcomes, yet advanced disease continues to carry a poor prognosis and durable responses remain uncommon [6,7,8]. For instance, objective response rates to single-agent checkpoint blockade in advanced HCC remain only approximately 16–20% [9,10]. These limitations

highlight the urgent need to elucidate the molecular mechanisms that drive HCC progression while simultaneously impairing anti-tumor immunity.

A central barrier to effective immunotherapy is the profoundly immunosuppressive tumor microenvironment (TME) of HCC [11,12,13]. Tumor cells and tumor-microenvironmental components frequently express PD-L1, which engages PD-1 on cytotoxic lymphocytes to induce functional impairment, exhaustion, and apoptosis [14,15]. In addition, FOXP3⁺ regulatory T cells (Tregs) accumulate within HCC tissue, where they suppress effector immune responses and promote immune escape [16,17]. Collectively, these processes establish an immunosuppressive milieu characterized by impaired CD8⁺ T-cell cytotoxicity, allowing tumor cells to evade immune surveillance [18,19]. Identifying tumor-intrinsic regulators that shape this immune landscape may therefore reveal therapeutic targets capable of simultaneously restraining tumor growth and restoring anti-tumor immunity.

Integrin β 1 (ITGB1), the most broadly expressed β -integrin subunit and a key component of major extracellular matrix receptors, is upregulated in HCC and promotes tumor-cell proliferation, migration, and invasion [20,21,22,23]. siRNA-mediated silencing of ITGB1 inhibits HCC cell growth and downregulates CDC42 expression, supporting the role of ITGB1 as an upstream regulator of a CDC42-dependent cell-cycle program [24]. CDC42, activated downstream of integrin signaling, regulates filopodia formation and directional cell motility and is associated with metastasis and poor outcomes in HCC [25,26]. Its downstream effector, p21-activated kinase 1 (PAK1), relays these signals to the actin cytoskeleton, reinforcing a migratory and invasive phenotype [27,28]. Beyond regulating cell motility, integrin signaling has increasingly been implicated in TME remodeling and immune evasion across multiple tumor types [29,30,31]. However, whether and how this axis is regulated in HCC remains largely unexplored.

Copines are calcium-dependent phospholipid-binding proteins that couple Ca²⁺ signals to membrane trafficking and a variety of downstream pathways [32]. Structurally, all Copine proteins contain two N-terminal C2 domains followed by a C-terminal von Willebrand factor type A (VWA), or integrin A-like, domain [32], suggesting an intrinsic connection to integrin-related biology. Copine 8 (CPNE8) remains a relatively understudied member of the Copine family [32]. The most comprehensive cancer-related evidence to date comes from gastric cancer, where CPNE8 is overexpressed, promotes metastasis through the focal-adhesion pathway, predicts poor survival, and is associated with a computationally predicted reduced benefit from immune-checkpoint blockade [33]. However, because copine family members may function as oncogenes in one tissue context but as tumor suppressors in another, these findings cannot be directly extrapolated to other ma-

lignancies. In HCC, the expression pattern and function of CPNE8 have, to our knowledge, not yet been reported.

We therefore set out to define the role of CPNE8 in HCC. By integrating analyses of the TCGA-LIHC cohort and cell-line models, we found that CPNE8 was consistently downregulated in HCC and weakly inversely associated with computationally estimated Treg infiltration. Gain- and loss-of-function experiments, together with ITGB1 rescue studies, revealed that CPNE8 reduces HCC cell viability, colony formation, migration, and invasion in association with reduced ITGB1 and CDC42 levels and decreased PAK1 phosphorylation. In an immunocompetent syngeneic tumor model, Cpne8 restoration constrained tumor growth, and the resulting smaller tumors exhibited lower ITGB1 expression and altered densities of immune marker-positive cells. Collectively, these findings identify CPNE8 as a candidate tumor suppressor in HCC while leaving unresolved whether its association with the immune microenvironment reflects a direct regulatory effect or is secondary to reduced tumor burden.

Materials and Methods

Bioinformatics Analysis of TCGA-LIHC Data

RNA-seq raw count data and corresponding clinical information from the TCGA-LIHC cohort (371 HCC tumor samples and 50 adjacent normal liver samples) were obtained from the Genomic Data Commons (GDC) portal using the R package TCGAbiolinks (v2.30.0, Bioconductor, <https://bioconductor.org>). Counts were variance-stabilizing transformed (VST) using DESeq2 (v1.42.0, Bioconductor) for visualization and downstream correlation analysis. Differential expression of CPNE8 between tumor and normal tissues was assessed using the Wilcoxon rank-sum test. Immune cell infiltration scores were estimated from TPM-normalized expression data using the CIBERSORT algorithm implemented in the IOBR R package (v0.99.9, Bioconductor) with 100 permutations. The Spearman correlation between CPNE8 expression and regulatory T cell (Treg) infiltration was calculated using the cor.test function. All analyses were performed using R version 4.5.2 (R Foundation for Statistical Computing, Vienna, Austria), and figures were generated with ggplot2 (v3.4.4, Bioconductor) and ggpubr (v0.6.0, Bioconductor).

Cell Lines and Culture Conditions

Four cell lines were used in this study: THLE-2 (normal human hepatocytes; CRL-2706, American Type Culture Collection, Manassas, VA, USA), human HCC cell lines Huh7 (SCSP-526) and Hep3B (SCSP-5045), and murine HCC cell line Hepa1-6 (SCSP-512). The three HCC cell lines were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). HCC cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; 11965092, Gibco, Thermo Fisher Scien-

tific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; 10099141, Gibco) and 1% penicillin-streptomycin (15140122, Gibco). THLE-2 cells were maintained in RPMI-1640 medium (11875093, Gibco) supplemented with 10% FBS and 1% penicillin-streptomycin. All cells were cultured at 37 °C in a humidified atmosphere containing 5% CO₂. Cell line identity was verified by short tandem repeat (STR) profiling, and mycoplasma testing was performed at regular intervals.

Lentiviral Transduction and Stable Cell Line Generation

Lentiviral vectors encoding human CPNE8 (pLV-CPNE8-OE, NM_153634.3), murine Cpne8 (pLV-Cpne8-OE, NM_025815.2), human ITGB1 (pLV-ITGB1-OE), shRNA targeting human CPNE8 (sh-CPNE8), and the corresponding empty vector or scrambled non-targeting control (sh-NC) were synthesized. The target sequence of sh-CPNE8 was 5'-AAGTAATTGATAATACTTTAAAT-3', and that of sh-NC was 5'-CAACAAGATGAAGAGCACCAA-3'. The functional titers of the lentiviral preparations were 1×10^8 transducing units (TU)/mL. Huh7 cells (for CPNE8 overexpression), Hep3B cells (for CPNE8 knockdown), and Hepa1-6 cells (for Cpne8 overexpression) were seeded at 2×10^5 cells/well in six-well plates and transduced at approximately 50–60% confluence. Lentiviral transduction was performed at a multiplicity of infection (MOI) of 20. Control cells were transduced with the corresponding control lentivirus at the same MOI. Polybrene (H8761, Solarbio, Beijing, China) was added at a final concentration of 8 µg/mL. After 24 h, the virus-containing medium was replaced with fresh complete medium. At 48 h post-transduction, cells were selected with 2 µg/mL puromycin (A1113803, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) for 7 days. Stable cell populations were maintained as pooled cultures, and overexpression or knockdown efficiency was confirmed by quantitative real-time PCR (qRT-PCR) and/or Western blotting before subsequent experiments.

RNA Extraction and qRT-PCR

Total RNA was extracted using a total RNA extraction reagent (DP424, TIANGEN, Beijing, China) according to the manufacturer's instructions. cDNA was synthesized from 1 µg of total RNA (G3337, Servicebio, Wuhan, China). qRT-PCR was performed using SYBR Green qPCR Master Mix (FP205, TIANGEN, Beijing, China) on an LightCycler96 system (Roche, Mannheim, Germany). Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequences used were as follows: human CPNE8: F 5'-CGCCTCCTCCCAATATGGAC-3', R 5'-TCCATCTCGGTCCCAGTCAT-3'; human

ITGB1: F 5'-CGCCGCGCGGAAAAGATG-3', R 5'-AAACACCAGCAGCCGTGTAA-3'; human GAPDH: F 5'-GTGGATATTGTTGCCATCAATGACC-3', R 5'-GCCCCAGCCTTCTTCATGGTGGT-3'; murine Cpne8: F 5'-ACAAGTGCTCCGTTCCCTCAG-3', R 5'-CAGGACACTGACACCTCCAC-3'; murine Gapdh: F 5'-AGGTCGGTGTGAACGGATTG-3', R 5'-TG TAGACCATGTAGTTGAGGTCA-3'. All primer pairs were validated by NCBI Primer-BLAST against the corresponding RefSeq transcripts (human CPNE8, NM_153634.3; human ITGB1, NM_002211.4; human GAPDH, NM_002046.7; murine Cpne8, NM_025815.2; murine Gapdh, NM_008084.4). Primer specificity was confirmed by single-peak melting-curve analysis and the presence of a single product of the expected size on agarose gel electrophoresis. Each sample was analyzed in triplicate, and five independent biological replicates were performed.

Western Blot

Cells were lysed in RIPA buffer (P0013J, Beyotime, Shanghai, China) supplemented with protease and phosphatase inhibitor cocktails (ST506, Beyotime, Shanghai, China) on ice for 30 min. Lysates were centrifuged at 12,000 ×g for 15 min at 4 °C, and protein concentrations were determined using a BCA Protein Assay Kit (P0012S, Beyotime, Shanghai, China). Equal amounts of protein (20–30 µg) were separated by 10% SDS-PAGE (P0015, Beyotime, Shanghai, China) and transferred onto polyvinylidene fluoride (PVDF) membranes (IPVH00010, Millipore, Burlington, MA, USA). Membranes were blocked with 5% non-fat milk in TBST for 1 h at room temperature and incubated with primary antibodies overnight at 4 °C. The primary antibodies used were anti-CPNE8 (human: ER63095, HUABIO, Woburn, MA, USA, 1:1000; mouse: CSB-PA007346, CUSABIO, Wuhan, China, 1:2000), anti-ITGB1 (12594-1-AP, Proteintech, Rosemont, IL, USA, 1:1000), anti-CDC42 (ET1701-7, HUABIO, Woburn, MA, USA, 1:1000), anti-phospho-PAK1 (85044-1-RR, Proteintech, Rosemont, IL, USA, 1:1000), anti-PAK1 (21401-1-AP, Proteintech, Rosemont, IL, USA, 1:1000), and anti-GAPDH (60004-1-Ig, Proteintech, Rosemont, IL, USA, 1:5000). After washing with TBST, membranes were incubated with HRP-conjugated secondary antibodies (ZB-2301/ZB-2305, ZS Golden Bridge, Beijing, China, 1:2000) for 1 h at room temperature. Protein bands were visualized using enhanced chemiluminescence reagent (WBKLS0500, Millipore, Burlington, MA, USA). p-PAK1, total PAK1 and GAPDH were detected on the same membrane by stripping and reprobing. Densitometric analysis was performed using ImageJ software (version 1.54b, National Institutes of Health (NIH), Bethesda, MD, USA). Band intensities were first normalized to GAPDH levels from the same membrane. For total (non-phosphorylated) proteins (CPNE8,

ITGB1, CDC42 and total PAK1), the GAPDH-normalized values were used directly. For the phosphorylated protein, the GAPDH-normalized p-PAK1 value was divided by the GAPDH-normalized total PAK1 value from the same sample. Because the GAPDH normalization term cancels during this calculation, the resulting value is equivalent to the p-PAK1/total PAK1 ratio and was reported accordingly. Total PAK1 was also analyzed independently as a GAPDH-normalized value to determine whether changes in the p-PAK1/total PAK1 ratio reflected altered phosphorylation rather than changes in total PAK1 abundance. All values were subsequently expressed relative to the mean value of the corresponding control group. Five independent biological replicates were performed.

Cell Counting Kit-8 (CCK-8) Cell Viability Assay

Cells were seeded into 96-well plates at a density of 3000 cells per well in 100 μ L of complete medium and allowed to adhere overnight. On day 5 after seeding, 10 μ L of CCK-8 reagent (MA0218-5, Meilunbio, Dalian, China) was added to each well, and plates were incubated at 37 $^{\circ}$ C for 2 h. Absorbance was measured at 450 nm using a microplate reader (Model 680, Bio-Rad Laboratories, Hercules, CA, USA). The mean absorbance of blank wells containing medium and CCK-8 reagent without cells (approximately 0.15) was subtracted from all readings before analysis. Each group was assessed in six replicate wells, and five independent experiments were performed.

Colony Formation Assay

Huh7 vector control (OE-NC) and OE-CPNE8 cells were seeded into 6-well plates at a density of 500 cells per well and cultured for 14 days, with the medium replaced every 3 days. Colonies were fixed with 4% paraformaldehyde (P1110, Solarbio, Beijing, China) for 15 min, stained with 0.1% crystal violet (G1064, Solarbio, Beijing, China) for 20 min, and gently washed with PBS. Colonies containing ≥ 50 cells were counted under an inverted microscope (IX73, Olympus, Tokyo, Japan). Each group was analyzed in triplicate wells, and five independent experiments were performed.

Transwell Migration and Invasion Assays

For migration assays, 5×10^4 cells suspended in 200 μ L of serum-free medium were seeded into the upper chambers of Transwell inserts (TCS-003-024, BIOFIL, Guangzhou, China). For invasion assays, the upper surface of the inserts was pre-coated with 50 μ L of Matrigel (1:8 dilution in serum-free medium, 356231, BD Biosciences, Franklin Lakes, NJ, USA) and incubated at 37 $^{\circ}$ C for 30 min to allow polymerization before cell seeding. The lower chambers were filled with 600 μ L of complete medium containing 10% FBS as a chemoattractant. After incubation at 37 $^{\circ}$ C for 24 h (migration) or 48 h (invasion), non-migrated or non-invaded cells on the upper surface were gently re-

moved using a cotton swab. Cells that migrated or invaded to the lower surface were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, and imaged under an inverted microscope (IX73, Olympus, Tokyo, Japan) at $\times 200$ magnification. Cells were counted in five randomly selected, non-overlapping fields per insert. Migration assays were performed in both the Huh7 overexpression and Hep3B knockdown cell lines, whereas invasion assays were performed only in the Huh7 overexpression cell line. Each group was evaluated using triplicate inserts, and five independent experiments were performed.

Rescue Experiments

For ITGB1 rescue experiments, Huh7 cells were co-transduced with CPNE8-OE or control empty vector (OE-NC) lentivirus together with ITGB1-OE or control empty vector (OE-NC) lentivirus, generating four groups including G1 (OE-NC + OE-NC), G2 (OE-CPNE8 + OE-NC), G3 (OE-NC + OE-ITGB1), and G4 (OE-CPNE8 + OE-ITGB1). Following puromycin selection, the four groups were subjected to Western blotting, CCK-8 assays (day 5), and Transwell migration assays as described above.

Subcutaneous Syngeneic Tumor Model in C57BL/6J Mice

A total of 16 six-week-old female C57BL/6J mice (body weight 18–20 g) were purchased from Beijing Vital River Laboratory Animal Technology and housed under specific pathogen-free (SPF) conditions at 22 ± 2 $^{\circ}$ C and $50 \pm 10\%$ relative humidity with a 12 h/12 h light/dark cycle. Mice had free access to food and water and were acclimatized for 7 days before the experiment. The sample size ($n = 8$ per group) was determined based on previous subcutaneous syngeneic tumor studies using this model and represented the minimum number considered sufficient to detect the expected difference in tumor volume while adhering to the principles of the 3Rs. Mice were assigned to two groups by simple randomization using a random number table, and cages were placed in a randomized order on the rack. Tumor measurements and all subsequent immunohistochemical quantification were performed by investigators blinded to group allocation. No animals were excluded from the analysis, and all 16 mice were included in the final dataset. Because Hepa1-6 cells are of murine origin, tumors generated in immunocompetent mice are referred to throughout this study as syngeneic (allograft) tumors rather than xenografts. All animal procedures were reviewed and approved by the Laboratory Animal Ethics Committee of Jiangsu Vocational College of Medicine (approval no. SYLL-2025-004) and were conducted in accordance with the institutional guidelines and applicable national regulations for the care and use of laboratory animals. The study is reported in accordance with the ARRIVE 2.0 guidelines.

Hepal-6 is a murine HCC cell line derived from the BW7756 hepatoma that originated in a C57L mouse. Because C57L and C57BL/6 mice share the H-2b haplotype, Hepal-6 cells are histocompatible with C57BL/6 recipients at the major histocompatibility complex level. This cell line is widely used as a syngeneic HCC model in C57BL/6 mice, where it forms progressively growing tumors that are responsive to immune-checkpoint blockade. C57BL/6J was chosen here because it is the recipient strain in which this model has been most extensively characterized, because the immune reagents required for the present analyses (CD8, Granzyme B, FOXP3 and PD-L1) are well validated in this background, and because the immunocompetent setting was essential for the aim of assessing the tumor immune microenvironment.

Stable Hepal-6 vector (OE-NC) or Hepal-6 Cpne8-OE cells were harvested during the logarithmic growth phase, resuspended in serum-free DMEM, and 1×10^6 cells in 100 μ L were subcutaneously inoculated into the right flank of each mouse. Mice were randomly assigned to two groups ($n = 8$ per group), including the OE-NC and OE-Cpne8 groups. Tumor volume was measured every 7 days using digital calipers (Mitutoyo, Kawasaki, Japan) and calculated as $V = 0.5 \times L \times W^2$, where L represents the longest diameter and W represents the perpendicular shortest diameter. On day 28 post-inoculation, all mice were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight; P3761, Sigma-Aldrich, St. Louis, MO, USA). After the absence of the pedal-withdrawal reflex was confirmed, mice were euthanized by cervical dislocation. Tumors were excised, weighed using an electronic balance, photographed, fixed in 4% paraformaldehyde, and embedded in paraffin for subsequent immunohistochemical analysis.

Immunohistochemistry (IHC)

Formalin-fixed paraffin-embedded sections (4 μ m) were deparaffinized in xylene and rehydrated through a graded ethanol series. Antigen retrieval was performed by heating sections in 10 mM sodium citrate buffer (pH 6.0; G1202, Servicebio, Wuhan, China) using a pressure cooker for 3 min. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 10 min, followed by blocking with 5% goat serum (G1208, Servicebio, Wuhan, China) for 30 min at room temperature. Sections were incubated with primary antibodies overnight at 4 °C. The primary antibodies used were Anti-CPNE8 (20097-1-AP, Proteintech, Rosemont, IL, USA, 1:200), Anti-ITGB1 (GB115173-100, Servicebio, Wuhan, China, 1:200), Anti-Ki67 (HA721115, HUABIO, Woburn, MA, USA, 1:300), Anti-CD8 (GB15068-100, Servicebio, Wuhan, China, 1:200), Anti-Granzyme B (HA751527, HUABIO, Woburn, MA, USA, 1:100), Anti-FOXP3 (GB150152-100, Servicebio, Wuhan, China, 1:100), and Anti-PD-L1 (A27937, Abclonal Technology, Wuhan, China, 1:200). Sections were

then incubated with HRP-conjugated secondary antibody (GB23303, Servicebio, Wuhan, China, 1:500) for 1 h at room temperature, developed with 3,3'-diaminobenzidine (DAB) substrate (G1212, Servicebio, Wuhan, China), and counterstained with hematoxylin (G1004, Servicebio, Wuhan, China).

All IHC staining was quantified using ImageJ (version 1.54b, NIH, Bethesda, MD, USA). For each marker, five randomly selected, non-overlapping, non-necrotic fields per tumor section were photographed at $\times 200$ magnification under identical illumination and exposure settings. Images were processed by color deconvolution using the H DAB vector to separate DAB and hematoxylin channels, and a single intensity threshold, determined from a subset of images and then kept constant, was applied to all images of the same marker.

The quantification method was selected according to the staining pattern of each marker. CD8, Granzyme B, FOXP3 and Ki67 label discrete cells or nuclei. For these markers, thresholded objects were separated by watershed segmentation and counted using the Analyze Particles function with marker-specific size and circularity filters (CD8 and Granzyme B, 15–150 μm^2 , circularity 0.30–1.00; FOXP3, 10–80 μm^2 , circularity 0.50–1.00; Ki67, 20–200 μm^2 , circularity 0.50–1.00). Objects touching the field border were excluded, and results were expressed as the number of positive cells or nuclei per mm^2 of imaged field. CPNE8, ITGB1 and PD-L1 exhibit diffuse cytoplasmic or membranous staining rather than discrete positive cells. Therefore, cell counting is not appropriate for these markers, and the proportion of DAB-positive area was quantified instead and expressed as the percentage of positive area per field.

For each marker, values obtained from the five fields of a tumor were averaged to generate a single value per mouse, resulting in a statistical sample size of $n = 8$ per group. All quantifications were performed independently by two observers blinded to group assignment, and the mean value of the two observers was used for analysis.

Statistical Analysis

Unless otherwise stated, all *in vitro* experiments were performed in five independent experiments. Technical replicates were averaged to generate one value for each independent experiment, and only these experiment-level values were used for statistical analysis. Therefore, the statistical sample size for *in vitro* experiments was $n = 5$, rather than the total number of wells, inserts, or microscopic fields. For qRT-PCR, the three technical reactions for each sample were averaged to obtain one value per independent experiment. For CCK-8 and colony-formation assays, replicate wells were averaged within each experiment. For Transwell assays, cell counts from the five fields of each insert were first averaged, followed by averaging across replicate inserts to obtain one value per independent

experiment. For Western blotting, each independently prepared cell lysate was considered one experimental replicate. In animal experiments, each mouse was considered one independent biological replicate ($n = 8$ per group). For IHC analysis, the five microscopic fields were averaged to generate one value for each tumor.

Statistical analyses were performed using GraphPad Prism (version 9.0, GraphPad Software, San Diego, CA, USA). The Wilcoxon rank-sum test was used to compare two independent groups in the TCGA-LIHC dataset, whereas Student's two-tailed t -test was used for comparisons between two groups in cell and animal experiments. One-way ANOVA followed by Tukey's post-hoc test was used for comparisons among more than two groups. Tumor growth curves were analyzed using two-way repeated-measures ANOVA, with group (OE-NC vs. OE-Cpne8) as the between-subjects factor and time as the within-subjects (repeated) factor, because tumor volume was measured repeatedly in the same animals. The Geisser-Greenhouse correction was applied because the assumption of sphericity was not made, and Šídák's multiple-comparisons test was used to compare the two groups at each time point. Endpoint tumor volume and tumor weight on day 28 were compared between groups using Student's two-tailed t -test. Spearman correlation analysis was performed to assess the association between CPNE8 expression and immune-cell infiltration in the TCGA-LIHC dataset. p values less than 0.05 were considered statistically significant.

Results

CPNE8 Is Downregulated in HCC and Shows a Weak Inverse Correlation With Estimated Regulatory T Cell Infiltration

To explore the clinical relevance of CPNE8 in HCC, we first analyzed its expression in the TCGA-LIHC cohort. CPNE8 mRNA levels were significantly lower in HCC tumor tissues ($n = 371$) than in adjacent normal liver tissues ($n = 50$; Wilcoxon rank-sum test, $p < 0.0001$; Fig. 1A), suggesting a potential tumor-suppressive role for CPNE8 in HCC.

Given the well-established involvement of integrin-mediated signaling in shaping the immunosuppressive tumor microenvironment of solid tumors, we further examined the relationship between CPNE8 expression and tumor-infiltrating immune cells estimated by CIBERSORT. CPNE8 expression showed a weak inverse correlation with the CIBERSORT-estimated proportion of regulatory T-cell (Treg) infiltration (Spearman $r = -0.20$, $p = 1.03 \times 10^{-4}$; Fig. 1B). Given the large sample size of the cohort ($n = 371$), this small p value reflects statistical significance rather than biological magnitude. The correlation explained approximately 4% of the variance in the estimated Treg proportion, and the association was therefore weak. We regard this observation as hypothesis-generating only and not as evidence that CPNE8 regulates Treg infiltration.

We further examined CPNE8 expression across HCC cell lines. Both qRT-PCR (Fig. 1C) and Western blotting (Fig. 1D) showed that CPNE8 levels were markedly lower in the HCC cell lines Huh7 and Hep3B than in the normal hepatocyte cell line THLE-2. Huh7 cells were selected for CPNE8 overexpression and Hep3B cells for CPNE8 knockdown in subsequent functional experiments.

Establishment of CPNE8 Overexpression and Knockdown Stable Cell Lines

To investigate the functional role of CPNE8 in HCC, we generated stable cell lines by lentivirus-mediated transduction. In Huh7 cells, ectopic CPNE8 expression (OE-CPNE8) significantly increased CPNE8 mRNA ($p < 0.001$, Fig. 2A) and protein levels ($p < 0.001$, Fig. 2B) relative to empty-vector (OE-NC) controls. In Hep3B cells, sh-CPNE8 significantly reduced CPNE8 mRNA ($p < 0.001$, Fig. 2C) and protein levels ($p < 0.001$, Fig. 2D) relative to sh-NC controls. These stable lines were used for the subsequent functional and mechanistic studies.

CPNE8 Reduces HCC Cell Viability, Colony Formation, Migration, and Invasion In Vitro

We next investigated the impact of CPNE8 on HCC malignant phenotypes *in vitro*. CCK-8 assays performed on day 5 revealed that CPNE8 overexpression significantly reduced Huh7 cell viability ($p < 0.001$, Fig. 3A), whereas CPNE8 knockdown significantly increased Hep3B cell viability ($p < 0.001$, Fig. 3B). Colony formation assays further demonstrated that CPNE8 overexpression significantly reduced the colony-forming capacity of Huh7 cells ($p < 0.001$, Fig. 3C). Colony-formation and Matrigel invasion assays were performed only in the overexpression setting. Transwell migration assays showed that CPNE8 overexpression significantly reduced Huh7 cell migration ($p < 0.001$, Fig. 3D), whereas CPNE8 knockdown significantly increased Hep3B cell migration ($p < 0.001$, Fig. 3E). In Matrigel-coated invasion assays, CPNE8 overexpression significantly reduced Huh7 cell invasion ($p < 0.001$, Fig. 3F). Collectively, these findings demonstrate that CPNE8 reduces HCC cell viability and migration in both gain- and loss-of-function settings, while additionally inhibiting colony formation and invasion in the overexpression setting.

CPNE8 Reduces ITGB1 and CDC42 Protein Levels and PAK1 Phosphorylation

To explore the molecular mechanism underlying CPNE8-mediated suppression of HCC malignant phenotypes, we examined key components of the ITGB1-CDC42 axis, an established driver of HCC progression, by measuring total ITGB1 and CDC42 protein levels and the phosphorylation status of their downstream effector PAK1. Western blot analysis revealed that CPNE8 overexpression in Huh7 cells significantly reduced the GAPDH-normalized protein

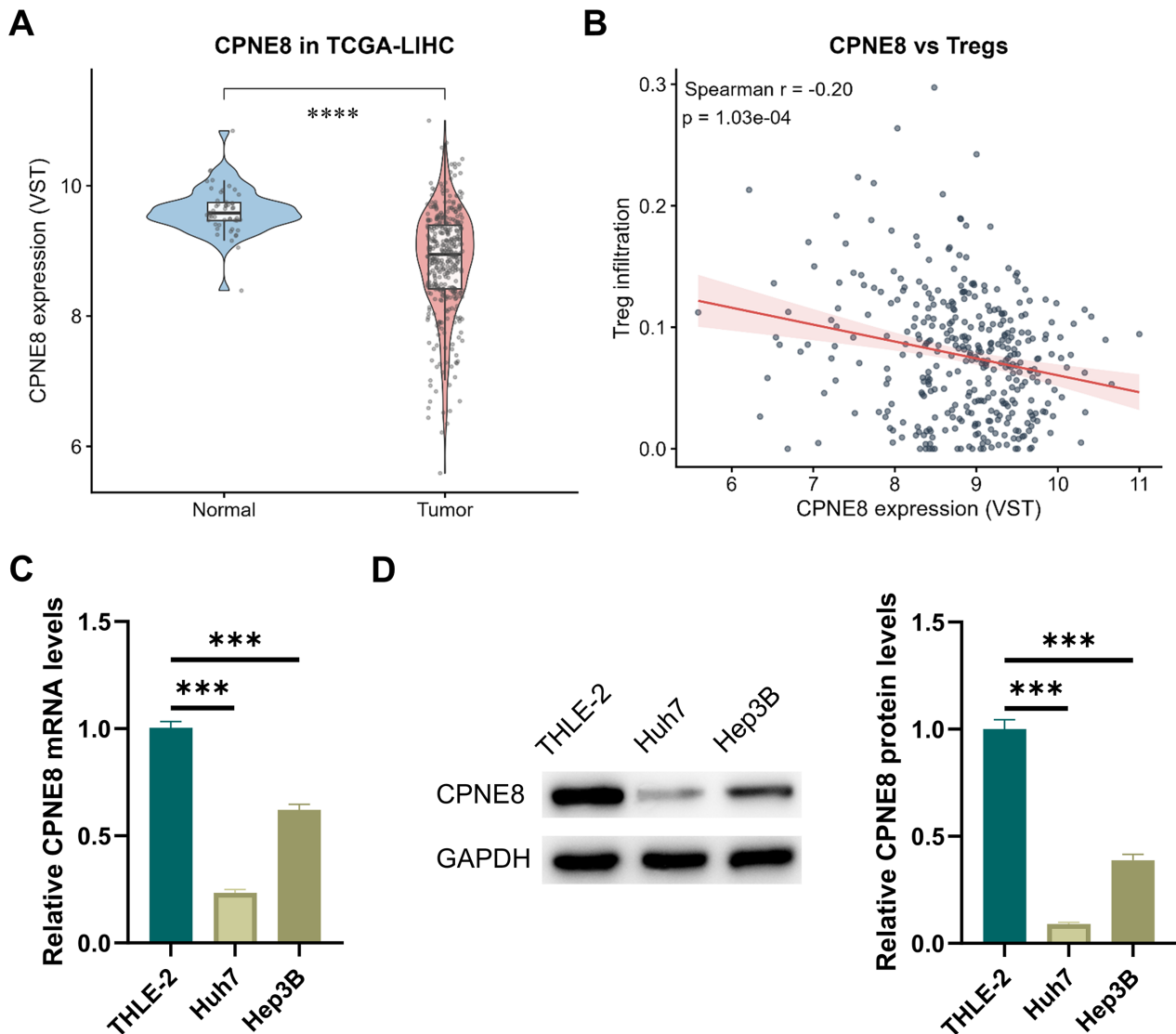


Fig. 1. CPNE8 is downregulated in HCC at both the mRNA and protein levels. (A) Violin and box plot of CPNE8 mRNA expression (variance-stabilizing-transformed) in TCGA-LIHC tumor ($n = 371$) versus adjacent normal liver tissues ($n = 50$). (B) Spearman correlation between CPNE8 expression and CIBERSORT-estimated regulatory T-cell (Treg) infiltration in TCGA-LIHC tumor samples. The regression line and 95% confidence band are shown. (C) Relative CPNE8 mRNA levels in THLE-2, Huh7, and Hep3B cells measured by qRT-PCR. (D) Representative Western blot and densitometric quantification of CPNE8 protein in the same cell lines (normalized to GAPDH). For (A) and (B), data are from the TCGA-LIHC cohort (371 tumor and 50 adjacent normal samples). For (C) and (D), data are presented as mean $\pm$ SD from five independent experiments. *** $p < 0.001$, **** $p < 0.0001$. CPNE8, copine 8; HCC, hepatocellular carcinoma; TCGA-LIHC, The Cancer Genome Atlas Liver Hepatocellular Carcinoma; CIBERSORT, Cell-type Identification By Estimating Relative Subsets Of RNA Transcripts; qRT-PCR, quantitative real-time PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SD, standard deviation.

levels of ITGB1 and CDC42, as well as the p-PAK1/total PAK1 ratio (all $p < 0.001$), whereas GAPDH-normalized total PAK1 levels did not differ significantly between the two groups ($p > 0.05$, Fig. 4A,B). Consistent with these protein-level changes, qRT-PCR showed that ITGB1 mRNA expression was significantly decreased upon CPNE8 overexpression ($p < 0.001$, Fig. 4C), indicating that CPNE8 represses ITGB1 at least partly at the transcriptional level.

Conversely, in Hep3B cells, CPNE8 knockdown significantly increased GAPDH-normalized levels of ITGB1, CDC42, as well as the p-PAK1/total PAK1 ratio (all $p < 0.001$), whereas GAPDH-normalized total PAK1 levels remained unchanged ($p > 0.05$, Fig. 4D,E). These changes were accompanied by a corresponding increase in ITGB1 mRNA expression ($p < 0.001$, Fig. 4F). Together, these findings indicate that CPNE8 reduces ITGB1 and CDC42

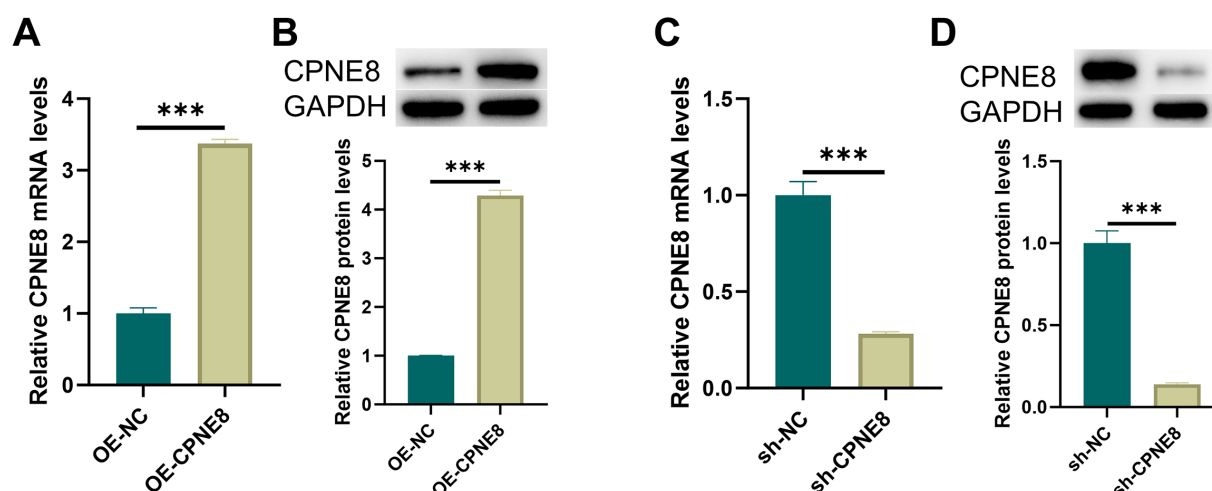


Fig. 2. Generation and validation of CPNE8-overexpression and -knockdown stable cell lines. (A,B) Validation of CPNE8 overexpression in Huh7 cells transduced with empty vector (OE-NC) or CPNE8-overexpression (OE-CPNE8) lentivirus, by qRT-PCR (A) and Western blotting (B) (normalized to GAPDH). (C,D) Validation of CPNE8 knockdown in Hep3B cells transduced with sh-NC or sh-CPNE8 lentivirus, by qRT-PCR (C) and Western blotting (D) (normalized to GAPDH). Data are presented as mean $\pm$ SD from five independent experiments. *** $p < 0.001$. OE-NC, overexpression negative control.

protein levels and decreases PAK1 phosphorylation in HCC cells. Because PAK1 is a canonical effector recruited and activated by GTP-bound CDC42, the reduction in the p-PAK1/total PAK1 ratio is consistent with, but does not directly demonstrate, reduced CDC42 activity. We did not measure GTP-loaded CDC42.

ITGB1 Overexpression Rescues the CPNE8-Mediated Suppression of HCC Phenotypes

To determine whether the tumor-suppressive activity of CPNE8 is functionally dependent on ITGB1, we performed a rescue experiment by co-transducing Huh7 cells with CPNE8- and ITGB1-overexpression constructs, generating four groups: G1 (OE-NC + OE-NC), G2 (OE-CPNE8 + OE-NC), G3 (OE-NC + OE-ITGB1), and G4 (OE-CPNE8 + OE-ITGB1). Western blot analysis confirmed successful manipulation of CPNE8 and ITGB1 expression in the respective groups. Ectopic ITGB1 expression in CPNE8-OE cells (G4) reversed the CPNE8-induced reduction in GAPDH-normalized CDC42 levels and of the p-PAK1/total PAK1 ratio (G4 vs G2, $p < 0.001$, Fig. 5A). GAPDH-normalized total PAK1 levels did not differ significantly among the four groups ($p > 0.05$, Fig. 5A). This restoration of CDC42 following ITGB1 re-expression is consistent with the established role of ITGB1 as an upstream regulator of CDC42 in HCC. ITGB1 overexpression also reversed the CPNE8-induced suppression of HCC cell migration (Transwell, Fig. 5B,C) and viability (CCK-8, Fig. 5D). Compared with G2 (CPNE8-OE), the G4 group (CPNE8-OE + ITGB1-OE) showed significantly restored migration and viability ($p < 0.001$). Together, these results indicate that ITGB1 re-expression is sufficient to reverse the CPNE8-induced phenotypes in HCC cells.

Cpne8 Overexpression Suppresses HCC Growth in Immunocompetent Mice

To test whether the tumor-suppressive effect of CPNE8 is reproducible in a genetic and species background independent of the human cell lines used above, we established stable murine HCC (Hepa1-6) cell lines and subcutaneously inoculated Hepa1-6 cells transduced with empty vector (OE-NC) or OE-Cpne8 into immunocompetent C57BL/6J mice. Successful Cpne8 overexpression in Hepa1-6 cells was confirmed by qRT-PCR ($p < 0.001$, Fig. 6A) and Western blotting ($p < 0.001$, Fig. 6B).

Tumor measurements showed that Cpne8 overexpression significantly suppressed tumor growth ($p < 0.001$, Fig. 6C,D). At the experimental endpoint (day 28), both tumor volume and tumor weight were significantly reduced in the OE-Cpne8 group compared with OE-NC controls ($p < 0.001$, Fig. 6D).

Immunohistochemistry of tumor tissues confirmed markedly increased CPNE8-positive area in the OE-Cpne8 group ($p < 0.001$; Fig. 6E), validating successful *in vivo* overexpression. Consistent with our *in vitro* mechanistic findings, the ITGB1-positive area was significantly reduced in the OE-Cpne8 group ($p < 0.001$, Fig. 6F), indicating that the CPNE8-ITGB1 relationship observed *in vitro* is also detectable *in vivo*. The density of Ki67-positive nuclei was likewise significantly lower in the OE-Cpne8 group ($p < 0.001$, Fig. 6G), in agreement with the reduced cell viability and colony formation observed *in vitro*.

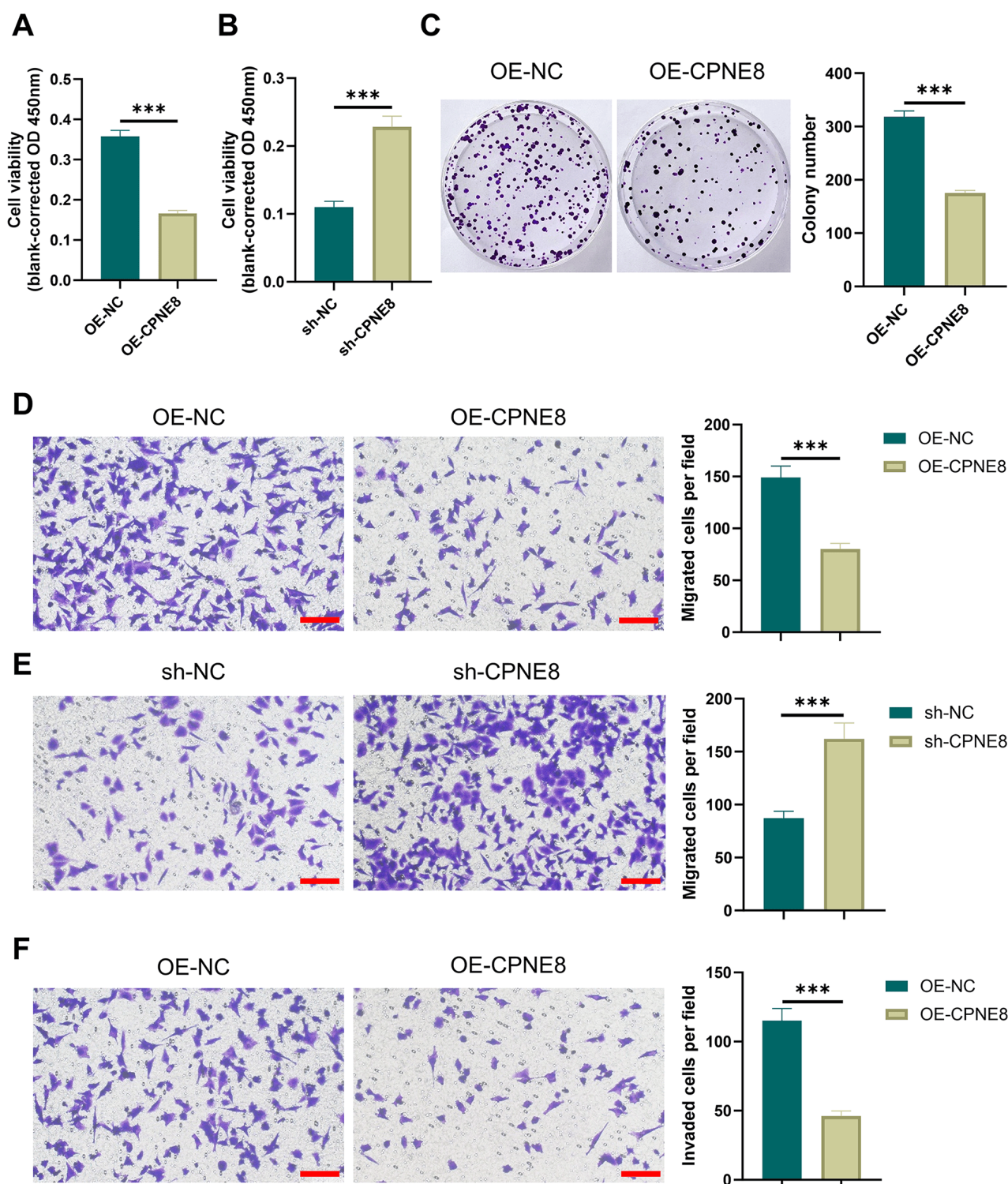


Fig. 3. CPNE8 reduces the viability, colony formation, migration, and invasion of HCC cells *in vitro*. (A) Viability of Huh7 cells transduced with empty vector (OE-NC) or CPNE8-OE, measured by CCK-8 assay. (B) Viability of Hep3B cells transduced with sh-NC or sh-CPNE8, measured by CCK-8 assay. (C) Representative images and quantification of colony formation by Huh7 OE-NC and CPNE8-OE cells. (D) Representative images and quantification of Transwell migration in Huh7 OE-NC and CPNE8-OE cells (scale bar: 100 μ m). (E) Representative images and quantification of Transwell migration in Hep3B sh-NC and sh-CPNE8 cells (scale bar: 100 μ m). (F) Representative images and quantification of Transwell invasion in Huh7 OE-NC and CPNE8-OE cells (scale bar: 100 μ m). Data are presented as mean $\pm$ SD from five independent experiments. *** p < 0.001. CCK-8, Cell Counting Kit-8.

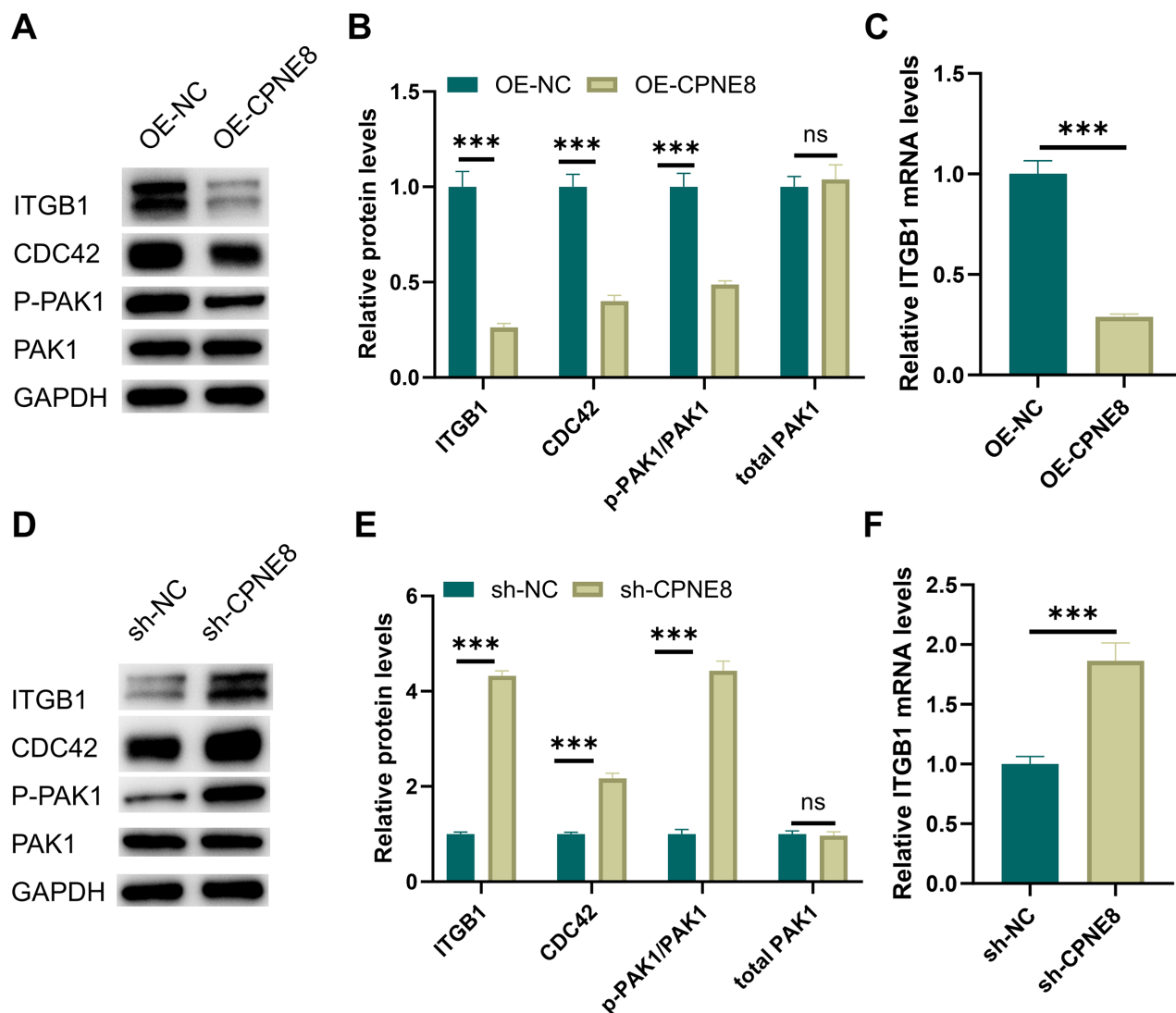


Fig. 4. CPNE8 reduces ITGB1 and CDC42 levels and PAK1 phosphorylation. (A) Representative Western blot of ITGB1, CDC42, p-PAK1, total PAK1, and GAPDH in Huh7 OE-NC and CPNE8-OE cells. (B) Quantification of ITGB1, CDC42 and total PAK1 (normalized to GAPDH), and of PAK1 phosphorylation, expressed as the ratio of GAPDH-normalized p-PAK1 to GAPDH-normalized total PAK1. (C) Relative ITGB1 mRNA levels in Huh7 OE-NC and CPNE8-OE cells, measured by qRT-PCR. (D) Representative Western blot of the same proteins in Hep3B sh-NC and sh-CPNE8 cells. (E) Quantification of (D). ITGB1, CDC42 and total PAK1 were normalized to GAPDH, and p-PAK1 is shown as the p-PAK1/total-PAK1 ratio. (F) Relative ITGB1 mRNA in Hep3B sh-NC and sh-CPNE8 cells. Data are presented as mean $\pm$ SD from five independent experiments. *** $p < 0.001$; ns, not significant. ITGB1, integrin $\beta 1$; CDC42, cell division cycle 42; p-PAK1, phosphorylated PAK1; PAK1, p21-activated kinase 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Cpne8 Overexpression Is Associated With Altered Immune-Marker Expression In Vivo

The OE-Cpne8 group showed a significantly higher density of CD8⁺ cells ($p < 0.001$, Fig. 7A) and of Granzyme B⁺ cells ($p < 0.001$, Fig. 7B), and a significantly lower density of FOXP3⁺ cells ($p < 0.001$, Fig. 7C). The PD-L1-positive area was also reduced ($p < 0.001$, Fig. 7D). Because each marker was assessed by single-marker immunohistochemistry, these values describe the abundance of marker-positive cells rather than their lineage identity or functional state.

These analyses were performed on tumors harvested at a single endpoint, at which tumor burden already differed markedly between the two groups. The present design therefore cannot distinguish an effect of Cpne8 on the immune microenvironment from a consequence of the smaller tumor burden itself, and no experiment reported here tests whether CPNE8 or ITGB1 remodels the microenvironment directly. We therefore describe these findings as immune-marker changes accompanying tumor suppression, rather than as evidence that Cpne8 remodels the tumor immune microenvironment.

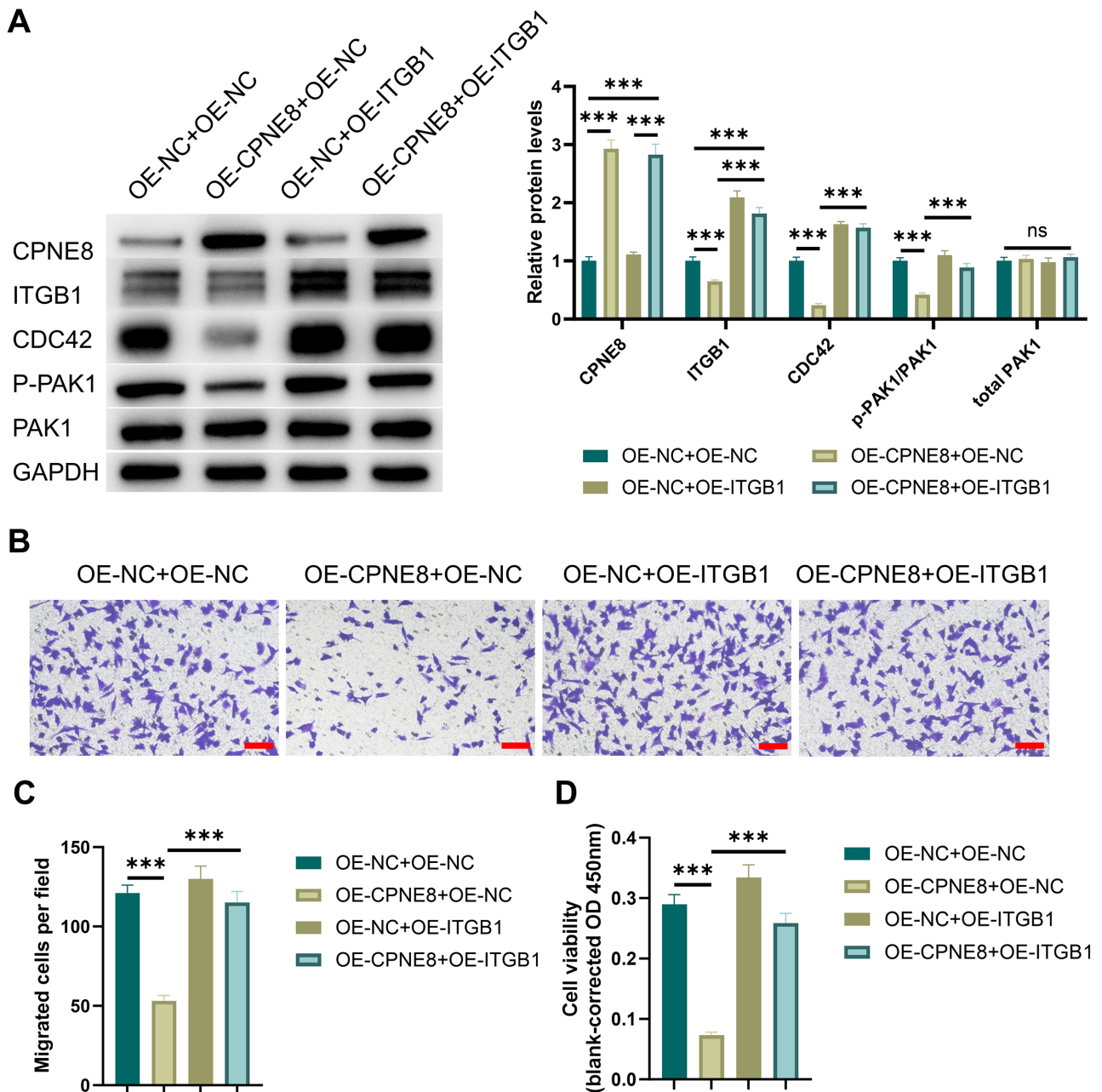


Fig. 5. ITGB1 overexpression rescues the tumor-suppressive effects of CPNE8 in HCC cells. (A) Representative Western blot and quantification of CPNE8, ITGB1, CDC42, p-PAK1, total PAK1, and GAPDH in Huh7 cells co-transduced with empty vector (OE-NC) or CPNE8-OE together with empty vector (OE-NC) or ITGB1-overexpression (OE-ITGB1) constructs. CPNE8, ITGB1, CDC42 and total PAK1 were normalized to GAPDH. PAK1 phosphorylation is presented as the p-PAK1/total-PAK1 ratio. Total PAK1 did not differ significantly among the four groups (ns). (B) Representative Transwell migration images of the four Huh7 groups (scale bar: 100 μ m). (C) Quantification of Transwell migration in the four Huh7 groups. (D) Cell viability measured by CCK-8 assay in the four Huh7 groups. Data are presented as mean $\pm$ SD from five independent experiments. *** p < 0.001; ns, not significant.

Discussion

In this study, CPNE8 emerged as a gene that is down-regulated in HCC and that, when restored, restrains the malignant behavior of HCC cells. This pattern was consistent across the TCGA-LIHC cohort and cell lines, and

CPNE8 re-expression reduced cell viability and colony formation and suppressed migration and invasion *in vitro*, as well as tumor growth *in vivo*. Mechanistically, CPNE8 restoration was associated with reduced ITGB1 and CDC42 levels and decreased phospho-PAK1, while ITGB1 re-expression was sufficient to reverse the CPNE8-induced

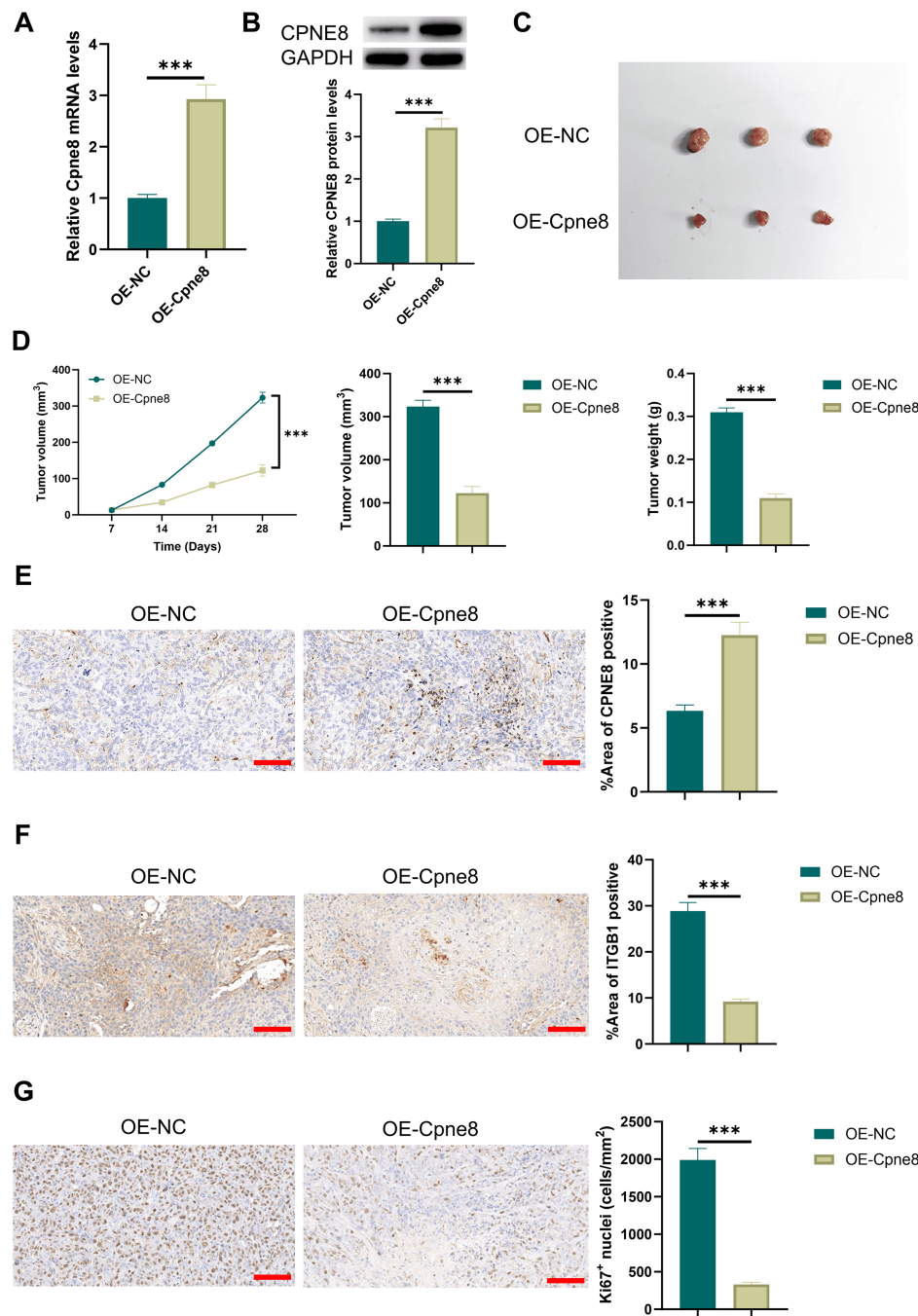


Fig. 6. Cpne8 overexpression suppresses HCC growth in immunocompetent C57BL/6J mice. (A,B) Validation of Cpne8 overexpression in murine Hepa1-6 cells transduced with empty vector (OE-NC) or OE-Cpne8 lentivirus, by qRT-PCR (A) and Western blotting (B) (normalized to GAPDH). (C) Representative images of subcutaneous Hepa1-6 OE-NC and OE-Cpne8 allografts at the endpoint. (D) Tumor growth curves (tumor volume measured every 7 days, $V = 0.5 \times L \times W^2$) and endpoint (day 28) tumor volume and tumor weight ($n = 8$ per group). Tumor growth curves were analyzed by two-way repeated-measures ANOVA with the Geisser-Greenhouse correction followed by Šidák's multiple-comparisons test. Endpoint tumor volume and weight were compared by Student's two-tailed *t*-test. (E–G) Representative IHC images and corresponding quantification of CPNE8 (E), ITGB1 (F), and Ki67 (G) in tumor tissues (scale bar: 100 μ m). CPNE8 and ITGB1 are expressed as the percentage of DAB-positive area. Ki67 is expressed as the number of positive nuclei per mm². Data are presented as mean $\pm$ SD. For (A) and (B), which were performed *in vitro*, $n = 5$ independent experiments. For (C–G), $n = 8$ mice per group, each mouse being treated as one independent biological replicate. *** $p < 0.001$. ANOVA, analysis of variance; IHC, Immunohistochemistry; DAB, 3,3'-diaminobenzidine.

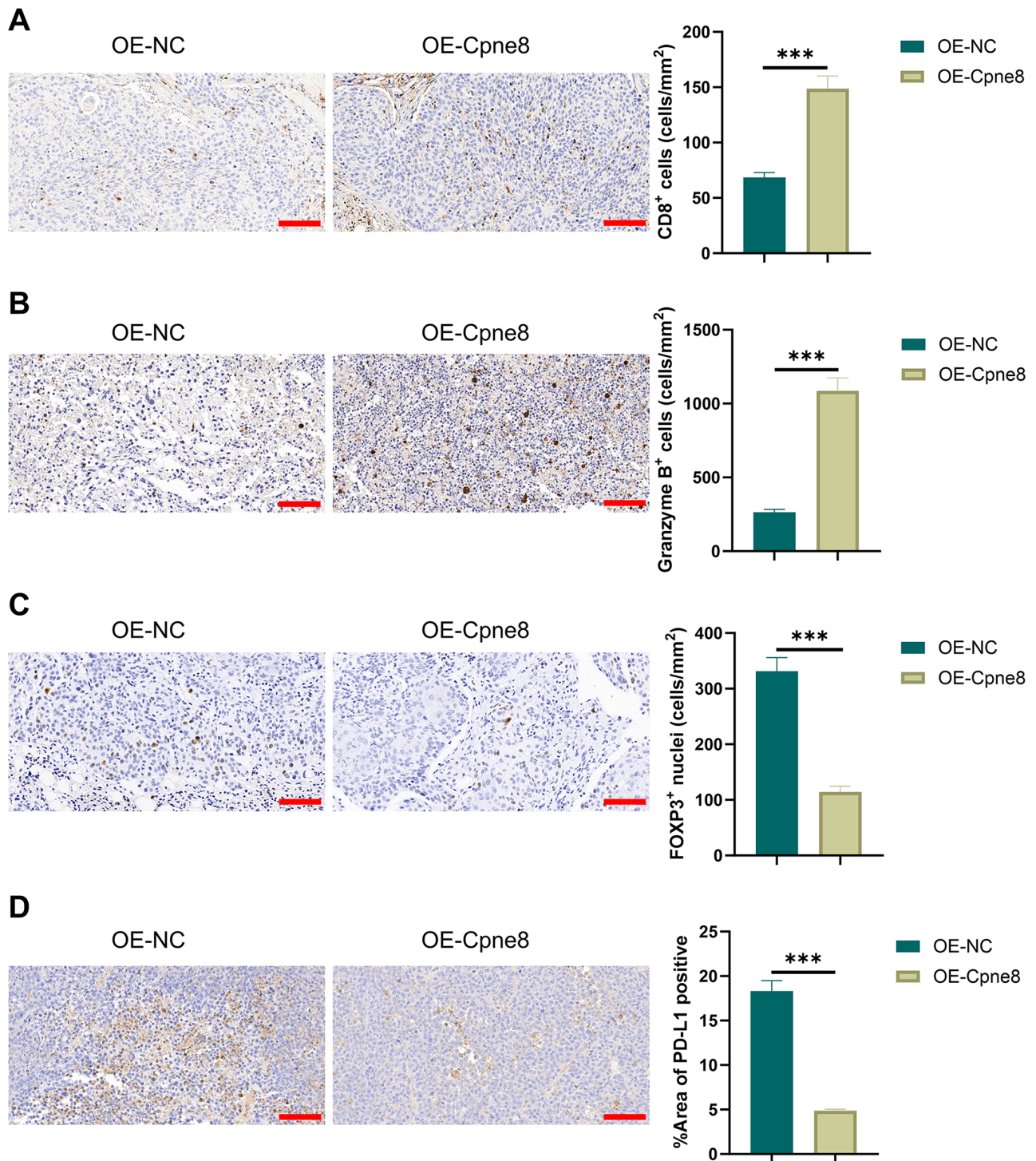


Fig. 7. Cpne8 overexpression is associated with altered immune-marker expression in the HCC allografts. (A–D) Representative IHC images and corresponding quantification of CD8 (A), Granzyme B (B), FOXP3 (C), and PD-L1 (D) in subcutaneous Hepa1-6 OE-NC and OE-Cpne8 tumors (scale bar: 100 μ m). CD8, Granzyme B and FOXP3 are expressed as the number of positive cells per mm². PD-L1 is expressed as the percentage of DAB-positive area. Data are presented as mean $\pm$ SD ($n = 8$ mice per group; for each tumor, five microscopic fields were averaged to yield one value). *** $p < 0.001$.

phenotypes. Taken together, these observations support a tumor-suppressive role for CPNE8 in HCC, although the present data do not define the full range of its activities.

One feature of our findings warrants explicit comment: they run opposite to the only prior functional study of CPNE8 in cancer. In gastric cancer, CPNE8 is overex-

pressed, promotes metastasis via the focal-adhesion pathway, and portends poor survival [33]. We do not regard this as a contradiction, but rather as an example of the context-dependent behavior that characterizes the copine family, whose members have been reported to act as oncogenes in some tissues and as suppressors in others [33,34]. It is worth noting that both the gastric-cancer study and our work converge on integrin/focal-adhesion signaling, albeit in opposite directions, which may reflect tissue-specific regulation of this pathway rather than a shared functional outcome. We emphasize, however, that our data pertain only to HCC, and no inference regarding other tumor types is intended.

The signaling changes we observed align with prior work without extending beyond it. Positioning CPNE8 upstream of reduced ITGB1 and CDC42 levels is consistent with evidence that ITGB1 promotes HCC progression and can regulate CDC42 expression [24,35]. This model also accords with the reported pro-tumorigenic role of CDC42 in HCC and the canonical function of CDC42-PAK1 signaling in actin-cytoskeletal remodeling and cell motility [26,27,28]. The parallel reduction in ITGB1 mRNA and protein levels suggests that CPNE8 acts, at least in part, at the transcript level. How a cytoplasmic, calcium- and phospholipid-binding protein such as CPNE8 produces this effect remains unresolved. CPNE8 is not a transcription factor, so this effect is most likely indirect, and the intermediate steps were not investigated here. Moreover, although ITGB1 re-expression restored CDC42 levels and PAK1 phosphorylation, we cannot exclude contributions from other ITGB1 effectors (for example, FAK or AKT) that were not assessed.

In immunocompetent mice, Cpne8-overexpressing tumors showed a higher density of CD8⁺ and Granzyme B⁺ cells, a lower density of FOXP3⁺ cells, and a smaller PD-L1-positive area. The most important qualification of these observations concerns causal direction. Tumors were analyzed at a single endpoint, by which time OE-Cpne8 tumors were substantially smaller. The changes in immune-marker abundance may therefore be secondary to reduced tumor burden through differences in necrosis, hypoxia, stromal architecture, and the ratio of stroma to tumor parenchyma, rather than evidence that CPNE8 or ITGB1 directly remodels the microenvironment. Nothing in the present design distinguishes these possibilities. The immune and signaling phenotypes were measured in parallel; no time-matched or size-matched comparison was performed, and the immune compartment itself was not manipulated. In the TCGA cohort, CPNE8 expression was inversely correlated, though weakly, with CIBERSORT-estimated Treg infiltration ($r = -0.20$), an association derived from bulk deconvolution that is likewise hypothesis-generating only. Establishing whether CPNE8 acts directly on the microenvironment directly will require harvesting size-matched tumors at earlier time points, depletion or reconstitution of specific immune

compartments (for example, CD8⁺ T-cell depletion or implantation of the same lines in T-cell-deficient hosts), and cell-intrinsic assays of immune-relevant molecules such as PD-L1 *in vitro*. Although integrin signaling has been linked to immunosuppression in other tumors, making such a connection plausible, plausibility alone is not evidence.

Our characterization of the tumor immune microenvironment relies on single-marker immunohistochemistry, which has intrinsic limitations. Quantifying the density of cells positive for a single marker establishes the abundance of marker-positive cells but does not define their lineage identity. CD8 is expressed by subsets of dendritic cells as well as cytotoxic T cells, Granzyme B is produced by natural killer cells and some myeloid populations in addition to CD8⁺ T cells, and FOXP3 can be transiently induced in activated conventional T cells in mice. Moreover, the presence of a marker does not establish functional activity. Granzyme B staining indicates that the protein is present but does not demonstrate that degranulation or target-cell killing has occurred. Multiparameter approaches, including flow cytometry of dissociated tumors, multiplex immunofluorescence enabling co-registration of CD3, CD8, FOXP3 and CD25, or single-cell transcriptomic profiling, together with functional readouts such as *ex vivo* cytotoxicity or cytokine production assays, would be required to establish the identity and activation state of the infiltrating populations. The immune findings reported here should therefore be interpreted as changes in the abundance of marker-positive cells accompanying tumor suppression, rather than as evidence that CPNE8 restores anti-tumor immune function.

Several further limitations should be acknowledged. Human evidence was limited to the public TCGA-LIHC dataset, without an independent patient cohort or survival data; therefore, the prognostic relevance of CPNE8 in HCC remains untested. A further limitation concerns the design of the functional experiments. Gain- and loss-of-function manipulations were assigned to different cell lines, Huh7 and Hep3B, which differ in differentiation status, viral background and proliferative capacity. A contribution of cell-line-intrinsic properties to the observed phenotypes therefore cannot be formally excluded. Several features of our data argue against this explanation. The two manipulations produced effects in opposite directions, which would be difficult to attribute solely to fixed differences between the cell lines. The same direction of effect was reproduced in murine Hepa1-6 cells in an immunocompetent host, representing a genetic and species background unrelated to either human line. In addition, ITGB1 re-expression alone reversed the CPNE8-induced phenotypes, indicating that these effects are transmitted through a defined signaling route rather than reflecting nonspecific differences between cell lines or shRNA off-target activity. Nevertheless, only a single shRNA sequence was used, and an shRNA-resistant rescue construct was not tested. Confirming the present

findings with an independent shRNA and with reciprocal gain- and loss-of-function experiments in the same cell line will be necessary, and we regard this as a priority for follow-up work. Finally, colony-formation and invasion assays were performed only in the overexpression setting; therefore, the reciprocal effects of CPNE8 loss on these two phenotypes have not been directly tested.

The allografts were subcutaneous rather than orthotopic or spontaneous and therefore only approximate the hepatic microenvironment. Although Hepa1-6 cells and C57BL/6J recipients share the H-2b haplotype, the cell line originated in the C57L strain, and residual disparities at minor histocompatibility loci cannot be excluded. Such disparities could, in principle, contribute an alloreactive component to the immune infiltrate observed here. Two features of our design limit, but do not eliminate, this concern. Both experimental groups were established from the same parental Hepa1-6 population and differed only in Cpne8 status, so any alloreactivity would be expected to affect both groups equally and could not, by itself, account for the between-group differences in CD8⁺, Granzyme B⁺ and FOXP3⁺ cells. In addition, any alloreactive component would be present in both groups from the time of implantation, whereas the marker differences we report emerged between groups that differed only in Cpne8 status. Even so, Hepa1-6 tumors are known to elicit a comparatively strong immune infiltrate, and the immune features of this model do not necessarily recapitulate those of human HCC, which is typically characterized by a more profoundly immunosuppressive microenvironment. The immune findings reported here should therefore be regarded as evidence that Cpne8 restoration is associated with changes in immune-marker abundance in this particular model, rather than as a direct prediction of the immune consequences of CPNE8 restoration in patients. Confirmation in additional immunocompetent models, such as orthotopic Hepa1-6 or RIL-175 tumors, or genetically engineered or carcinogen-induced HCC models on a pure C57BL/6 background, will be required.

Moreover, a limitation of our mechanistic analysis is that we quantified total CDC42 protein rather than the GTP-bound active fraction. A reduction in total CDC42 constrains the pool available for activation but does not, by itself, establish that CDC42 signaling output is diminished, since GTP loading is governed by guanine nucleotide exchange factors and GTPase-activating proteins independently of protein abundance. The concomitant decrease in PAK1 phosphorylation is consistent with reduced CDC42 activity because PAK1 is a canonical CDC42 effector whose activation loop is phosphorylated upon binding to GTP-CDC42. However, PAK1 can also be activated by RAC1 and CDC42-independent inputs, making this readout supportive rather than definitive. Direct measurement of GTP-loaded CDC42 by effector pull-down or G-LISA assays will be required to establish whether CPNE8 modu-

lates CDC42 activity in addition to its abundance. As noted above, the immune observations are associative and were obtained at a single time point when tumor burden already differed between groups; therefore, a secondary origin cannot be excluded. Our conclusions are accordingly confined to HCC and the models employed. In addition, the *in vitro* effects of CPNE8 were assessed mainly by a single-time-point CCK-8 assay, which reflects metabolic activity and viable cell number rather than cell division per se. We have therefore described these results as changes in cell viability. Direct proliferation assays, such as EdU incorporation or time-course cell counting, will be needed to confirm an anti-proliferative effect of CPNE8.

Conclusion

In summary, our data indicate that CPNE8 is down-regulated in HCC and can suppress the malignant phenotype of HCC cells in association with reduced ITGB1 and CDC42 protein levels and decreased PAK1 phosphorylation. Its restoration also coincides with an altered immune-marker profile in a syngeneic immunocompetent model, although the present design cannot attribute this change to a direct action of CPNE8 on the microenvironment. These findings identify CPNE8 as a candidate tumor suppressor in HCC and provide a rationale for further investigation of the CPNE8-ITGB1 relationship. Mechanistic dissection of how CPNE8 regulates ITGB1, causal testing of its immune effects, and validation in larger, outcome-annotated cohorts will be required before the biomarker or therapeutic potential of CPNE8 can be assessed.

Availability of Data and Materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions

XFZ and LMW designed the research study. XFZ and JM performed the research. LMW and JM analyzed the data. XFZ drafted the article and all authors contributed to the important editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

All animal experiments were reviewed and approved by the Laboratory Animal Ethics Committee of Jiangsu Vocational College of Medicine (approval no. SYLL-2025-004) and were conducted in accordance with the institu-

tional guidelines for the care and use of laboratory animals. The study is reported in accordance with the ARRIVE 2.0 guidelines.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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