

PLEKHA4 knockdown induces apoptosis in melanoma cells through the MAPK and β -catenin signaling pathways

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Abstract. Malignant melanoma (MM) is a highly aggressive subtype of skin cancer characterized by a poor prognosis, particularly in the advanced stages. Despite advancements in targeted therapy and immunotherapy, the survival rates for MM remain low, underscoring the need for new therapeutic targets. Pleckstrin homology domain-containing family A member 4 (PLEKHA4), which has regulatory functions in pivotal cellular processes, has emerged as a potential target in melanoma. The present study aimed to investigate the role of PLEKHA4 in melanoma progression, focusing on its influence on the MAPK and Wnt/ β -catenin signaling pathways. Bioinformatics analysis revealed that PLEKHA4 was upregulated in melanoma tissues, whereas PLEKHA4 knockdown in melanoma cell lines (A375 and A2058) significantly inhibited cell proliferation and migration, enhanced apoptosis and inhibited tumor growth *in vivo*. Mechanistic studies demonstrated that PLEKHA4 may exert its effects by modulating the MAPK signaling pathway through interactions with key proteins, including ERK, JNK and MEK. Additionally, PLEKHA4 was shown to impact apoptosis by regulating caspase-3, COX2 and p65. Additionally, β -catenin nuclear translocation was affected via the Wnt pathway. Moreover, PLEKHA4 knockdown reduced cMyc ubiquitination, consequently promoting its degradation. The present findings suggested that PLEKHA4 could promote melanoma cell proliferation by regulating both the MAPK and Wnt/ β -catenin pathways, thereby proposing

PLEKHA4 as a promising therapeutic target for MM. Further studies are warranted to elucidate the mechanisms underlying PLEKHA4-mediated modulation of cMyc ubiquitination.

Introduction

Malignant melanoma (MM) is a highly malignant type of cancer characterized by aggressive behavior and rapid metastasis to distant sites. Existing melanoma treatments include surgical resection, chemotherapy and radiotherapy (1). Despite notable advancements in immunotherapy, targeted agents and oncolytic viral therapy, the 5-year survival rate is 50% for patients with advanced melanoma (2). Therefore, there is a critical need to investigate novel therapeutic targets for the treatment of MM.

Melanoma is commonly associated with mutations in the Ser/Thr kinase BRAF (50%), the small GTPase NRAS (25%) or the RAS regulator neurofibromin 1 (14%), leading to enhanced RAS/MAPK signaling (3). This pathway, involving RAS, RAF, MEK and ERK, serves crucial roles in melanoma, thus indicating that it may be a prominent therapeutic target of significant interest (4). MEK serves as a key relay in the pathway, passing signals from RAF to ERK. Pharmacological inhibition of MEK can disrupt this signaling relay, thereby impeding ERK activation and effectively arresting the aberrant signaling cascade that fuels cancer proliferation (5). Notably, preclinical and clinical studies have highlighted the RAS/RAF/MAPK pathway as a key therapeutic target, particularly in the era of precision medicine (1-3,6).

Wnt/ β -catenin signaling, which regulates cell proliferation, is frequently hyperactive in cancer, including melanoma (7). In the canonical β -catenin-dependent pathway, Wnt ligands bind to Frizzled receptors on the cell surface, triggering Dishevelled (DVL) recruitment and disruption of the β -catenin destruction complex (8), its translocation into the nucleus and subsequent alteration of gene expression, particularly affecting TCF/LEF target genes. In cancer, this signaling cascade upregulates genes such as cyclin D1 and cMyc, driving G₁/S cell cycle progression, and promoting tumor growth and malignancy (7). The involvement of Wnt signaling in melanoma pathogenesis remains a topic of ongoing discussion, with its precise contributions subject to debate (7,9). Numerous studies have

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demonstrated that Wnt/ β -catenin signaling serves a role in facilitating tumor initiation and progression in melanomas harboring mutations in BRAF and NRAS (10-12). A previous study using an engineered mouse model also linked Wnt signaling to the transformation of melanocyte stem cells into melanoma in BRAF and PTEN mutants (13). Notably, it has been observed that the efficacy of BRAF inhibition is enhanced in scenarios where β -catenin levels are decreased (14).

Pleckstrin homology domain-containing family A member 4 (PLEKHA4) has a key role in the landscape of cancer biology, such as in glioma (15). This biomolecule performs crucial functions in cancer advancement and prognosis, delineated by its diverse mechanisms of action. It is involved in tumor microenvironment remodeling, particularly through the recruitment and polarization of M2 macrophages (15,16). In glioblastoma, PLEKHA4 is involved in the modulation of apoptotic regulators and inhibits apoptosis (15). Additionally, PLEKHA4 has been implicated in promoting cancer cell proliferation in melanoma through the activation of the Wnt/ β -catenin signaling pathway, a key regulator of cell proliferation and differentiation (17). In a BRAF-mutant melanoma xenograft model (18), inducible PLEKHA4 knockdown exhibited additive effects when combined with the clinically used BRAF V600D/E inhibitor encorafenib (19). These findings suggested the therapeutic potential of targeting PLEKHA4 in melanoma, particularly involving the MAPK pathway. Since β -catenin levels contribute to the inhibition of BRAF, an upstream component of the MAPK pathway, a comprehensive examination of both the MAPK and Wnt/ β -catenin pathways is imperative in MM. The present study aimed to assess the role of PLEKHA4 in MAPK and Wnt/ β -catenin pathways, as well as the underlying mechanisms in melanoma.

Materials and methods

Bioinformatics analysis. Pan-cancer RNA sequencing data from 51 datasets were obtained from The Cancer Genome Atlas (TCGA) (<https://portal.gdc.cancer.gov>) and Genotype-Tissue Expression (<https://www.gtexportal.org/home/>). The data were processed using R software (v.4.2.1) (<https://cran.r-project.org/bin/windows/base/old/4.2.1/>) and visualized with the 'ggplot2' package (v.3.3.6; <https://cran.r-project.org/src/contrib/Archive/ggplot2/>). Statistical analyses were performed using the Wilcoxon rank-sum test. Additionally, the expression profile of PLEKHA4 in melanoma was examined using the Gene Expression Profiling Interactive Analysis (GEPIA) platform (<http://gepia.cancer-pku.cn>). Furthermore, the gene expression profiles GSE3189 (20) and GSE8401 (21) were obtained from the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>), both of which are based on the GPL96 platform. The GSE3189 data were collected from 7 normal skin, 18 nevi and 45 melanoma samples, whereas the GSE8401 data were collected from 31 primary melanoma and 52 melanoma metastasis tissues from patients. Data from the GEO database were downloaded using 'GEOquery' (v.2.64.2; <https://bioconductor.org/packages/release/bioc/html/GEOquery.html>) and were normalized with the 'normalizeBetweenArrays' function from the 'limma' package (v.3.52.2; <https://www.bioconductor.org/packages/release/bioc/html/limma.html>). All gene expression data were calibrated, standardized and log₂-transformed. The results

were visualized using 'ggplot2' (v.3.3.6) and 'ComplexHeatmap' (v.2.13.1; <https://github.com/jokergoo/complexheatmap>). Principal component analysis was performed using R package ggplot2 (<https://cran.r-project.org/web/packages/ggplot2/index.html>). The expression of PLEKHA4 across various pathological and histological grades was analyzed using R and was visualized with 'ggplot2'. RNAseq data from TCGA-Skin Cutaneous Melanoma (SKCM) project (portal.gdc.cancer.gov/projects/TCGA-SKCM) was obtained using the STAARpipeline (<https://github.com/xihaoli/STAARpipeline>) from TCGA database and extracted in transcripts per million (TPM) format. Kyoto Encyclopedia of Genes and Genomes (KEGG) combined with Gene Ontology (GO) analyses were performed using 'clusterProfiler' (v.4.4.4; <https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html>) and GOplot (v.1.0.2; <https://cran.r-project.org/web/packages/GOplot/index.html>). Correlation analysis was conducted by downloading TCGA-SKCM dataset, as aforementioned. Results were statistically analyzed using Spearman's correlation coefficient. The interaction of PLEKHA4 and other proteins was analyzed using GeneMANIA (<http://genemania.org>). The overall survival analysis was conducted using the GEPIA platform, incorporating both the Kaplan-Meier method and the log-rank test.

Cell culture. Human melanoma cells A375, A2058 and SK-MEL-3 cells were obtained from the National Collection of Authenticated Cell Cultures. A375, A2058 and SK-MEL-3 cells were maintained in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS and 1% penicillin-streptomycin (both from Biological Industries; Sartorius AG) at 37°C in a 5% CO₂ incubator. For drug treatment, lithium chloride (LiCl; Selleck Chemicals) was added at 5 mM to activate the Wnt/ β -catenin pathway, with cells incubated under the same conditions for 12 h at 37°C in a 5% CO₂ incubator. MG132 (cat. no. S2619; Selleck Chemicals) was added at concentrations of 0, 5, 10 and 20 μ M and incubated at 37°C in a 5% CO₂ incubator for 24 h to conduct the ubiquitination assay.

Cell transduction and transfection. A375 and A2058 cells were transduced with PLEKHA4-targeting short hairpin RNA (shRNA) lentiviruses. The shRNA sequences were as follows: shPLEKHA4 [K7453 LV3(H1/GFP&Puro)-PLEKHA4-Homo-2851; target sequence: 5'-GCGAGTCACTCTGCTACAATC-3'], forward, 5'-GATCCGCGAGTCACTCTGCTACAATCTTCAAGAGAGATTGTAGCAGAGTGACTCGCTTTTGTG-3' and reverse, 5'-AATTCAAAAAAGCGAGTCACTCTGCTACAATCTCTCTTGAAGATTGTAGCAGAGTGACTCGCG-3'; and a negative control (NC) (LV3-shNC; target sequence: 5'-GTTCTCCGACGTGTACAGT-3'), forward, 5'-GATCCGTTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTTCGGAGAAC TTTTGTG-3' and reverse 5'-AATTCAAAAAAGTTCTCCGAACGTGTACGTTCTCTTGAACGTGACACGTTTCG GAGACG-3'. These lentiviruses were provided by Shanghai GenePharma Co., Ltd.

A 3rd-generation system facilitated lentiviral transduction. Briefly, 293T cells (National Collection of Authenticated Cell Cultures) were used for viral production, utilizing recombinant shuttle and packaging plasmids (pGag/Pol, pRev and pVSV-G) (Addgene, Inc.). Plasmid concentration and purity were

confirmed by ultraviolet absorption, ensuring an A260/A280 ratio between 1.8 and 2.0. The recombinant and packaging plasmids were mixed in an 8:4:4:4 μg ratio (shPLEKHA4 or NC:pGag/Pol:pRev:pVSV-G), added to 293T cells and transfected for 4–6 h at 37°C with 5% CO using EndoFectinTM MAX (GeneCopoeia, Inc.). The medium was then refreshed and incubation continued for 72 h. The 293T cell cultures were maintained in a 37°C incubator with 5% CO₂ to support optimal growth. After 72 h, the supernatant was collected, filtered through a 0.45- μm filter to remove debris and concentrated by ultracentrifugation at 70,000 $\times$ g for 2 h at 20°C. Pellets were resuspended in 100 μl 1X HBSS (Gibco; Thermo Fisher Scientific, Inc.), and another 100 μl was added, yielding a final volume of 200 μl . The solution was vortexed at low speed for 15–30 min, briefly spun, and aliquoted into 20- μl portions for storage at -20°C (up to 1 month) or -80°C (long-term), avoiding more than three freeze-thaw cycles.

Lentiviral infection proceeded immediately post-preparation. The virus titers were: shPLEKHA4 (LV3-PLEKHA4-Homo-2851), 7×10^8 TU/ml; and shNC (LV3-shNC), 9×10^8 TU/ml. Multiplicity of infection values for infection were set at 5 and 10 for shPLEKHA4 and shNC in A375 cells, and at 10 and 15 in A2058 cells, respectively. The lentiviruses were added to the cells with 2 $\mu\text{g}/\text{ml}$ polybrene to boost infection efficiency and incubation was continued for 24 h. Post-transduction, cells underwent selection with 1 $\mu\text{g}/\text{ml}$ puromycin for 7 days, with the same concentration maintained thereafter. Infection efficiency was validated through western blotting, performed immediately after the selection period along with other assays.

To induce PLEKHA4 overexpression in SK-MEL-3 cells, pIRES2-EGFP-PLEKHA4 and the control plasmid (pIRES2-EGFP-empty) were purchased from Shanghai GenePharma Co., Ltd. Transfection was performed using EndoFectin MAX. For transfection in a 6-well plate, 2.5 μg DNA and 5–12.5 μl EndoFectin Max were diluted separately in 125 μl medium. After allowing both solutions to sit for 5 min, they were gently mixed and incubated for 5–20 min to form the DNA-EndoFectin complex. The complex was then added to SK-MEL-3 cells cultured in a 6-well plate, once the cells had reached 80% confluence, and the cells were transfected for 24 h at 37°C. Protein expression was detected 24–48 h post-transfection.

Cell proliferation assay. A375 and A2058 cells were seeded in 96-well plates at a density of 3×10^3 cells/well and were cultured for 0, 24, 48, 72 and 96 h. Following this, cells were treated with 10 μl Cell Counting Kit (CCK)-8 solution (Shanghai Yeasen Biotechnology Co., Ltd.) and incubated for 1 h at 37°C. Absorbance was then recorded at 450 nm. Each experiment was repeated at least three times.

Wound healing assay. Transduced A375 and A2058 cells were plated in 6-well plates at a density of 5×10^3 cells/well and grown to 90% confluence. Subsequently, the cells were incubated overnight in serum-free medium. The cell monolayers were then mechanically wounded using a 10- μl pipette tip. Images of the wounds were captured at 0 and 24 h using a Nikon Eclipse Ti-S/L100 inverted phase contrast fluorescence microscope (Nikon Corporation) with a 10 $\times$ objective. The

wound was measured by ImageJ bundled with 64-bit Java 8 [version (ij154-win-java8); National Institutes of Health] and calculated with SPSS 29 (IBM Corp.).

Colony formation assay. Transduced A375 and A2058 cells were plated in 6-well plates at a density of 5×10^3 cells/well and were cultured for 7 days. After incubation, the cells were rinsed twice with PBS at room temperature, fixed with 4% paraformaldehyde for 15 min and stained with Giemsa (both from Beijing Solarbio Science & Technology Co., Ltd.) for 20 min at room temperature. The cells were then washed twice with PBS. Colonies, defined as groups of ≥ 50 cells, were counted using ImageJ software [bundled with 64-bit Java 8 (ij154-win-java8), National Institutes of Health].

Quantitative proteomics analysis. Cell samples were transferred to a 1.5-ml centrifuge tube and lysed with DB lysis buffer (8 M urea, 100 mM TEAB, pH 8.5). The solution was then alkylated with sufficient iodoacetamide. Protein quantity was assessed using a Bradford protein quantitation kit. The proteins underwent enzymatic hydrolysis and salt removal. Subsequently, the samples were processed with tandem mass tag (TMT) labeling reagent (Thermo Fisher Scientific Inc.). The separated peptides were analyzed by Q ExactiveTM HF-X mass spectrometer, with an ion source of Nanospray FlexTM (ESI), spray voltage of 2.3 kV and ion transport capillary temperature of 320°C. The full scan ranged from 350 to 1,500 m/z with a resolution of 60,000 (at m/z 200), the automatic gain control (AGC) target value was 3×10^6 and the maximum ion injection time was 20 msec. The 40 most abundant precursors in the full scan were selected and fragmented by higher energy collisional dissociation and analyzed in tandem mass spectrometry, where resolution was 45,000 (at m/z 200) for 10 plex, the AGC target value was 5×10^4 , the maximum ion injection time was 86 msec, the normalized collision energy was set at 32%, the intensity threshold was 1.2×10^5 , and the dynamic exclusion parameter was 20 sec. The spectra from each run were analyzed individually against the UniProt database (<https://www.uniprot.org/>) using Proteome Discoverer (Thermo Fisher Scientific, Inc.). Liquid chromatography analysis was performed with the EASY-nLCTM 1200 UHPLC system (Thermo Fisher Scientific, Inc.). Identified peptide-spectrum matches and proteins were retained with a false discovery rate (FDR) of no more than 1.0%. The protein quantitation results were then statistically analyzed using unpaired Student's t-test. Proteins that showed significant differences in quantity between the experimental and control groups ($P < 0.05$ and $|\log_2 \text{FC}| > 0.5$) were defined as differentially expressed proteins. Finally, KEGG analysis was utilized to analyze the protein families and pathways.

Flow cytometric analysis. Cells were trypsinized without EDTA, centrifuged at 300 $\times$ g for 5 min at 4°C, washed twice with chilled PBS, and resuspended in 100 μl 1X Binding Buffer (Shanghai Yeasen Biotechnology Co., Ltd.). Annexin V-FITC and PI Staining Solution (Shanghai Yeasen Biotechnology Co., Ltd.) were added, and the cells were incubated in the dark at room temperature for 10–15 min. Subsequently, 400 μl 1X Binding Buffer (Shanghai Yeasen Biotechnology Co., Ltd.) was added on ice. Samples were analyzed on a CytoFLEX SRT flow cytometer (Beckman Coulter, Inc.) within 1 h, with FlowJo V8 (FlowJo; BD Biosciences) used for data analysis.

Ubiquitination assay. The ubiquitination assay was performed using the Ubiquitylation Assay Kit (cat. no. ab139467; Abcam). Protein lysates from A375shNC, A375shPLEKHA4, A375shPLEKHA4, A2058shNC, A2058shPLEKHA4 and A2058shPLEKHA4 cells following treatment with 0, 5, 10 and 20 μ M MG132 were subjected to the ubiquitination assay. To prepare the reaction, the E1 stock solution was first diluted 1:20 in 1X ubiquitylation buffer and 5 μ l was added to a 50- μ l reaction solution (including E1 Enzyme, E2 Enzyme, Ubiquitin, 10X Ubiquitylation Buffer, ATP Solution, E3 Enzyme). Next, the E2 stock solution was diluted 1:40 in 1X ubiquitylation buffer and 5 μ l was added to the reaction solution, and ubiquitin was diluted 1:20 in 1X ubiquitylation buffer and 5 μ l was added to the reaction solution. Subsequently, 3.5 μ l 10X ubiquitylation buffer and 5 μ l 2 mM ATP solution were added to the reaction solution, with the amount depending on the substrate concentration, and the volume was made up to 48.5 μ l with water. Finally, 1.5 μ l 0.05 mg/ml E3 was added to generate a final volume of 50- μ l reaction mixture, which was incubated at room temperature for 1 h. After incubation, western blot analysis was performed.

Western blotting. Briefly, genetically transduced or transfected A375, A2058 and SK-MEL-3 cells were seeded in 6-well plates at a density of 1×10^6 cells/well and were cultured in DMEM supplemented with 10% FBS until they reached ~90% confluence. Protein lysates were extracted using RIPA buffer (Beijing Solarbio Science & Technology Co., Ltd.), and nuclear proteins were isolated with the Nuclear and Cytoplasmic Extraction Kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Protein concentration was assessed using the BCA Protein Assay Kit (Beyotime Institute of Biotechnology). Proteins (30 μ g/lane) were separated by SDS-PAGE on 8-10% gels, transferred to PVDF membranes and blocked with 5% non-fat dry milk (MilliporeSigma) for 1 h at room temperature, before overnight incubation with primary antibodies at 4°C. The primary antibodies used included PLEKHA4 (cat. no. NBP1-56679; Novus Biologicals; Bio-Techne), β -actin (cat. no. GB12001-100; Wuhan Servicebio Technology), phosphorylated (p)-p38 MAPK (cat. no. AF4001; Affinity Biosciences), p38 MAPK (cat. no. AF6456; Affinity Biosciences), p-JNK1/2/3 (cat. no. AF3318; Affinity Biosciences), JNK1/2/3 (cat. no. AF6318; Affinity Biosciences), p-cJUN (cat. no. AF3095; Affinity Biosciences), c-Jun (cat. no. AF6090; Affinity Biosciences), p-MEK1/2 (cat. no. AF8035; Affinity Biosciences), MEK (cat. no. AF6385; Affinity Biosciences), p-ERK (cat. no. AF1015; Affinity Biosciences), ERK1/2 (cat. no. AF0155; Affinity Biosciences), COX2 (cat. no. AF7003; Affinity Biosciences), p-NF- κ B p65 (cat. no. AF2006; Affinity Biosciences), NF- κ B p65 (cat. no. AF5006; Affinity Biosciences), cleaved-caspase-3 (cat. no. AF2006; Affinity Biosciences), caspase-3 (cat. no. AF6311; Affinity Biosciences) cMyc (cat. no. 10828-1-AP; Proteintech Group), ubiquitin polyclonal antibody (cat. no. 10201-2-AP; Proteintech Group), β -catenin (cat. no. AF6266; Affinity Biosciences), p-GSK3 β (cat. no. sc-373800; Santa Cruz Biotechnology), GSK3 β (cat. no. sc-377213; Santa Cruz Biotechnology), cyclin D1 (cat. no. AF0931; Affinity Biosciences) and Lamin B1 (cat. no. sc-374015; Santa Cruz Biotechnology), each diluted

1:1,000 in Primary Antibody Dilution Buffer (Beyotime Institute of Biotechnology). Membranes were then incubated with HRP-conjugated anti-rabbit and anti-mouse secondary antibodies (cat. nos. RGAR001 and RGAM001; Proteintech Group, Inc.) diluted 1:5,000 in Tris-buffered saline-0.1% Tween 20 for 2 h at room temperature. Blots were visualized with ECL Western Blotting Substrate (Beijing Solarbio Science & Technology Co., Ltd.) using the Shenhua Science Technology Co., Ltd. system. Protein grayscale values were semi-quantified with ImageJ (version: ij154-win-java8). Each experiment was performed at least three times.

Co-immunoprecipitation. Upon reaching a confluence of ~90%, cells were lysed with RIPA buffer (Beijing Solarbio Science & Technology Co., Ltd.) and subjected to co-immunoprecipitation to investigate protein interactions. Protein concentrations were determined using the Bradford assay. A cMyc (cat. no. 10828-1-AP; Proteintech Group, Inc.) antibody was diluted to a final concentration of 10 μ g/ml with whole cell lysates free of debris (to clear debris, the lysates were centrifuged at 20,000 $\times$ g for 20 min at 4°C, and the sediments were discarded). Subsequently, 400 μ l whole lysate-antibody complex was added to 25 μ l Protein A/G Magnetic Beads (cat. no. HY-K0202; MedChemExpress). The mixture was centrifuged at 120 $\times$ g for 30 sec at 4°C and the supernatant was discarded. The beads were washed 3-4 times with 1 ml RIPA lysis buffer, and underwent further centrifugation at 500-1,000 rpm for 30 sec at 4°C before the supernatant was discarded. Two elutions of the pellet were performed using 40 μ l 0.10 M glycine and 0.05 M Tris-HCl (pH 1.5-2.5) elution buffer with 500 mM NaCl. The eluates were then combined and neutralized with 10X PBS buffer (pH 6.8-7.2) to a final concentration of 1X. Finally, the collected eluates were analyzed by western blotting to identify specific protein interactions.

In vivo tumor growth analysis. Male nude mice (average weight, 14 g; age, 4 weeks) were obtained from the Experimental Animal Center of Yanbian University (Yanji, China) and were randomly divided into two groups (n=5/group): The shNC and shPLEKHA4 groups. The mice were kept in a pathogen-free environment at 25°C with 30% humidity, under a 12-h light/dark cycle, and were provided unrestricted access to cobalt-60-sterilized feed and autoclaved water. Animal health and behavior were monitored daily. Each mouse was injected subcutaneously with 200 μ l of a solution containing 5×10^6 A375 cells transduced with either shNC or shPLEKHA4 in the right flank. Before the subcutaneous injection, mice were given 50 mg/kg sodium pentobarbital via the intraperitoneal route as an anesthetic to minimize suffering. Tumor growth was tracked every 2 days. The humane endpoints were as follows: Euthanasia was performed if tumor weight surpassed 10% of body weight or tumor diameter exceeded 20 mm; however, none of these criteria were met before day 15 when all mice were sacrificed. The two groups of total 10 mice (n=5/group) were euthanized, and no mice were euthanized or found dead prior to day 15. Mice were euthanized by cervical dislocation after anesthesia with an intravenous dose of 70 mg/kg sodium pentobarbital, with death confirmed by cessation of respiration and heartbeat for >5 min.

Hematoxylin and eosin staining. Subcutaneous tumors were fixed in 10% formalin at room temperature for 24 h, dehydrated with graded ethanol and embedded in paraffin. Tumor samples were then sectioned into 4- μ m slices, baked at 56°C overnight, and stained with an H&E Stain kit (Beijing Solarbio Science & Technology Co., Ltd.), following the manufacturer's instructions with all procedures conducted at room temperature (25°C). Briefly paraffin was removed by immersing the slides in xylene for two to three changes, each lasting 5 min and rehydration was achieved through a graded ethanol series. The slides were then rinsed in distilled water for 1-2 min. Hematoxylin staining was conducted at room temperature for 5-10 min, followed by rinsing in running tap water for 5 min. Excess hematoxylin was removed using 1-2 dips in acid alcohol, and the slides were immediately rinsed again under running tap water. A bluing reagent (0.1% ammonia solution) was then applied for 1-2 min and the sections were immersed in eosin staining solution for 1-3 min, followed by a final rinse in running tap water for 5 min. Images were captured using a Nikon Eclipse Ti-S/L100 inverted phase contrast fluorescent microscope with a 10x objective.

Statistical analysis. Statistical analysis was performed using an unpaired Student's t-test for two-group comparisons, and one-way ANOVA with Tukey's post hoc test for multiple group comparisons. SPSS 26.0 (IBM Corp.) was used for data analysis, with results presented as the mean $\pm$ SD. Each experiment was repeated at least three times. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

PLEKHA4 is upregulated in melanoma. The expression of PLEKHA4 in different human cancer tissues was assessed using RNA sequencing data. PLEKHA4 was widely expressed in nearly all tissue types, with notably higher expression in melanoma tissues than in normal skin tissues from healthy individuals (Fig. 1A and B). This expression was also confirmed by GEPIA in which tumor tissues displayed a distinct gene expression pattern compared to that in normal skin tissues from healthy individuals (Fig. 1C). Subsequently, gene expression profiles from the GSE3189 and GSE8401 datasets were retrieved from the GEO database. As shown in Fig. 1D, tumor tissues displayed a distinct gene expression pattern compared to that in normal skin tissues from healthy individuals. The heatmap and volcano plots highlighted distinct genes in melanoma tissues (Fig. 1E and F), with the heatmap specifically indicating the upregulation of PLEKHA4 (Fig. 1E). Furthermore, as depicted in Fig. 2A, metastatic tumor tissues exhibited a pronounced gene expression profile relative to primary tumor tissues. The volcano plot and heatmap in Fig. 2B and C revealed distinct genes in melanoma metastatic tissues; however, PLEKHA4 was not among them, suggesting that PLEKHA4 may not be involved in melanoma metastasis. Additionally, PLEKHA4 expression across different pathological stages showed no significant variation (Fig. 2D-G), and its expression was not directly associated with overall survival outcomes (Fig. 2H), further suggesting that PLEKHA4 may not be involved in metastasis. To further explore the role of PLEKHA4 in melanoma, KEGG combined

with GO analyses were performed. The results highlighted an upregulation of 'beta-catenin binding', suggesting activation of the Wnt signaling pathway in melanoma tissues (Fig. 1G). Additionally, the heatmap across different pathological stages (Fig. 2D) revealed high expression levels of PLEKHA4 along with key MAPK signaling genes (MAPK1, MAPK3 and MAPK12), and Wnt signaling genes (CTNNB1 and GSK3B).

PLEKHA4 knockdown inhibits the proliferation of melanoma cells. PLEKHA4 knockdown experiments were conducted in A375 and A2058 melanoma cell lines; notably, a significant reduction in PLEKHA4 protein expression was detected in the A375 and A2058 cells lines following shRNA transduction (Fig. 3A). Subsequent assessments through CCK-8, colony formation and wound healing assays were conducted to evaluate cell proliferation and migration. Knockdown of PLEKHA4 resulted in the inhibition of cell proliferation, colony formation and migration (Fig. 3B-D). Subsequently, subcutaneous tumor growth models were established to assess the effect of PLEKHA4 on tumor development. Notably, PLEKHA4 knockdown significantly reduced tumor growth (Fig. 4A-C). Tumors in the A375shPLEKHA4 group grew more slowly than those in the A375shNC group (Fig. 4B), and tumor weight in the A375shPLEKHA4 group was lower compared to that in the A375shNC group (Fig. 4C). Following tumor collection, histological analysis was conducted through H&E staining. Cells in the shPLEKHA4 group exhibited lighter staining, were smaller and showed greater heterogeneity compared with those in the shNC group (Fig. 4D).

PLEKHA4 knockdown inhibits MAPK signaling in melanoma. TMT proteomics analysis has evolved as an important technology for investigating abnormal signaling and therapeutic responses in cancer. As shown in Fig. 5A, shPLEKHA4-transduced A375 cells exhibited a distinct proteomic profile compared with those transduced with shNC. Subsequently, KEGG enrichment analysis was conducted using the TMT proteomics data. As depicted in Fig. 5B, the differentially expressed proteins were enriched in the 'MAPK signaling pathway'. Utilizing GeneMANIA, the interactions of PLEKHA4 with proteins in the MAPK pathway were further explored (Fig. 5C). PLEKHA4 was found to have physical interactions with MAPK1 (ERK), MAPK9 (JNK) and MAP2K2 (MEK2). Additionally, correlation analysis unveiled a positive relationship between PLEKHA4 expression and MAPK1 (ERK2), MAPK3 (ERK1) and JUN (cJUN) (Fig. 5D-F) Although the r-values were < 0.3 , the P-values indicated statistical significance, suggesting that the relationship is unlikely to be random and warrants further investigation. Weak correlations may still hold biological significance, especially in multi-factorial systems where individual contributions are inherently subtle. Additionally, this is a gene-level correlation, and further validation at the protein level through western blotting is necessary to confirm these findings. Subsequent validation through western blotting demonstrated that following PLEKHA4 knockdown, the levels of p-P38, p-JNK, p-cJUN, p-MEK and p-ERK were diminished (Fig. 6A). In addition, an overexpression assay was conducted to examine the effects of PLEKHA4 overexpression on cells with low PLEKHA4 levels. The results indicated that PLEKHA4

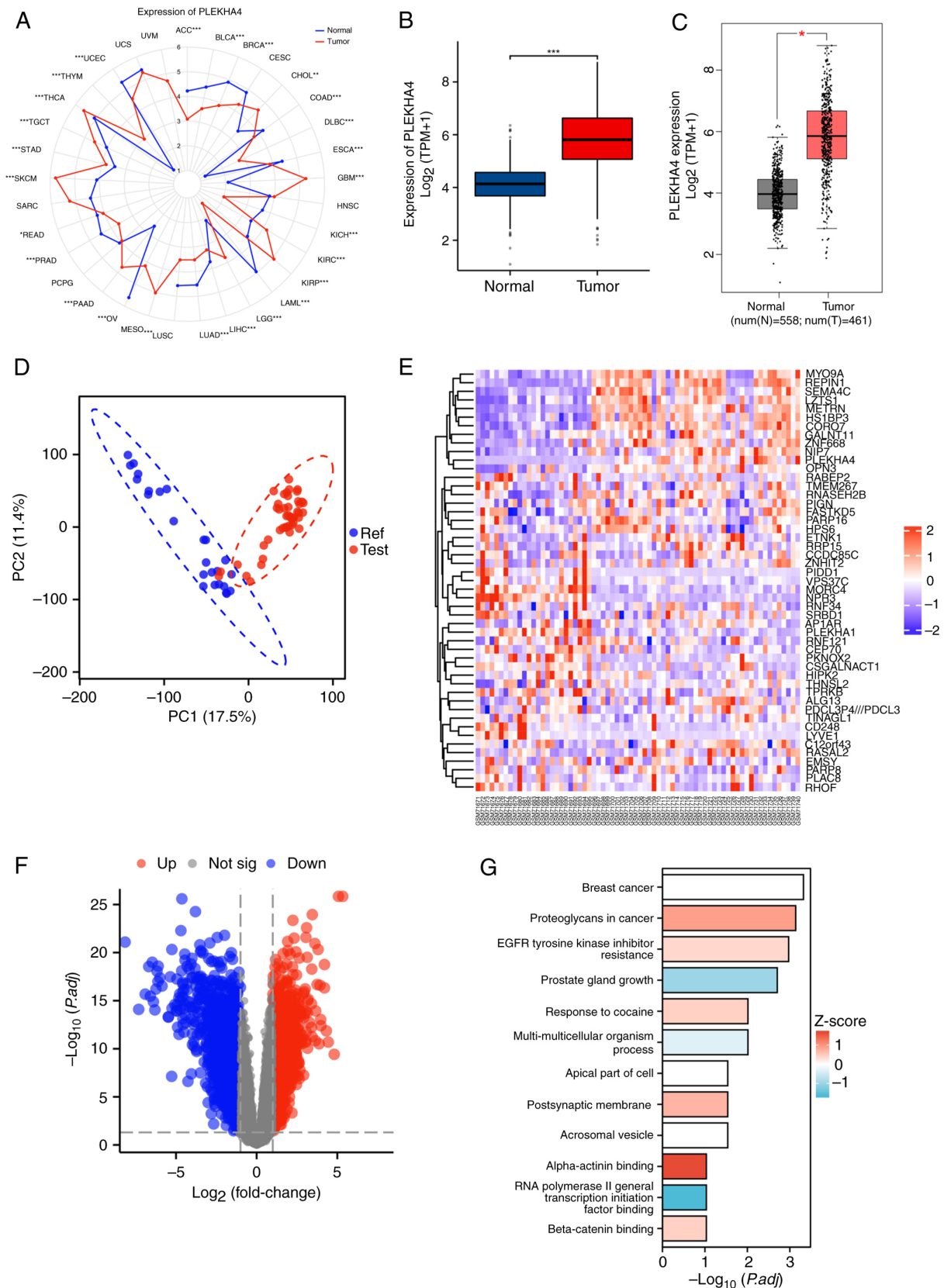


Figure 1. Comprehensive analysis of PLEKHA4 mRNA Expression and related gene profiles in normal and melanoma tissues using multi-dataset approaches. (A) PLEKHA4 mRNA expression in various normal and tumor tissues was analyzed by R (v.4.2.1) and visualized by ‘ggplot2’ (v.3.3.6) based on TCGA and GTEx databases. (B) PLEKHA4 mRNA expression in melanoma tissues was analyzed and visualized by R (v.4.2.1) and visualized by ‘ggplot2’ (v.3.3.6) based on TCGA and GTEx database. The statistical significance was calculated with the Wilcoxon test (* $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$). (C) Expression profile of PLEKHA4 in melanoma was analyzed using Gene Expression Profiling Interactive Analysis. (D) Principal component analysis of GSE3189 showed that melanoma tissues exhibited a unique gene expression profile compared to normal tissue. (E) Heatmap of significantly different genes in melanoma tissues from GSE3189. (F) Volcano plot highlighted the differentially expressed genes in melanoma tissues from GSE3189. (G) Kyoto Encyclopedia of Genes and Genomes enrichment analysis of GSE3189 data showed that β -catenin binding signaling was activated in melanoma tissues. PLEKHA4, pleckstrin homology domain-containing family A member 4; TCGA, The Cancer Genome Atlas; GTEx, Genotype-Tissue Expression.

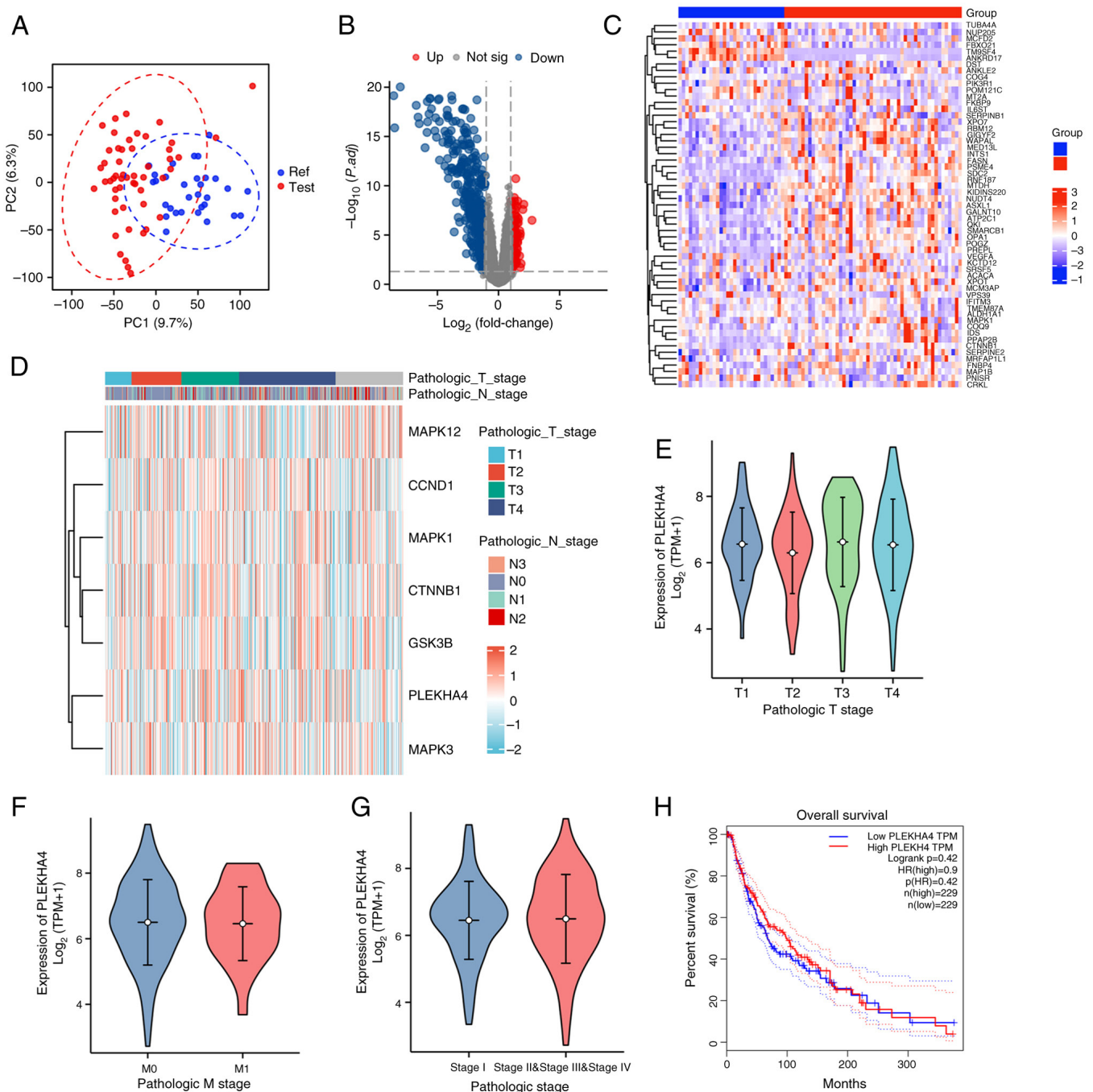


Figure 2. Gene expression and prognostic analysis of PLEKHA4 in melanoma. (A) Principal component analysis of GSE8401 revealed that metastatic tumor tissues exhibited variation in gene expression profiles compared with primary tumor tissues. (B) Volcano plot highlighted the differentially expressed genes in melanoma tissues from GSE8401. (C) Heatmap of significantly different genes in melanoma tissues from GSE8401. (D) Heatmap of gene expression in different pathological stages. (E) PLEKHA4 expression in different pathologic T stages. (F) PLEKHA4 expression in different pathologic M stages. (G) PLEKHA4 expression in different pathologic stages. (H) Association of PLEKHA4 expression with overall survival in melanoma. PLEKHA4, pleckstrin homology domain-containing family A member 4; TPM, transcripts per million.

overexpression successfully upregulated MAPK signaling in SK-MEL-3 cells (Fig. 6B). Collectively, these results indicated that PLEKHA4 may affect the MAPK pathway in melanoma.

Melanoma cell apoptosis is induced after PLEKHA4 knockdown. Quantitative analysis of alterations in the A375 cell proteome following PLEKHA4 gene silencing was conducted using mass spectrometry (Fig. 7A and B). A total of 6,206 proteins were quantified, with 133 peptides that

reached an FDR-corrected $P < 0.05$; among these, 23 were upregulated >5 -fold and 111 were downregulated <5 -fold in the shPLEKHA4 group compared with in the shNC group (Table SI). Notably, these dysregulated peptides encompassed sites known to regulate various apoptotic and proliferative molecules. Specifically, the substantial reduction of caspase-3 in PLEKHA4 knockdown cells, was associated with apoptosis (Table SI). Additionally, a significant reduction was observed in COX2 levels in PLEKHA4 knockdown cells (Table SI),

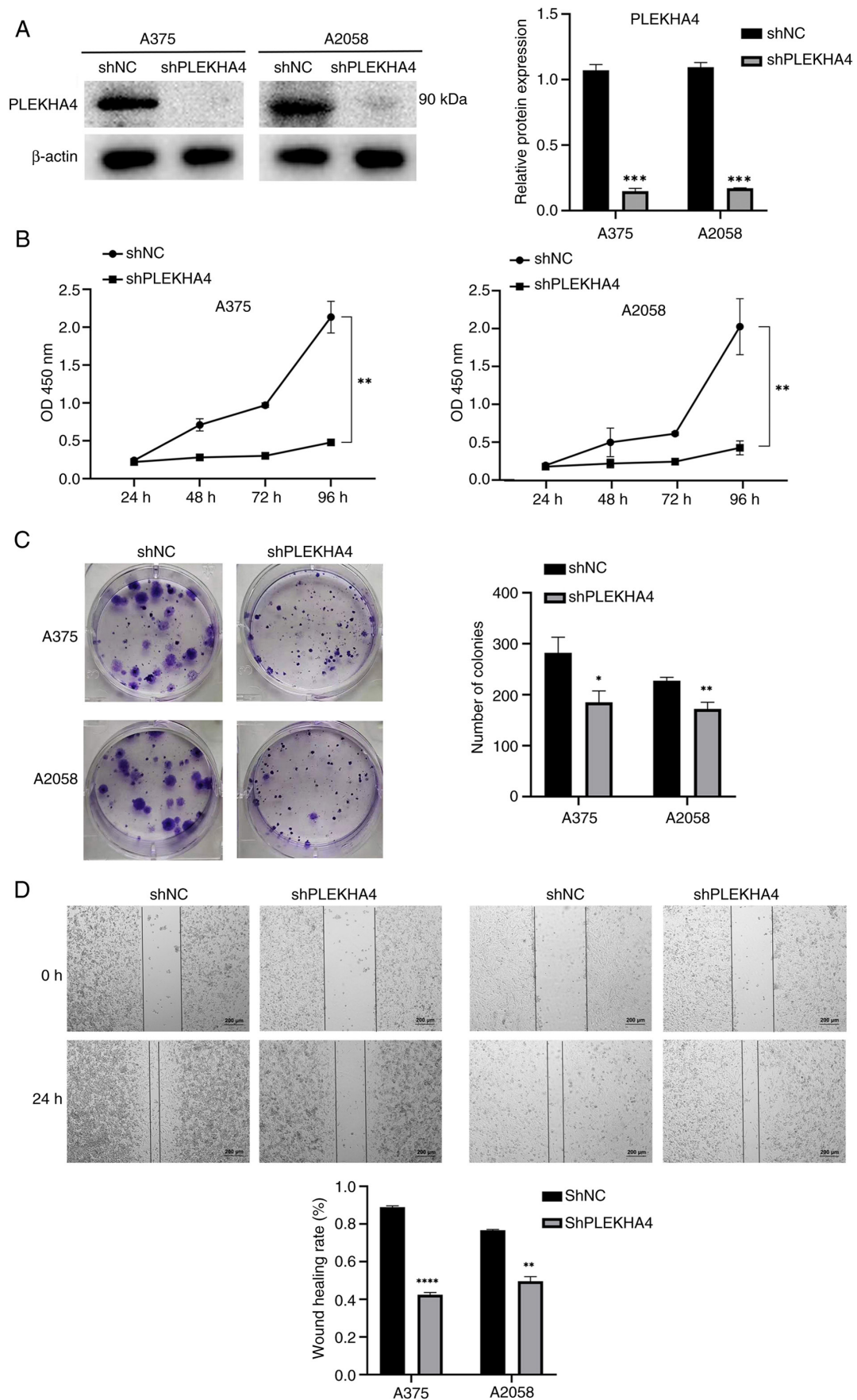


Figure 3. Functional analysis of PLEKHA4 knockdown in melanoma cell lines. (A) Western blotting was performed to confirm the knockdown of PLEKHA4 in A375 and A2058 melanoma cell lines. (B) Proliferation of PLEKHA4 knockdown cells was detected by Cell Counting Kit-8 assay. (C) Knocking down PLEKHA4 suppressed the colony-forming ability of A375 and A2058 cells. (D) Knocking down PLEKHA4 suppressed the migratory ability of A375 and A2058 cells. ****P<0.0001, ***P<0.001, **P<0.01 and *P<0.05 vs. shNC. PLEKHA4, pleckstrin homology domain-containing family A member 4; sh, short hairpin; NC, negative control.

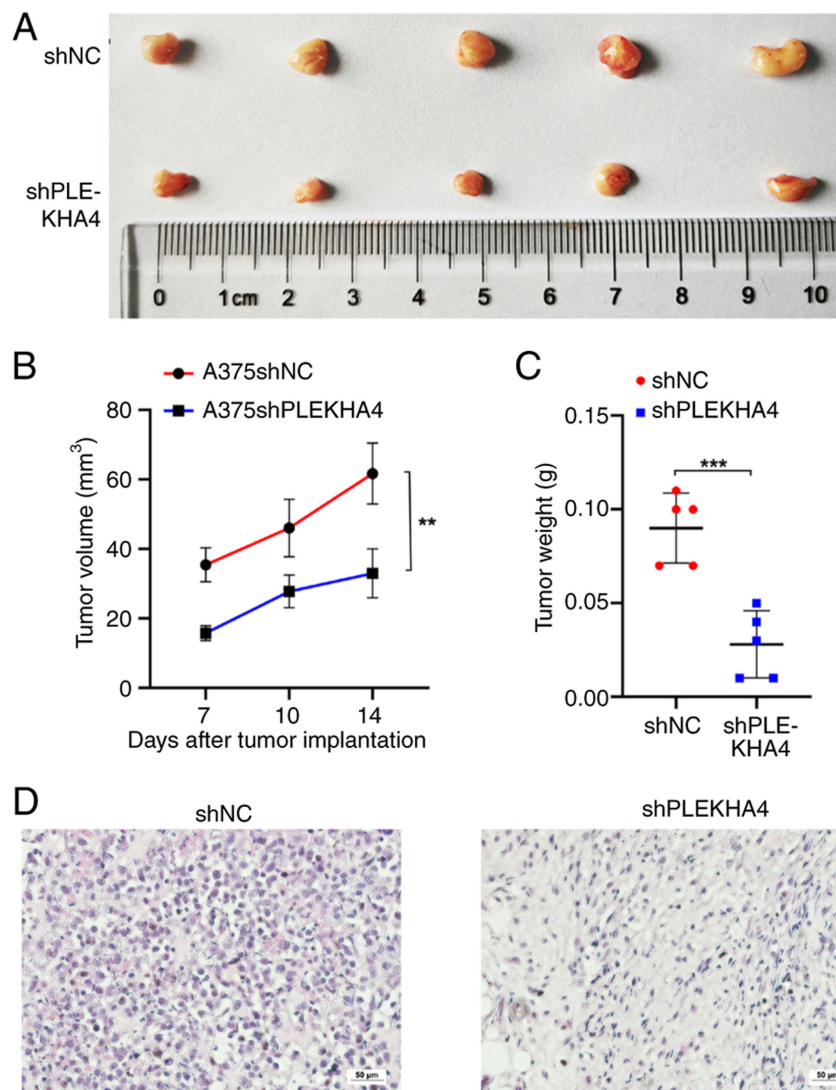


Figure 4. PLEKHA4 knockdown inhibits subcutaneous tumor growth. (A) Knocking down PLEKHA4 suppressed the growth of subcutaneous A375 tumors. (B) Tumor volume was calculated at the indicated intervals. (C) Tumor weight was measured. (D) Histology of tumors stained with hematoxylin and eosin. *** $P < 0.001$, ** $P < 0.01$ vs. shNC. PLEKHA4, pleckstrin homology domain-containing family A member 4; sh, short hairpin; NC, negative control.

consistent with the findings from GeneMANIA (Fig. 5C). Subsequent expression correlation analysis revealed a correlation between NFKB1 (p65) and CASP3 (with genes in the MAPK pathways at the mRNA level, consistent with the outcomes depicted in GeneMANIA (Fig. 7C). To confirm the aforementioned results, western blotting was conducted to assess protein levels in shNC cells and PLEKHA4 knockdown cells. The results showed that the protein levels of COX2 and p-p65 were reduced, whereas the levels of cleaved caspase-3 were increased (Fig. 7D). Subsequently, flow cytometry was performed to compare the apoptotic effects between the shNC and shPLEKHA4 groups. The findings indicated an elevation in apoptosis in the shPLEKHA4 cells (Fig. 7E).

PLEKHA4 modulates Wnt/ β -catenin signaling in melanoma cells. Given the upregulation of Wnt signaling-related proteins observed in melanoma (Fig. 2D), a correlation analysis focusing on β -catenin was initially performed. The analysis revealed significant correlations between CTNNB1 and MAPK1, MAPK8, and MAPK14, highlighting

a potential interaction between β -catenin and these MAPK family members (Fig. 8A). Subsequently, Wnt signaling proteins (p-GSK3 β , β -catenin and cyclin D1) were examined and their levels were shown to be reduced following PLEKHA4 knockdown (Fig. 8B). The nuclear translocation of β -catenin is crucial for initiating gene transcription and promoting tumorigenesis. Upon PLEKHA4 knockdown, β -catenin levels decreased in both total and nuclear fractions, while cytosolic β -catenin levels significantly increased (Fig. 8B and C). These findings indicated that PLEKHA4 may promote the nuclear translocation of β -catenin. To further investigate its effect on the Wnt/ β -catenin pathway, melanoma cells were treated with the Wnt signaling activator LiCl following PLEKHA4 knockdown. Western blotting revealed that LiCl reversed the changes induced by PLEKHA4 knockdown (Fig. 8D). Additionally, an overexpression assay in SK-MEL-3 cells demonstrated that PLEKHA4 overexpression successfully upregulated Wnt/ β -catenin signaling proteins (Fig. 8E). These findings suggested that PLEKHA4 may regulate Wnt/ β -catenin signaling in melanoma cells.

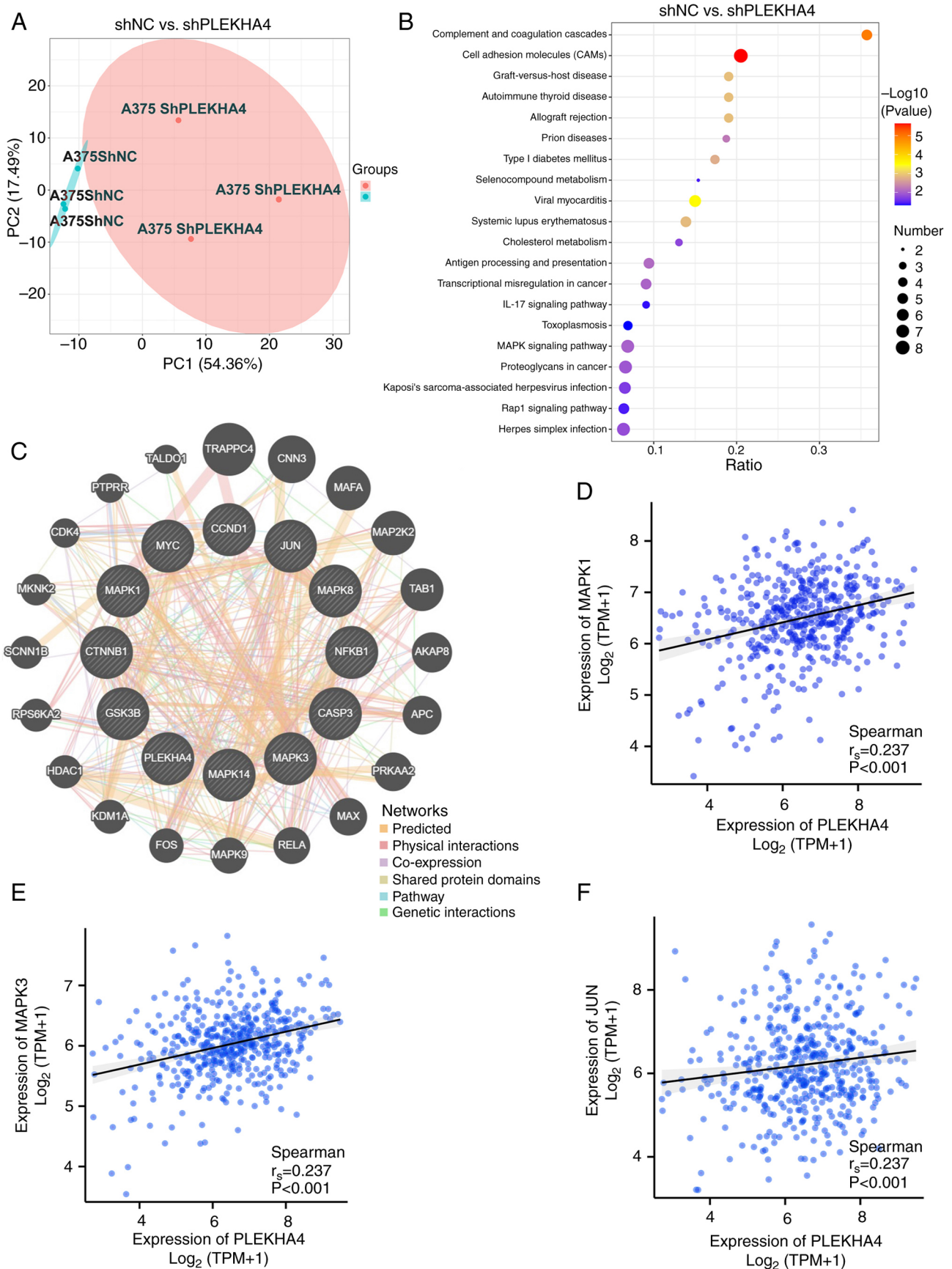


Figure 5. PLEKHA4 knockdown suppresses MAPK signaling. (A) Tandem mass tag proteomics was performed. Principal component analysis using the reporter ion $\log_2$ ratios of all analyzed proteins showed that shPLEKHA4 A375 cells had a distinct proteomic profile compared with shNC cells. (B) Kyoto Encyclopedia of Genes and Genomes enrichment analysis showed that MAPK signaling was inhibited by PLEKHA4 knockdown. (C) Integrated functional association networks of PLEKHA4, β -catenin-related, MAPK-related and MYC genes. (D) Correlation analysis between PLEKHA4 and MAPK1. (E) Correlation analysis between PLEKHA4 and MAPK3. (F) Correlation analysis between PLEKHA4 and JUN. PLEKHA4, pleckstrin homology domain-containing family A member 4; sh, short hairpin; NC, negative control.

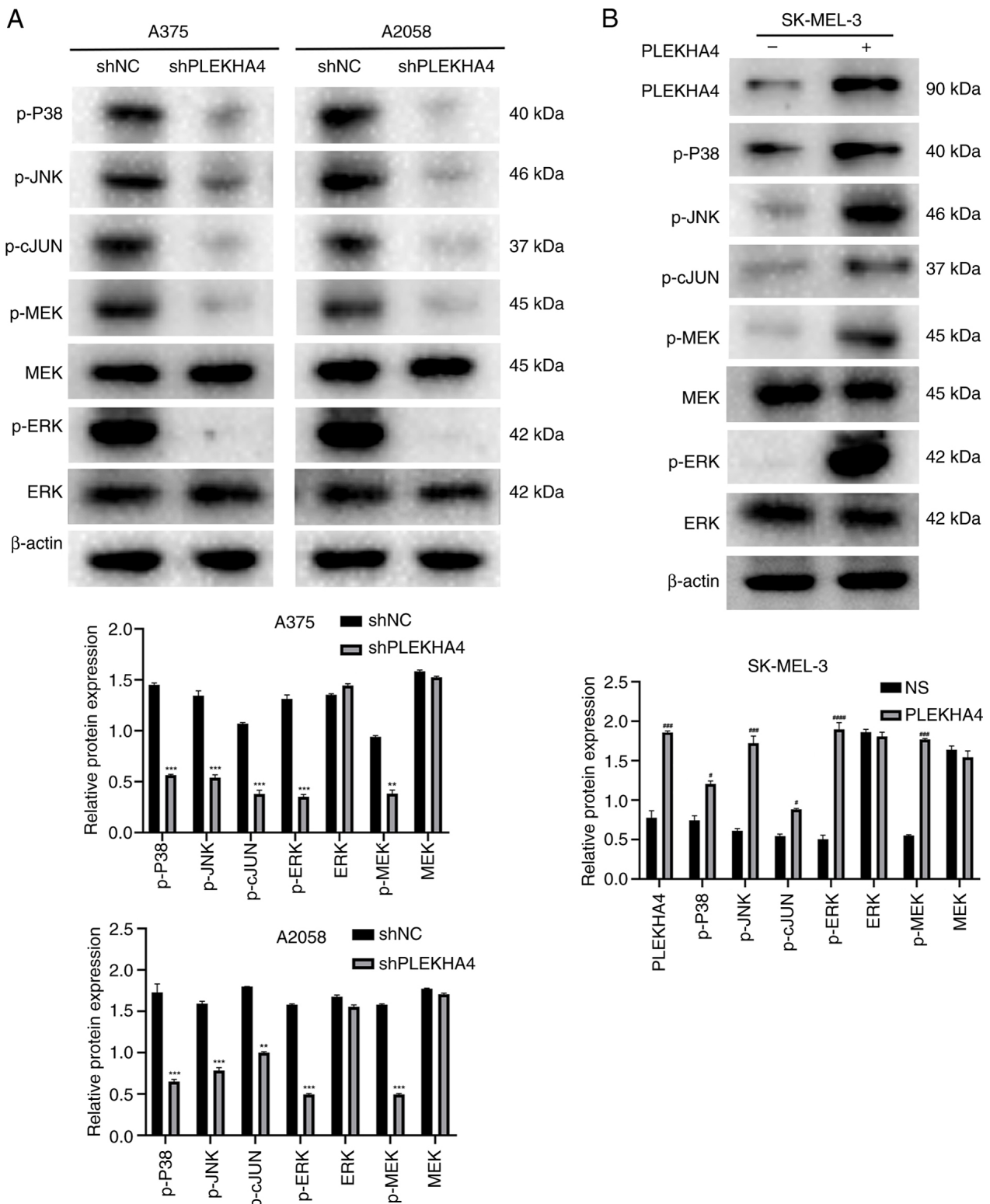


Figure 6. Impact of PLEKHA4 knockdown and overexpression on MAPK pathway protein expression in melanoma cells. (A) Western blotting of proteins in the MAPK pathway after PLEKHA4 knockdown ** $P < 0.01$, *** $P < 0.001$ vs. shNC. (B) Western blotting of proteins in the MAPK pathway after PLEKHA4 overexpression. * $P < 0.05$, *** $P < 0.001$ and **** $P < 0.0001$ vs. NS. PLEKHA4, pleckstrin homology domain-containing family A member 4; sh, short hairpin; NC, negative control; NS, empty vector; p-, phosphorylated.

cMyc ubiquitination is inhibited after PLEKHA4 knockdown. cMyc is dysregulated in ~70% of human cancer cases, with substantial evidence linking aberrant cMyc expression to both tumor initiation and maintenance (18). GeneMANIA results indicated that cMyc has physical interactions with multiple

members in MAPK pathway (Fig. 5C). Subsequently, a correlation analysis was performed and it was revealed that MYC was positively correlated with MAPK1 and MAPK14 (Fig. 9A). Subsequent western blot analysis was conducted to investigate cMyc expression following the inhibition of

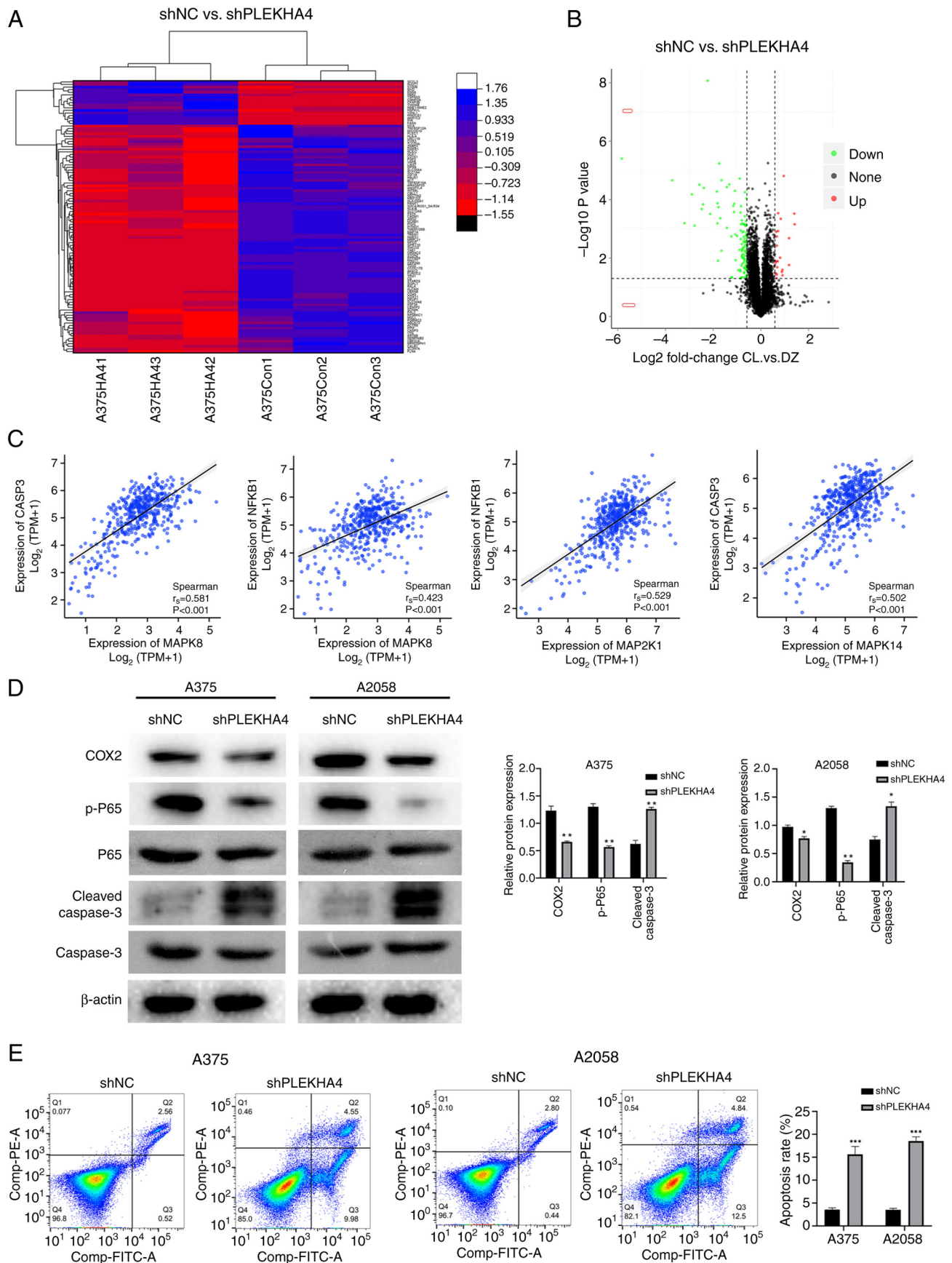


Figure 7. Analysis of PLEKHA4 knockdown effects on proteomic profiles and apoptosis in melanoma cells. (A) Expression results of significantly different proteins from tandem mass tag proteomics. (B) Volcano plot of tandem mass tag proteomics highlighting the differentially expressed proteins in shPLEKHA4 vs. shNC. (C) Correlation of NFKB1 and CASP3 with MAPK8, MAP2K1 and MAPK14. (D) Western blotting of PLEKHA4 knockdown cells showed changes in the protein expression levels of COX2, p-P65 and cleaved caspase-3. (E) Apoptosis was determined by flow cytometry in shNC and shPLEKHA4 melanoma cells. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. shNC. PLEKHA4, pleckstrin homology domain-containing family A member 4; sh, short hairpin; NC, negative control; p-, phosphorylated.

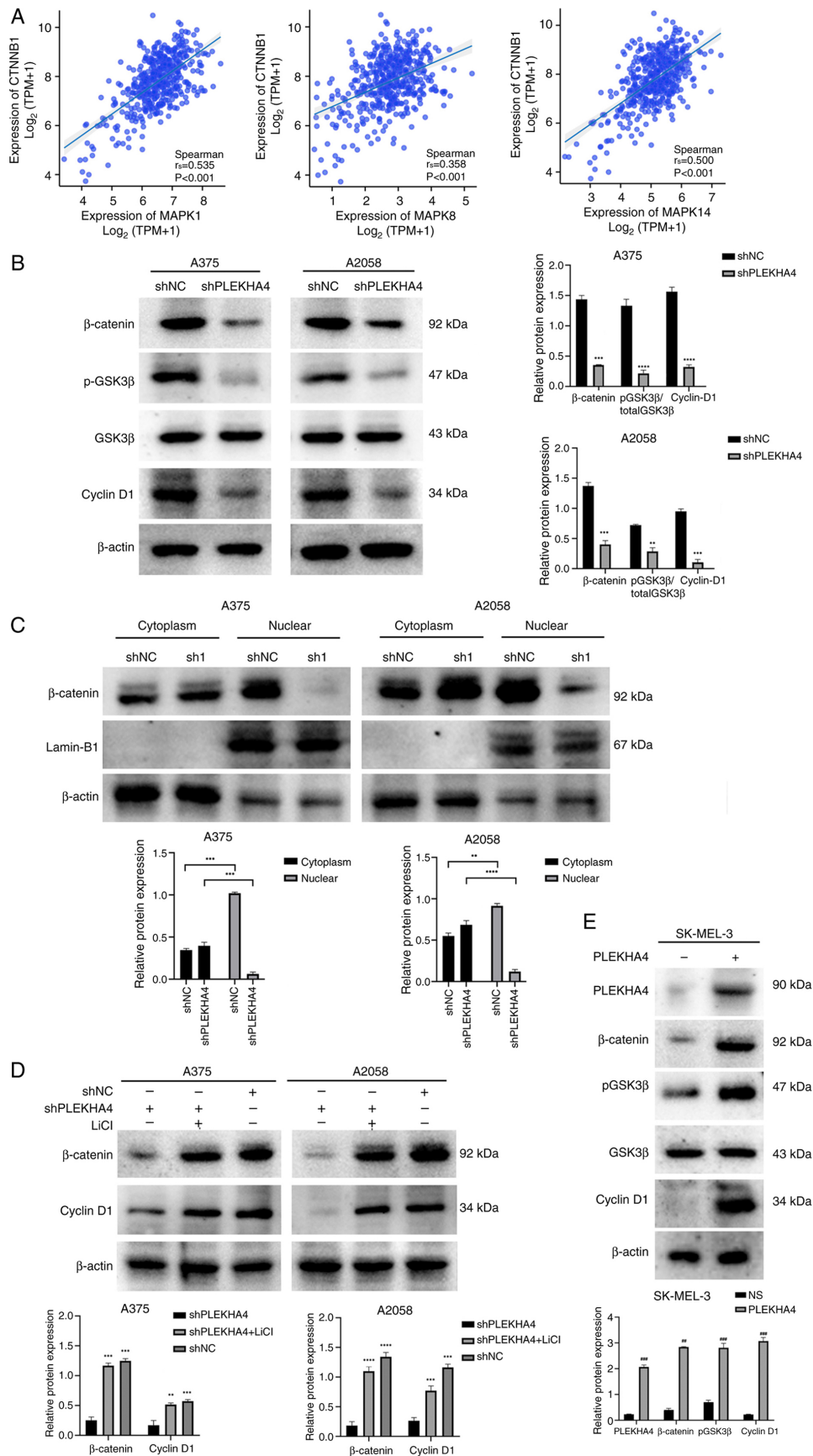


Figure 8. PLEKHA4 regulates the β -catenin pathway in melanoma. (A) Correlation of CTNNB1 with MAPK1, MAPK8 and MAPK14 in melanoma. (B) Western blotting revealed that PLEKHA4 knockdown reduced the expression of β -catenin, p-GSK3 β and cyclin D1. (C) PLEKHA4 knockdown affected the subcellular localization of β -catenin in A375 and A2058 cells. (D) LiCl treatment reversed the protein expression changes induced by PLEKHA4 knockdown. (E) Overexpression of PLEKHA4 in SK-MEL-3 cells increased the levels of β -catenin, p-GSK3 β and cyclin D1. ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ vs. shNC or as indicated. # $P < 0.01$, ## $P < 0.001$ vs. NS. PLEKHA4, pleckstrin homology domain-containing family A member 4; sh, short hairpin; NC, negative control; NS, empty vector; p-, phosphorylated; LiCl, lithium chloride.

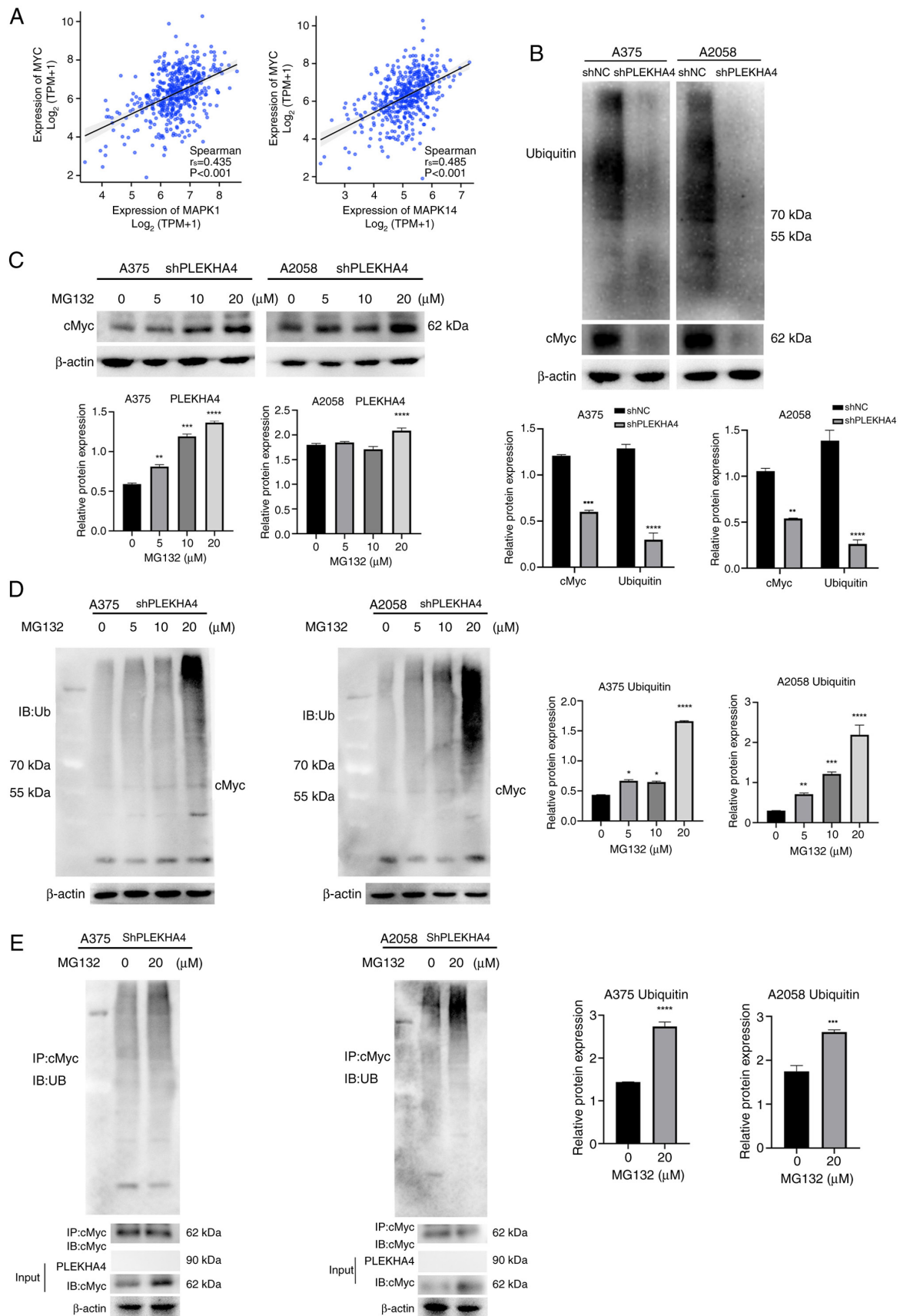


Figure 9. PLEKHA4 knockdown modulates cMyc expression and ubiquitination. (A) Correlation analysis of MYC with MAPK1 and MAPK14. (B) PLEKHA4 knockdown reduced cMyc expression. ** $P<0.01$, *** $P<0.001$ and **** $P<0.0001$ vs. shNC. (C) PLEKHA4 knockdown cells were treated with different concentration of MG132. Western blotting showed that MG132 reversed cMyc expression compared to the shPLEKHA4 group; higher concentrations of MG132 resulted in higher cMyc expression. ** $P<0.01$, *** $P<0.001$ and **** $P<0.0001$ vs. MG132 0 μ M. (D) PLEKHA4 knockdown cells were treated with different concentration of MG132. Western blotting showed that MG132 reversed ubiquitination. (IB:Ub). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ and **** $P<0.0001$ vs. MG132 0 μ M. (E) MG132 reversed cMyc ubiquitination was verified by co-immunoprecipitation. *** $P<0.001$ and **** $P<0.0001$ vs. MG132 0 μ M. PLEKHA4, pleckstrin homology domain-containing family A member 4; sh, short hairpin; NC, negative control.

MAPK signaling in PLEKHA4 knockdown melanoma cells. PLEKHA4 knockdown markedly reduced cMyc and ubiquitin expression at the protein level (Fig. 9B). cMyc, as an unstable protein, has a half-life of <30 min in non-transformed cells and is rapidly degraded mainly by the ubiquitin-proteasome pathway (19). To examine whether cMyc was stabilized through the ubiquitin-proteasome pathway, shPLEKHA4 melanoma cells were treated with different concentrations of the proteasome inhibitor MG132 (0, 5, 10 and 20 μ M). cMyc protein expression was rescued by different concentrations of MG132 (Fig. 9C), suggesting that cMyc degradation could be inhibited via the ubiquitin-proteasome pathway. A higher concentration of MG132 resulted in a higher level of cMyc protein. Subsequently, a ubiquitination assay was employed to detect the influence of PLEKHA4 on cMyc ubiquitination. As revealed in Fig. 9D, MG132 markedly upregulated cMyc polyubiquitination, with higher MG132 concentrations resulting in elevated levels. Furthermore, co-immunoprecipitation was conducted to test cMyc polyubiquitination. As shown in Fig. 9E, cMyc and ubiquitin were detected; however, PLEKHA4 was not detected. This suggests that the PLEKHA4 does not directly interact with cMyc polyubiquitination; however, knockdown of PLEKHA4 resulted in decreased cMyc expression and its ubiquitination, thus suggesting that PLEKHA4 may regulate cMyc and its polyubiquitination indirectly, possibly through another molecule. In Fig. 9E, although PLEKHA4 was knocked down, MG132 still increased cMyc ubiquitination in its absence, thus indicating that while PLEKHA4 may influence cMyc ubiquitination, other pathways or factors may also be involved in regulating this process. Collectively, these results strongly suggested that cMyc is degraded and ubiquitinated by the proteasome system, which may be related to molecules other than PLEKHA4.

Discussion

Initially, it was observed that cell proliferation was inhibited following PLEKHA4 knockdown. To further investigate the role of PLEKHA4, additional analyses, including proteomics analysis, were conducted.

Proteomics analysis revealed that PLEKHA4 knockdown led to changes in proteins associated with the MAPK pathway and apoptosis. Subsequently, GeneMANIA analysis was performed. The GeneMANIA database combines genomics and proteomics data from sources such as the GEO, BioGRID, Ensembl and Pathway Commons. To validate the results from GeneMANIA, correlation analyses were conducted. The analyses were performed using RNA sequencing data from TCGA-SKCM project, a widely recognized and reliable database. The authors recognize the need for further experiments to validate molecular interactions and plan to explore these mechanisms in future studies. Furthermore, the bioinformatics investigations unveiled the activation of the MAPK and β -catenin pathways in melanoma, and the correlation analysis revealed the interaction between β -catenin and MAPK pathway genes in melanoma. Notably, up to 90% of melanoma cases exhibit aberrant MAPK pathway activation, disrupting the cell cycle and impeding apoptosis (22). Western blotting revealed that PLEKHA4 knockdown affected both the expression and activation of ERK, while also suppressing p38, JNK and cJUN activation. In cancer

cells, aberrant protein expression in the JNK and p38 MAPK pathways is frequently observed. Research has indicated that elevated JNK and p38 MAPK signaling can promote tumor growth and facilitate cancer cell invasion (23,24). Conversely, it has also been suggested that downregulation of p38 MAPK signaling in tumor cells contributes to anoikis resistance and enhances the survival of circulating cancer cells (24). Therefore, the role of JNK and p38 MAPK signaling in cancer remains a topic of debate.

ERK can act as an antiapoptotic agent by downregulating proapoptotic proteins and upregulating antiapoptotic proteins through transcriptional and post-translational mechanisms (25). Multiple studies have indicated that ERK can influence mitochondria to trigger cytochrome c release via pro-apoptotic molecules such as Bax and/or p53 (25,26). Inhibition of the ERK pathway has been shown to reduce Bax expression induced by cisplatin or H_2O_2 (27,28). The TMT proteomics results showed changes in the expression of the apoptosis-related proteins COX2 and caspase-3. COX2 prolongs the G_1 phase in cancer cells, thereby suppressing apoptosis (29,30). Caspase-3 acts as a critical effector caspase downstream in the apoptotic signaling pathway, executing the final stages of programmed cell death (31). While suppressing proliferation is key in cancer therapy, the data of the present study showed that PLEKHA4 knockdown markedly induced cell apoptosis. Quantitative proteomics revealed significant changes in apoptosis-related proteins after PLEKHA4 knockdown, highlighting the role of cell death pathways. This focus aligns with the goal of exploring mechanisms that actively eliminate cancer cells rather than just slowing their growth. In the present study, PLEKHA4 knockdown reduced COX2 and increased cleaved caspase-3 expression, suggesting the role of MAPK in regulating apoptosis through interactions with COX2 and caspase-3. Activation of the MAPK pathway can lead to the induction of NF κ B signaling (32,33). NF κ B activation often inhibits apoptosis by promoting the transcription of anti-apoptotic genes (34). The present study demonstrated that downregulation of the MAPK pathway inhibited NF κ B activation. Therefore, these results suggested that knocking down PLEKHA4 may stimulate melanoma cell apoptosis through MAPK signaling.

PLEKHA4 enhances Wnt signaling by inhibiting the cullin-3 E3 ubiquitin ligase complex, reducing DVL polyubiquitination (35). Silencing PLEKHA4 can impact the expression of cyclin D and cMyc (36), an effect that the present study confirmed and could be reversed by the Wnt activator LiCl, highlighting the pivotal role of PLEKHA4 in Wnt/ β -catenin signaling in melanoma. Additionally, PLEKHA4 knockdown reduced p-GSK-3 β protein levels. GSK-3 β , known to aid cancer therapy resistance by enhancing DNA repair (37), has been widely studied, although its role in melanoma chemoresistance necessitates further elucidation.

Increased Wnt/ β -catenin activity can raise cMyc levels, promoting tumor growth (36,37). Additionally, cMyc serves as a recognized downstream effector of the MAPK signaling pathway (38). Correlation analysis further validated the association between cMyc and MAPK-related proteins. Evaluation of the effects of PLEKHA4 knockdown on cMyc expression revealed diminished cMyc levels, aligning with the results of a previous study that used a similar melanoma cell line (38). Notably, cMyc has a central role in almost every

aspect of the oncogenic process, orchestrating proliferation, apoptosis, differentiation and metabolism (39-42). Although PLEKHA4 was knocked down in the present study prior to co-IP analysis, a small amount of expression remained. Notably, the co-IP results did not detect a direct interaction between cMyc and PLEKHA4. Instead, it was observed that c-Myc expression was restored upon the addition of the proteasome inhibitor MG132, thus suggesting that PLEKHA4 likely influences other components of the proteasomal pathway rather than directly interacting with c-Myc to regulate its degradation. These results suggested that cMyc is degraded and ubiquitinated through the proteasome system. Typically, cMyc is a short-lived protein marked for breakdown (43,44), likely involving molecules other than PLEKHA4. Treatment with the proteasome inhibitor MG132 effectively reversed the degradation of cMyc following PLEKHA4 knockdown; however, after PLEKHA4 knockdown, reduced levels of cMyc ubiquitination were observed compared with in the shNC group. In cells, high levels of ubiquitination do not always indicate increased protein degradation. Ubiquitination can serve various functions depending on the type of ubiquitin chains attached. For example, K48-linked chains generally target proteins for degradation, whereas K63-linked chains are involved in non-degradative roles, such as signaling and localization (45,46). Additionally, deubiquitinating enzymes can regulate ubiquitination by removing ubiquitin from proteins, creating cycles of ubiquitination and deubiquitination that raise ubiquitination levels without leading to efficient degradation (47,48). Furthermore, proteins may be rapidly ubiquitinated but slowly degraded due to delayed recognition by the proteasome, or they may accumulate in a ubiquitinated state when the proteasome is saturated, such as under cellular stress (49,50). Finally, some proteins employ feedback mechanisms to control their stability, remaining ubiquitinated yet stable until receiving a specific degradation signal (51). Collectively, these factors contribute to the complex relationship between ubiquitination and protein degradation. We aim to delve deeper into the relationship between cMyc and its ubiquitination in the future, along with investigating the roles of Wnt/ β -catenin and MAPK signaling pathways in regulating cMyc ubiquitination.

In conclusion, PLEKHA4 is upregulated in melanoma, promoting cell proliferation. By contrast, its knockdown triggers apoptosis through the MAPK and β -catenin pathways, and enhances cMyc degradation. These findings highlight the role of PLEKHA4 in melanoma progression and suggest its potential as a therapeutic target. In future studies, we aim to investigate the mechanisms by which PLEKHA4 regulates cMyc degradation and ubiquitination, as well as the roles of JNK and p38 MAPK signaling in melanoma.

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Availability of data and materials

The data generated in the present study may be found in the PRIDE database under accession number PXD053608 or at the following URL: <http://www.ebi.ac.uk/pride/archive/projects/PXD053608>. All other data generated in the present study may be requested from the corresponding author.

Authors' contributions

YY, GA and SC performed the experiments. DX made substantial contributions to data analysis. XL and LD made substantial contributions to the bioinformatics analysis, drafted the manuscript, critically reviewed the manuscript for important intellectual content and constructed the figures. LL and DX made substantial contributions to the conception or design of the work. LL and DX 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

The present study was approved by the Laboratory Animal Ethics Committee of Yanbian University (approval no. YD20230911021). Yanbian University Hospital is affiliated with Yanbian University and all ethical approvals for Yanbian University Hospital are issued by Yanbian University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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