Supplementary Materials

Materials and Methods

Cell lines and cell culture

The human melanoma cell line (A375), human melanocyte cell line (PIG1), human keratinocyte cell line (HaCaT), and human embryonic kidney cell line (293FT) were obtained from the American Type Culture Collection (ATCC, USA), and the human melanoma cell line (MV3) was obtained from the University Hospital Nijmegen. A375, PIG1, and HaCaT cells were cultured in DMEM (Gibco, Thermo Fisher Scientific), and MV3 cells were cultured in RPMI-1640 (Gibco, Thermo Fisher Scientific). Both media were supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin (P/S; BC). The 293FT cells were cultured in DMEM containing 10% fetal bovine serum, 2% glutamine, 1% non-essential amino acids, 1% sodium pyruvate, and 1% G418 (Invitrogen, USA). All cells were cultured in a 37°C incubator containing 5% CO_2.

Drug treatment

Kuwanon A (KA) (Cat. No.: PS013354) was purchased from Chengdu Push Biotechnology Co., Ltd. (Chengdu, China) and dissolved in dimethyl sulfoxide (DMSO, Cat. No.: D2650-100ML, Sigma-Ald, Beijing, China) at 100 mmol/L as the stock solution. Cisplatin (CDDP, Cat. no. HY-17394, MCE, USA) was dissolved in N, N-dimethylformamide (DMF, Cat. No.: HY-Y0345, MCE, USA).

Cell viability assay

A total of 1000 A375 or MV3 cells and 200 μL of complete medium were added to each well of a 96-well plate, incubated overnight, and treated with different concentrations of KA or CDDP for 48 h. An equal amount of DMSO or DMF was used as the negative control. Cell viability was determined using a cell counting kit-8 (CCK-8) assay. At the indicated time points, cells were incubated for 2 h with 100 μL of medium and 10 μL of CCK-8 (TargetMol, USA) according to the manufacturer's instructions. The absorbance was measured at 490 nm using an enzyme marker (MDSpecteaMax iD5, USA). Finally, the IC50 values were calculated using

GraphPad Prism 8. Each experiment was performed independently and repeated three times.

EdU/DAPI staining

Next, 2×10^4 logarithmic growth phase A375 or MV3 cells were seeded in 24-well plates, cultured overnight, and treated with KA for 48 h. EdU staining was performed according to the manufacturer's instructions (Beyotime, Shanghai, China). Cells were firstly incubated with 10 μM EdU for 2 h, then fixed with 4% paraformaldehyde (PFA) for 15 min, incubated with 0.3% Triton X-100 for 10 min, incubated with 5% bovine serum albumin (BSA) for 1 h, and incubated with Click reaction cocktails for 30 min. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) for 30 min at 25°C before observation under a microscope.

Plate clone formation and soft agar assays

Briefly, 1000 cells (A375 or MV3) were cultured overnight in 6-well plates (37°C, 5% CO_2). The cells were cultured alone or in combination with CDDP in a medium containing KA for 6 days. They were fixed with 4% PFA for 15 min, stained with 0.1% crystal violet for 20 min, and washed three times with phosphate-buffered saline (PBS) for 5 min each. Finally, images were acquired using a digital scanner. The soft agar experiments were performed as previously described. Each experimental group was conducted independently in triplicate.

For the soft agar assay, 2 × DMEM and 1.2% agarose were mixed at a 1:1 ratio in a tube and added to a 6-well plate. One thousand cells were mixed in 1 × DMEM, mixed with 2 × DMEM and 1.2% agarose at a ratio of 2:1:1, and added to the solidified lower gel as the upper gel. After culturing for 25–35 days, images were scanned using a scanner, and colonies were counted.

Wound-healing assay

Cells were cultured in 24-well plates and grown to 80–90% confluence. Scratch lines were etched on the monolayer of the cells with a 200 μL pipette tip. Subsequent cultures were performed using a serum-free medium containing 10 μM KA with DMSO as control. The wound widths were examined and imaged using a microscope at 0, 12, 24, and 48 h. The percentage of wound closure was calculated as follows:

(initial wound width × designated time-wound width)/ initial wound width × 100%. Each experiment was performed independently and repeated three times.

Migration transwell assay

An 8 mm Transwell chamber (Corning, Beijing, China) was used for the migration experiments. First, 200 μL serum-free medium containing 1 × 10^5 cells and 20 μM KA was added to the upper chamber, with DMSO as the control. The upper chamber was placed in the lower chamber, which was filled with 500 μL of medium containing 10% FBS. After incubation for 16 h and 24 h for the migration and invasion assays, respectively, the non-migrating and non-invasive cells remaining in the upper chamber were carefully wiped. Finally, the lumen was stained with a crystal violet staining solution (Beyotime), and the stained cells were quantified under an inverted microscope (Nikon). Each experiment was performed independently and repeated three times.

Flow cytometry assay

For the cell cycle assay, A375 and MV3 cells were treated with KA for 48 h, with equal amounts of DMSO used as negative controls. Cell samples were collected and fixed with 70% ethanol at 4°C for 24 h. Cells were centrifuged and resuspended in pre-cooled PBS and stained with propidium iodide and RNase A for 1 h at 37°C, protected from light. The cells were identified using flow cytometry. Each group comprised three replicates. Data were analyzed using Modfit software.

Western blot analysis

KA- or equivalent DMSO-treated cells were harvested using a cell scraper, centrifuged at 800 rpm for 5 min, washed with pre-cooled PBS, and the supernatant was aspirated. Cells were lysed using pre-cooled RIPA lysis buffer containing a phosphatase inhibitor and phenylmethylsulfonyl fluoride for 30 min, and the protein concentration was measured using the BCA Protein Assay Kit. A 5 × loading buffer was added at a 1:4 ratio, and the mixture was heated in a water bath at 100°C for 10 min. Subsequently, transblotting and SDS-PAGE were performed. After blocking the PVDF membranes with 5% TBST diluted in skim milk powder for 2 h at room temperature, the membranes were incubated overnight at 4°C on a shaker with the following primary antibodies: tubulin (1:50,000, Cat. no. 66031-1-lg, Proteintech,

Wuhan, China) and *CDK2* (1:2000, Cat. No. 10122-1-AF; Proteintech, Wuhan, China) and *CDK4* (1:1000; Cat. no. sc-56277, Santa Cruz, USA), *CDK6* (1:1000, Cat. no. sc-53638, Santa Cruz Biotechnology), *Cyclin D1* (1:1000, Cat. No.: A19038, ABclonal, Wuhan, China), *P27* (1:4000, Cat. No. 10355-1-AP, Proteintech, Wuhan, China), *N-cadherin* (1:10000, Cat. no. 22018-1-AP, Proteintech, Wuhan, China) and *MMP2* (1:2000, Cat. no. 66366-1-lg; Proteintech, Wuhan, China), β-catenin (1:1000; Cat. no. sc-376841, Santa Cruz Biotechnology), HA-Tag (1:5000, Cat. No.: AE008, ABclonal). The PVDF membranes were washed with TBST and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG for 2 h at 25°C. Finally, protein bands were visualized using an ECL reagent (Beyotime, China) and a WB detection system (FUSION FX. EDGE, France).

Quantitative real-time PCR

Briefly, cells were collected by trypsin digestion, and total RNA was extracted using TRIzol reagent according to the manufacturer's instructions. Supplementary Table 1 lists the primers used in this study. *ACTB* was used as the internal reference. Finally, mRNA expression levels were calculated as described in PMID: 11846609.

Ubiquitination and turnover assays

HA-Ub and Flag-β-catenin plasmids were transferred into 293FT cells for the ubiquitination assay. Cells were collected and subjected to immunoprecipitation (co-IP) and WB analyses after 8 h of incubation in MG132 (20 mg/mL, Sigma-Aldrich), as previously reported. For the protein turnover assay, cells were treated with KA or DMSO, then extracted and incubated with 100 g/mL cycloheximide for the indicated time periods before WB analysis.

Transfection and infection

To overexpress β-catenin in melanoma cells, we cloned human β-catenin full-length cDNA into pcdhh-cmvmcs-ef1-gfp+Puro lentiviral vector (Unibio, Chongqing, China). Liposomes, packaging plasmids (pLP1, pLP2, pLP/VSVG), and OE-β-catenin were transferred into 293FT cells using Lipofectamine™ 2000 Transfection Reagent (Thermo Fisher), according to the manufacturer's instructions, with the empty vector used as a negative control. Melanoma cell lines were infected with the harvested lentiviruses and screened with 4 mg/mL puromycin (Sigma-Aldrich) to produce stably

transfected cell lines.

Tumor xenograft assay

Sixteen 1-month-old female BALB/C-nu mice were maintained in a specific pathogen-free chamber. Each mouse was subcutaneously injected with 1×10^6 MV3 cells. Seven days after injection (when the tumor was palpable), the mice were randomly divided into control, KA (20 $mg.kg.day^{-1}$), CDDP (4 $mg.kg.day^{-1}$), and KA + CDDP groups, with four mice in each group. The corresponding intraperitoneal injections were administered daily. KA was dissolved in corn oil (Changshouhua, Sanxing Group, Binzhou, China), and CDDP was dissolved in saline solution. The length and width of the tumors were measured every 2 days using an electronic caliper, and the weight of the mice was measured using an electronic balance. Tumor volume was calculated as follows: volume = tumor length $\times$ width^2 $\times$ $\pi/6$. At the end of the experiments, the mice were euthanized, and the tumors and organs were excised, weighed, and immersed in 4% PFA. This study was approved and supervised by the

Animal Ethics Committee of Hebei Medical University (No: IACUC-Hebmu-2024072).

H&E staining

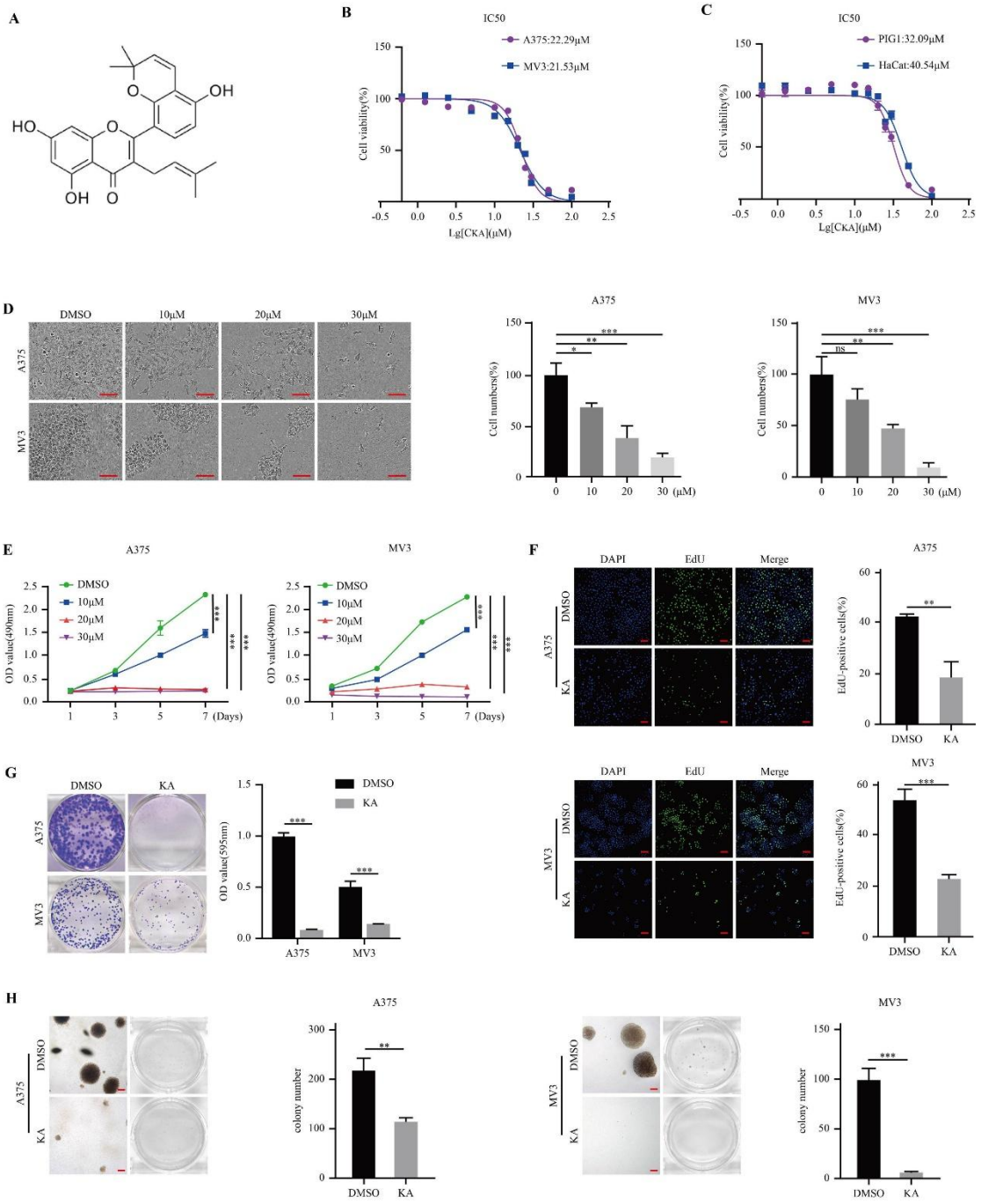
After the experiments, the mice were euthanized, and their hearts, livers, spleens, lungs, kidneys, and stomachs were completely removed. Tissue samples were sequentially fixed, dehydrated, embedded, and sectioned. The sections were stained with an H&E staining kit (Beyotime) according to the manufacturer's instructions.

Immunohistochemistry

Tumors were embedded in paraffin, deparaffinized, dehydrated, and treated with antigens. The sections were incubated with Ki-67 (1:200; Cat. No.: MA5-14520, Invitrogen) or β-catenin (1:200; Cat. no.: sc-376841; Santa Clara, CA, USA) overnight at 4°C, washed with PBS, and incubated with a secondary antibody. Finally, the sections were stained with DAB and dehydrated, and images were obtained under a microscope.

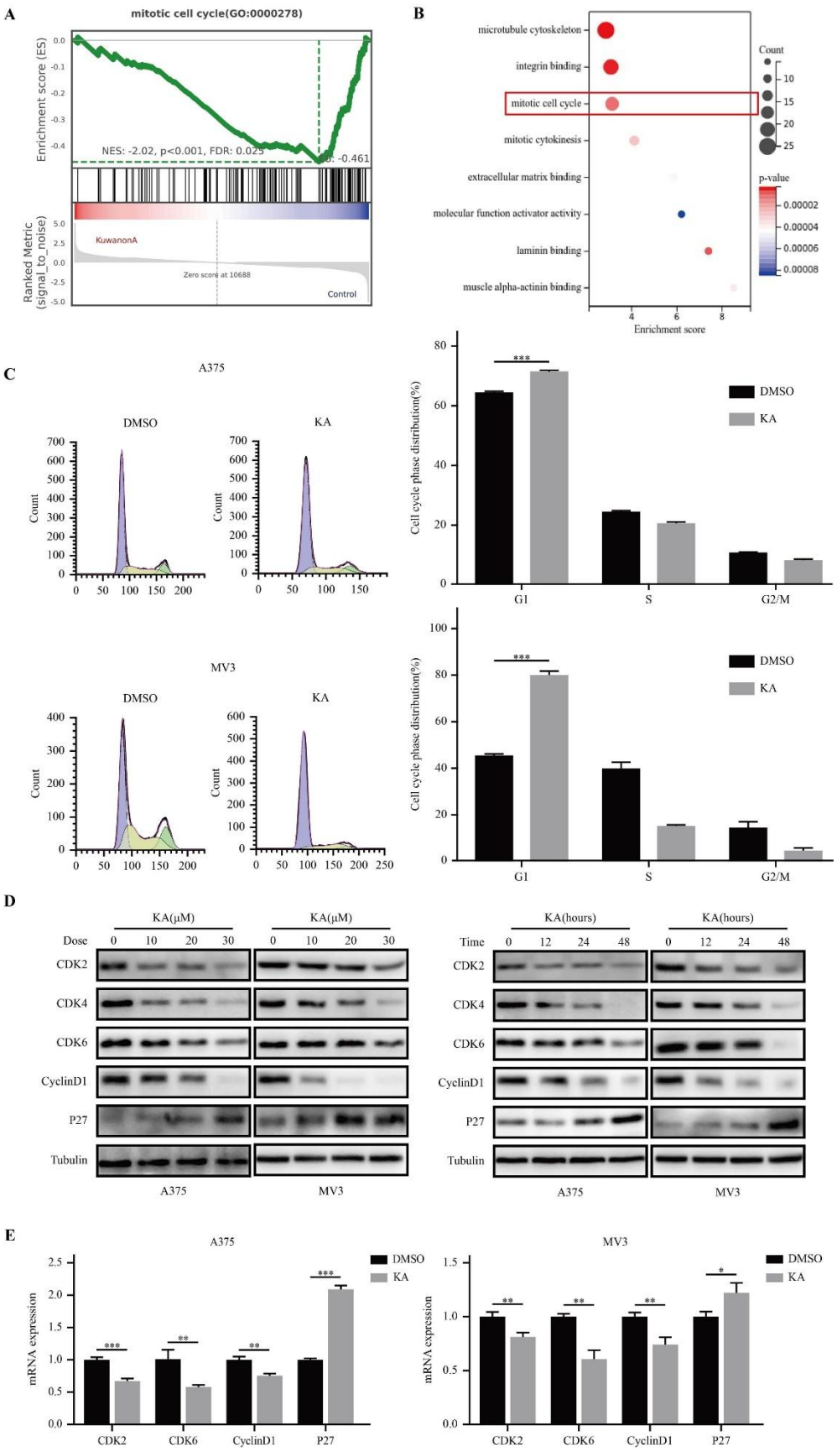
Statistical analysis

All data were independently collected three times and analyzed using GraphPad Prism 8. Results are presented as mean ± standard deviation. Statistical significance was assessed using one-way ANOVA or unpaired Student's *t*-test, with *P*-value < 0.05 indicating significance (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). The efficacy of the combination was evaluated using Jin's formula [q = E (A+B)/(EA + EB − EA × EB)], where EA and EB are the inhibition rates of drugs A and B, respectively, E (A + B) is the combination inhibition rate, $q < 0.85$ indicates an antagonistic effect, $0.85 \leq q < 1.15$, superimposed effect, and $q \geq 1.15$, synergistic effect.



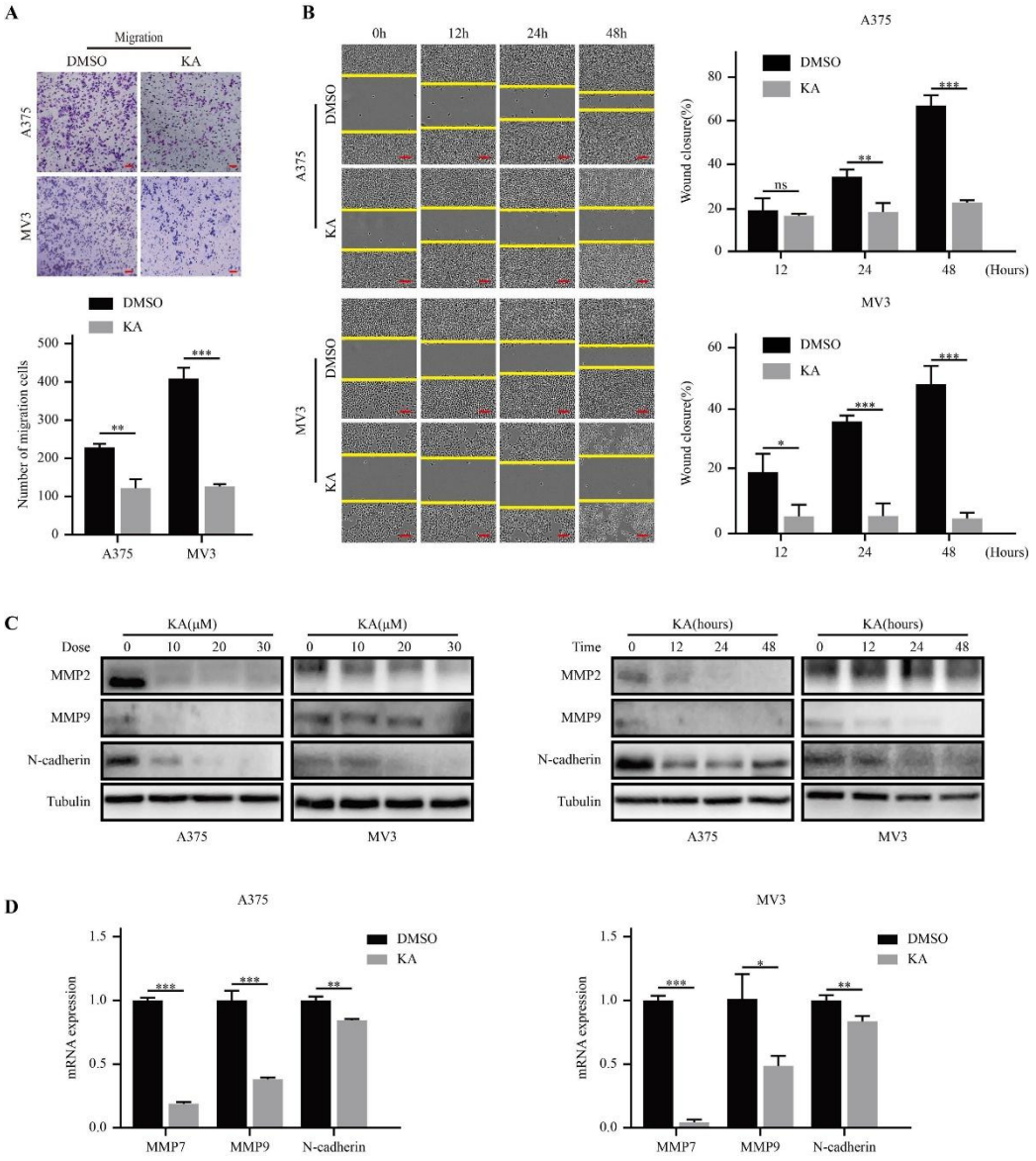
Supplementary Figure 1: KA inhibits melanoma cell proliferation *in vitro*. (A) Chemical structure of Kuwanon A. (B and C) Melanoma cells (A375 and MV3), immortalized melanocytes (PIG1), and human keratinocytes (HaCaT) were treated with a series of indicated concentrations of KA for 48 h. Cell viability was detected using CCK-8 assay. The IC50 values of KA in the tested cells were marked. DMSO was used as the control. (D) Changes in the number of A375 and MV3 cells after 48 h incubation with KA or DMSO. Scale bar = 100 μm. (E) Viability of A375 and MV3 cells after treatment with 10, 20, or 30 μmol/L KA. DMSO was used as the control. (F) A375 and MV3 cells were treated with KA or

DMSO for 48 h, their proliferation rates were measured with EdU, and the EdU positive rate was calculated. Scale bar = 100 μm. (G) A375 and MV3 cells were treated with KA for 6 days in a plate colony formation assay, and the OD value at 595 nm was measured to indicate the number of cells. (H) Soft agar assay was used to detect the colony-forming ability of A375 and MV3 melanoma cell lines. Scale bar = 100 μm. Each experiment was repeated independently, at least in triplicates. Data are expressed as mean ± SD. Student's *t*-test and one-way ANOVA were performed to analyze significance. **P* < 0.05, ***P* < 0.01, ****P* < 0.001; NS: non-significant.

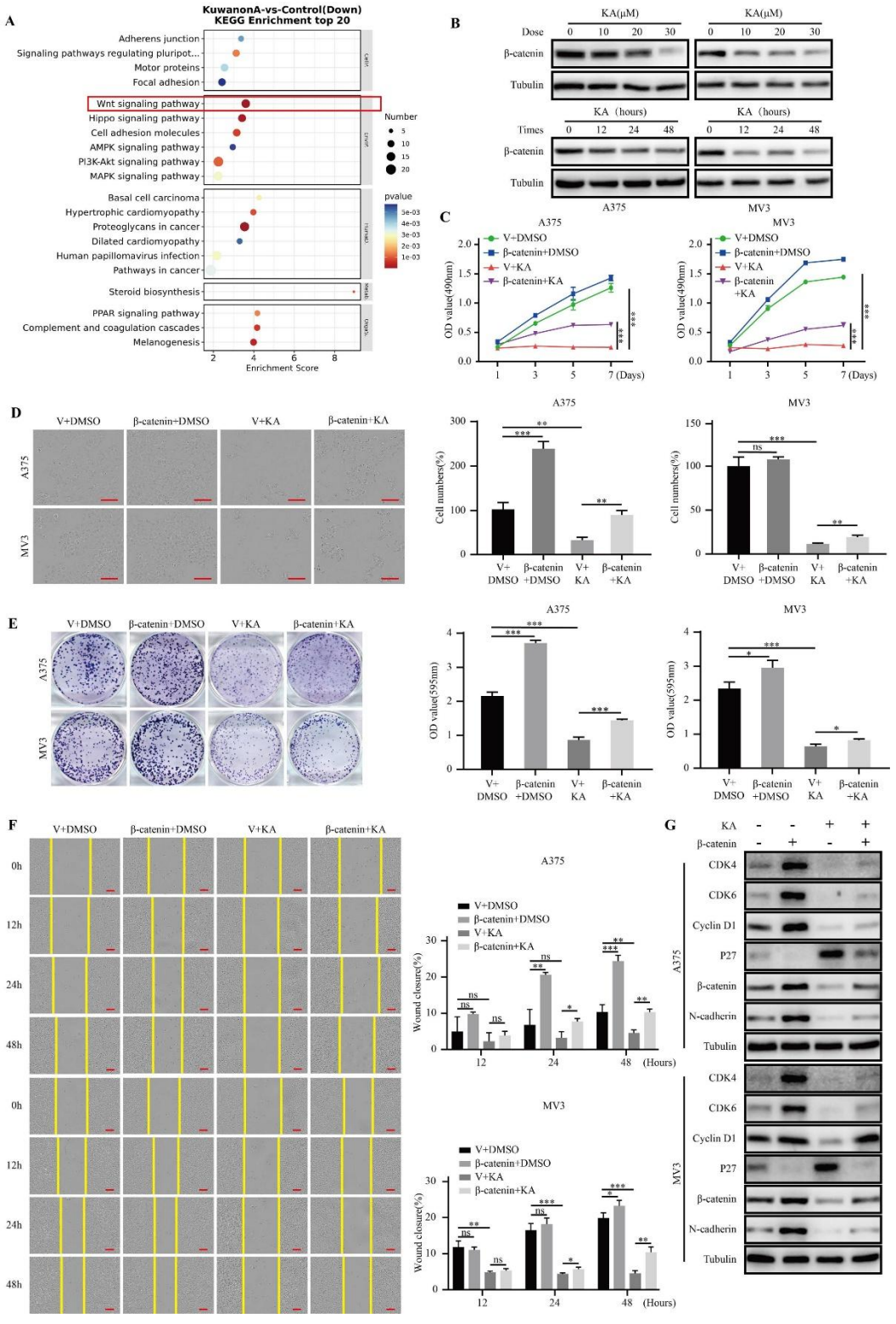


Supplementary Figure 2: KA-induced melanoma cell cycle arrest at G0/G1 phase.

(A and B) GSEA and KEGG analysis of downregulated genes in MV3 cells after KA treatment, based on RNA-seq data. Changes in the mitotic cell cycle pathway are shown. DMSO was used as the control. (C) Cell cycle was detected using flow cytometry after treating A375 and MV3 cells with the IC50 concentration of KA for 48 h, followed by fluorescent dye propidium iodide (PI) staining. DMSO was used as the control. (D) Protein expression levels of *CDK2*, *CDK4*, *CDK6*, *Cyclin D1*, and *P27* in A375 and MV3 cells treated with KA for indicated times were detected by western blot. DMSO was used as the control. (E) mRNA expression levels of *CDK2*, *CDK6*, *Cyclin D1*, and *P27* in A375 and MV3 cells treated with KA for 48 h were assessed by RT-qPCR. DMSO was used as the control. Data are expressed as mean ± SD. Student's *t*-test was performed to determine significance. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

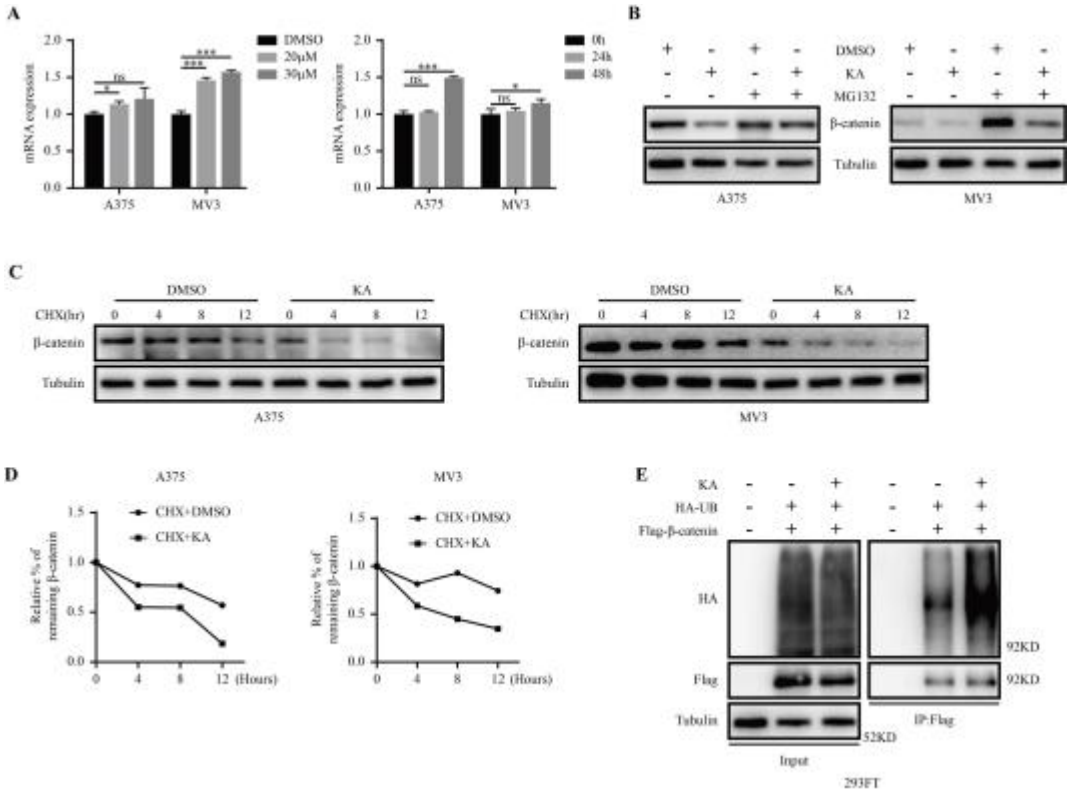


Supplementary Figure 3: KA inhibited melanoma cell migration *in vitro*. (A) Migration assay of A375 and MV3 cells treated with KA or DMSO. Scale bar = 100 μm. (B) Changes in the percentage of trabecular closure of A375 and MV3 cells under KA treatment. DMSO was used as the control. Scale bar = 100 μm. (C) Protein expression levels of *MMP2* and *N-cadherin* in A375 and MV3 cells treated with KA were detected by WB. DMSO was used as the control. (D) mRNA expression levels of *MMP7*, *MMP9*, and *N-cadherin* in A375 and MV3 cells treated with KA for 48 h were detected by RT-qPCR. DMSO was used as the control. Data are expressed as mean ± SD. Student's *t*-test was performed to assess significance. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.



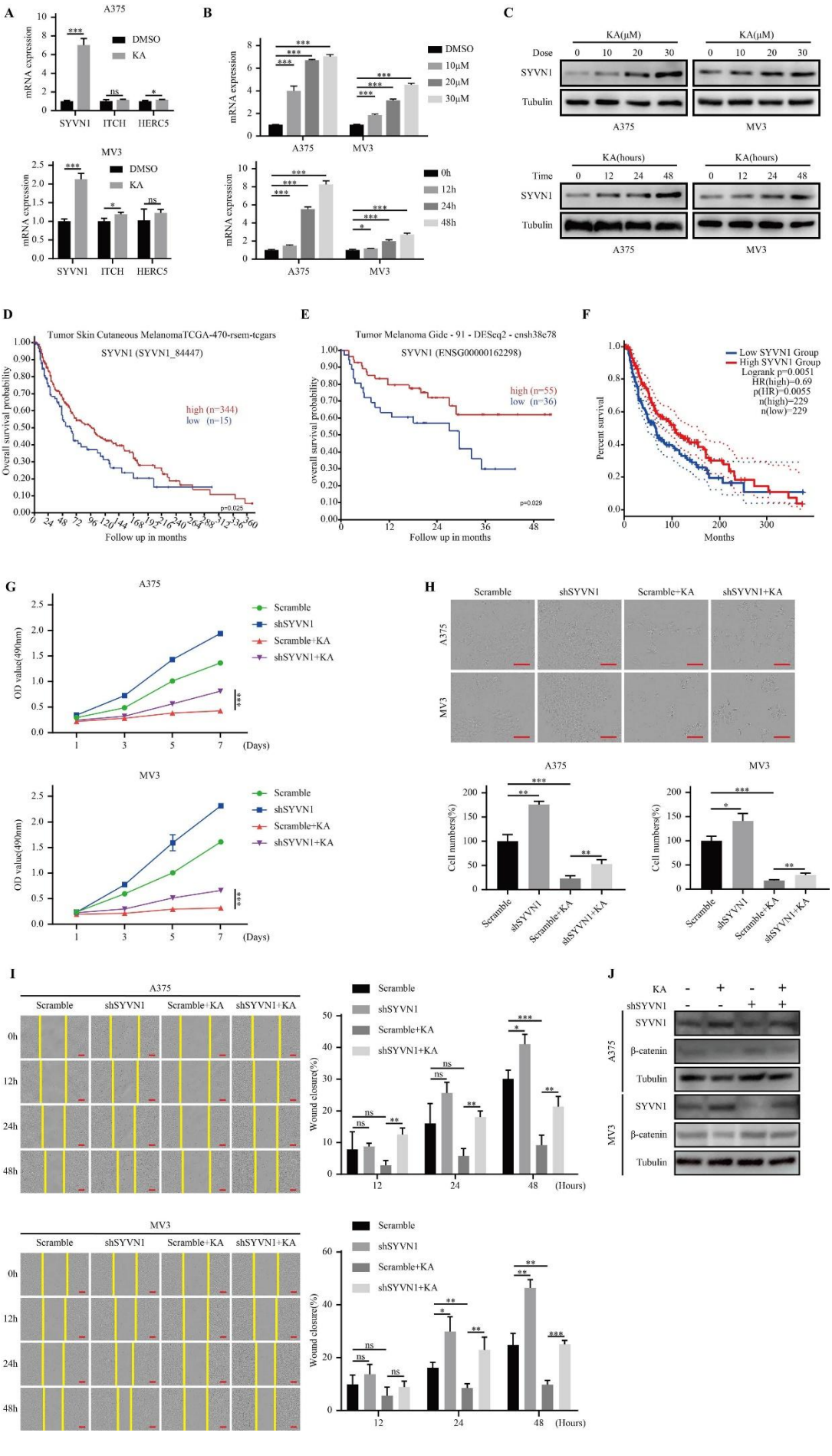
Supplementary Figure 4: KA downregulates β-catenin expression in melanoma cells, and overexpression of β-catenin partially reverses the inhibitory effect of KA on melanoma cells. (A) KEGG analysis of downregulated genes in MV3 cells after KA treatment (determined by RNA-seq data). The top 20 KEGG pathway process terms

based on fold enrichment are shown. DMSO was used as the control. (B) The protein expression levels of β-catenin in A375 and MV3 cells treated with KA were detected by western blot. DMSO was used as the control. (C) CCK-8 assay was used to detect cell proliferation of β-catenin overexpressing cells treated with KA or DMSO for the indicated times. DMSO was used as the control. (D) β-catenin overexpressing A375 and MV3 cells were treated with KA or DMSO for 48 h. Photographs were taken for observation, and cells were counted. Scale bar = 100 μm. (E) Plate colony assay was performed in β-catenin overexpressing A375 and MV3 cells treated with KA or DMSO for 6 days. The number of cells was indicated by the OD value at 595 nm. (F) Wound-healing assay was performed on normal or β-catenin overexpressing A375 and MV3 cells, and the healing of scratches was observed and counted at the indicated times. Scale bar = 100 μm. (G) The expression of cell cycle- and migration-related proteins in β-catenin overexpressing A375 and MV3 cells were detected by western blot. Cells were treated with KA or DMSO for 48 h. Data are expressed as mean ± SD. Student's *t*-test and one-way ANOVA were used to determine significance. * $P< 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns: non-significant.

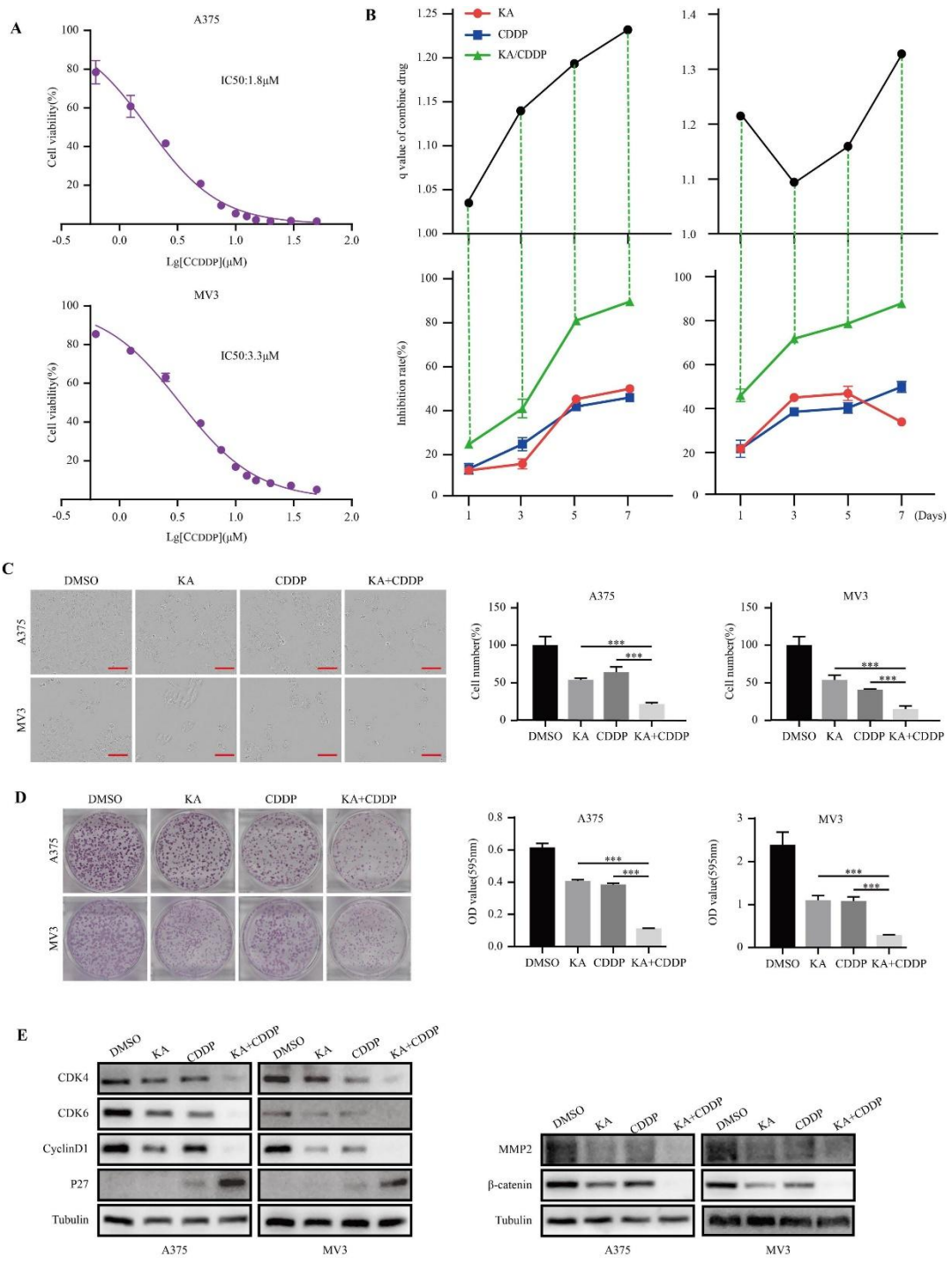


Supplementary Figure 5: KA decreased the protein value stability of β-catenin through the ubiquitin-proteasome pathway. (A) The mRNA expression levels of

β-catenin in A375 and MV3 cells treated with the indicated concentrations of KA for the indicated times were detected by RT-qPCR. DMSO was used as the control. (B) Protein expression of β-catenin in cells treated with KA or DMSO for 48 h was assessed in the presence or absence of MG132. (C and D) A375 and MV3 cells were pretreated with KA or DMSO, respectively, and subsequently treated with the protein synthesis inhibitor cycloheximide (CHX) for 0, 4, 8, and 12 h to block protein synthesis. β-catenin protein levels were detected by western blot. Microtubule proteins, which serve as reference proteins for the loading control of the same blot, were placed below the corresponding target proteins. Protein levels were calculated. (E) The indicated plasmids were transfected with 293FT cells and treated with KA or DMSO. Ubiquitinated β-catenin protein was pulled down with an anti-Flag antibody and immunoblotted with an anti-HA antibody. DMSO was used as the control. Data are expressed as mean ± SD. Student's t-test was used to assess significance. $*P < 0.05$, $***P < 0.001$.

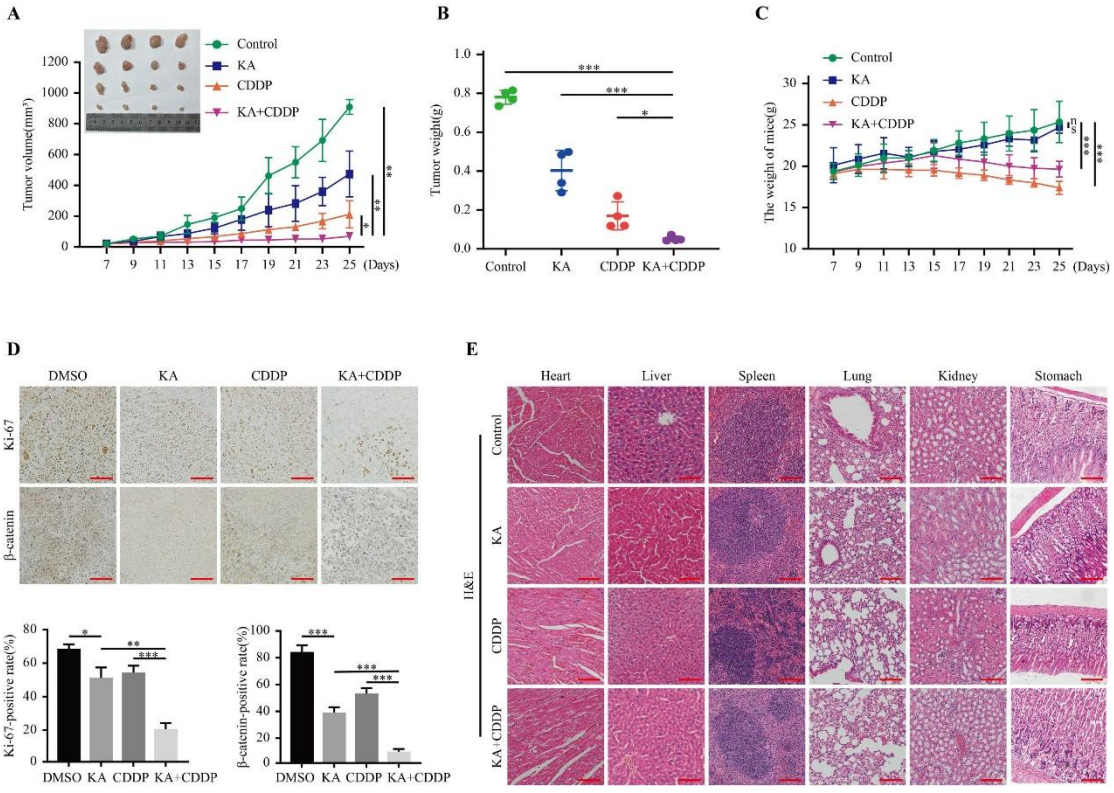

A
A375
mRNA expression
DMSO
KA
SYVN1
ITCH
HERC5
MV3
B
DMSO
10μM
20μM
30μM
0h
12h
24h
48h
C
KA(μM)
Dose
SYVN1
Tubulin
KA(hours)
Time
D
Tumor Skin Cutaneous MelanomaTCGA-470-rsem-tcgars
SYVN1 (SYVN1_84447)
high (n=344)
low (n=15)
Overall survival probability
Follow up in months
E
Tumor Melanoma Gide - 91 - DESeq2 - ensh38e78
SYVN1 (ENSG00000162298)
high (n=55)
low (n=36)
F
Low SYVN1 Group
High SYVN1 Group
Logrank p=0.0051
HR(high)=0.69
p(HR)=0.0055
n(high)=229
n(low)=229
Percent survival
Months
G
OD value(490nm)
Scramble
shSYVN1
Scramble+KA
shSYVN1+KA
(Days)
H
Cell numbers(%)
I
Wound closure(%)
(Hours)
J
β-catenin

Supplementary Figure 6: Kuwanon A (KA) decreases the protein stability of β-catenin through *SYVN1*. (A) The mRNA expression levels of three E3 ubiquitin-ligase enzymes in A375 and MV3 cells treated with KA for 48 h were detected by RT-qPCR. (B) The mRNA expression levels of *SYVN1* in A375 and MV3 cells treated with KA were detected by RT-qPCR. DMSO was used as the control. (C) The protein expression levels of *SYVN1* in A375 and MV3 cells treated with KA. DMSO was used as the control. (D and E) The association between *SYVN1* expression and the overall survival rate of patients with melanoma in the R2 database. (F) The effect of *SYVN1* expression level on percent survival in the GEPIA database. (G) CCK-8 assay was used to detect cell proliferation of sh*SYVN1*-downregulation cells treated with KA or DMSO for the indicated times. DMSO was used as the control. (H) sh*SYVN1*-downregulated A375 and MV3 cells were treated with KA or DMSO for 48 h. Photographs were taken for observation and counted. Scale bar = 100 μm. (I) Wound-healing assay was performed on normal or sh*SYVN1*-downregulated A375 and MV3 cells, and the healing of scratches was observed and counted at the indicated times. Scale bar = 100 μm. (J) Western blot assays were performed to examine the protein expression level of *SYVN1* and β-catenin in sh*SYVN1*-downregulated cells after treatment with KA or DMSO for 2 days. DMSO was used as the control. Data are expressed as mean ± SD. Student's *t*-test and one-way ANOVA were performed to analyze significance. $^{**}P < 0.01$, $^{***}P < 0.001$, NS: non-significant.



Supplementary Figure 7: KA enhances the chemosensitivity of melanoma cells to CDDP *in vitro*. (A) Melanoma cells (A375 and MV3) were treated with a gradient concentration of CDDP for 48 h. Cell viability was detected by CCK-8 assay. Semi-inhibitory concentrations of CDDP on two melanoma cell lines are indicated. DMSO was used as the control. (B) A375 and MV3 cells were treated with KA (10 μmol/L), or CDDP (1 μmol/L for A375 and 1.5 μmol/L for MV3), or their combination for 0, 1, 3, 5, and 7 days. Cell viability was measured using the CCK-8

assay. The q-value of the combination was calculated. (C) A375 and MV3 melanoma cells were treated with KA (10 μmol/L) /CDDP (1 μmol/L for A375 and 1.5 μmol/L for MV3) or in combination for 48 h, and cell growth was observed and counted. Scale bar = 100 μm. (D) A375 and MV3 melanoma cells were treated with KA (10 μmol/L), or CDDP (1 μmol/L for A375, 1.5 μmol/L for MV3), or their combination for 6 days for plate colony formation assay. (E) A375 and MV3 melanoma cells were treated with KA (10 μmol/L), or CDDP (1 μmol/L for A375, 1.5 μmol/L for MV3), or their combination for 48 h. Western blot was performed to detect the expression of cell cycle-associated and migration-associated proteins. Data are expressed as mean ± SD. Student's *t*-test was performed to analyze significance. ***$P < 0.001$.



Supplementary Figure 8: KA enhanced the chemosensitivity of melanoma to CDDP in vivo without obvious organ toxicity. (A) The volume of MV3 xenograft tumors in nude mice was measured and photographed at the end of the experiments with 20 mg/kg KA or 4 mg/kg CDDP or in combination. (B) At the end of the experiments, mice were sacrificed, and tumors were dissected and weighed. (C) The weight of the mice used in this experiment was measured. (D) Ki-67 and β-catenin expression in xenograft tumors was detected using immunohistochemical staining. (E) H&E staining of heart, liver, spleen, lungs, kidneys, and stomach of mice treated with KA

or CDDP or their combination. Scale bar = 100 μm. Data are expressed as mean ± SD. Student's *t*-test and one-way ANOVA were performed to analyze significance. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, Ns: non-significant.

Supplementary Table 1: Primers used for reverse transcription-quantitative PCR.

Gene	NCBI Reference Sequence	Sequence (5′-3′)
ACTB	NM_001101.5	F:CATGTACGTTGCTATCCAGGC R:CTCCTTAATGTCACGCACGAT
CDK2	NM_001290230.2	F:CCAGGAGTTACTTCTATGCCTGA R:TTCATCCAGGGGAGGTACAAC
CDK6	NM_001145306.2	F:GCTGACCAGCAGTACGAATG R:GCACACATCAAACAACCTGACC
Cyclin D1	NM_053056.3	F:GCTGCGAAGTGGAAACCATC R:CCTCCTTCTGCACACATTTGAA
P27	NM_004064.5	F:AACGTGCGAGTGTCTAACGG R:CCCTCTAGGGGTTTGTGATTCT
MMP7	NM_002423.5	F:GAGTGAGCTACAGTGGGAACA R:CTATGACGCGGGAGTTTAACAT
MMP9	NM_004994.3	F:TGTACCGCTATGGTTACACTCG R:GGCAGGGACAGTTGCTTCT
N-cadherin	NM_001792.5	F:TCAGGCGTCTGTAGAGGCTT R:ATGCACATCCTTCGATAAGACTG
CTNNB1	NM_001330729.2	F:AAAGCGGCTGTTAGTCACTGG R:CGAGTCATTGCATACTGTCCAT
SYVN1	NM_172230.3	F:GCTCACGCCTACTACCTCAAA R:GCCAGACAAGTCTCTGTGACG
HERC5	NM_016323.4	F:GGTGAGCTTTTTGCCTGGG R:TTCTCCGGCAGAAATCTGAGC
ITCH	NM_001324198.2	F:TGATGATGGCTCCAGATCCAA R:GACTCTCCTATTTTCACCAGCTC