



# SAV1, regulated by HERC4, inhibits the proliferation, migration, and invasion of hepatocellular carcinoma

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**Background:** Hepatic carcinoma is one of the most malignant cancers worldwide. Salvador 1 (SAV1) plays a key role in a variety of human carcinogenesis. This study investigated the role of SAV1 and HERC4 in hepatocellular carcinoma (HCC).

**Methods:** SAV1 and HERC4 expressions in HCC tissues were examined using RT-qPCR assay. The regulatory effect of HERC4 on SAV1 was verified by co-immunoprecipitation (Co-IP), RT-qPCR, Western blot, and immunofluorescent assays in HEP3B and Huh 7 cell lines. In addition, functional experimental verification was performed through Edu staining, colony formation, and Transwell assay. Finally, Xenograft tumor model was finally used in nude mice.

**Results:** Clinical features showed significant difference with SAV1 and HERC4 expression. HERC4 was found to be upregulated, while SAV1 was downregulated in HCC. Patients with high HERC4 or low SAV1 had a worse prognosis. Results showed that HERC4 could notably decreased the expression level of SAV1 in HCC cells. Our results showed that overexpression HERC4 could reverse the inhibitory effects of SAV1 on HCC cell proliferation, migration, and invasion. SAV1 overexpression repressed tumor growth and enhance caspase 3 expression.

**Conclusion:** SAV1 can be directly downregulated by HERC4, indicating that the HERC4/SAV1 axis might have great promise for targeted therapies of HCC.

**Keywords:** Hepatocellular carcinoma (HCC); HERC4, Salvador 1 (SAV1); migration; invasion

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## Introduction

Primary hepatic carcinoma is one of the most malignant cancers worldwide (1). Hepatocellular carcinoma (HCC) accounts for 70% to 90% of primary hepatic carcinoma (2). It has the fifth highest incidence of cancer in the world and the third highest death rate (3). There were 782,000 new cases worldwide and 745,000 patients died in 2012 (4). Currently, surgical resection is the preferred treatment for

HCC, while chemoradiotherapy, interventional therapy, and immunotherapy are not preferred treatments (5,6). However, the clinical diagnosis is still dominated by advanced HCC due to the insidious onset of HCC and the absence of obvious early symptoms. Only 10–15% of patients with advanced HCC are suitable for surgical treatment, and the 5-year survival rate is only 5–9%, while the 5-year survival rate of patients who are diagnosed early and undergo hepatectomy increase to 69% (7,8). How to

effectively prevent or delay the progression of HCC is a hot and difficult topic in current studies. Therefore, it is of great significance to further explore the pathogenesis of HCC and identify the key targets for regulating the HCC process.

Abnormal gene expression and dysregulation of signal transduction networks can generate pathological proliferation, apoptosis, migration, and invasion of cells, and further accelerate cancer development (9-11). The ubiquitin proteasome system (UPS) has been found to be essential in regulating the dynamic balance of proteins *in vivo* and thereby affecting a series of physiological processes (12,13). In addition, growing evidence has suggested that UPS dysfunction is closely related to the development of cancer (14,15). The E3 ubiquitin ligase is an enzyme with specific recognition of substrate proteins, and is significant part of the ubiquitination pathway (16,17). The E3 ubiquitin ligase is mainly divided into three types: HECT, TING, and u-box domain families (18). Among them, HERC4 belongs to the HECT family, and its gene is located on chromosome 10q21.3. Findings have suggested that HERC4 has significant clinical significance in several types of cancers including cervical cancer (19), lung cancer (20), and breast cancer (21,22). Moreover, research has also confirmed that HERC4 can expedite proliferation and migration of HCC (23). However, the downstream regulatory mechanism of HERC4 in HCC has not been elucidated.

The Hippo signaling pathway has been actively studied in recent years as it affects cell proliferation, apoptosis, and metastasis (24). Also, it has an important regulatory function in the development of cancer, stem cell function, tissue regeneration, and cell contact inhibition (25-28). Salvador 1 (SAV1, also known as WW45), a core kinase component of the Hippo signaling pathway, plays an extensive and prominent role in a variety of human carcinogenesis (29,30). For instance, SAV1 can inhibit tumor growth of colorectal cancer (29) and a decrease of SAV1 can accelerate the pathological progression of high-grade clear cell renal cell carcinoma (30). However, the possible mechanism and related function of SAV1 in HCC is not yet clearly established. Our preliminary experiment found that HERC4 could down-regulate the level of SAV1 in HCC. However, it is not clear whether HERC4 can directly bind to SAV1 and participate in HCC processes.

We further identified the levels and regulatory relationships of HERC4 and SAV1 in HCC. In addition, we investigated the influences of SAV1 overexpression on proliferation, migration, and invasion of HCC by

being regulated by HERC4. Therefore, it is an extremely attractive hypothesis that HERC4 and SAV1 might be new therapeutic targets for HCC.

## Methods

### *HCC tumor samples*

HCC and para-carcinoma non-tumor tissues (15 pairs) were harvested from HCC patients who were diagnosed at the Second Affiliated Hospital of Zhejiang Chinese Medical University from May 2017 to April 2018. These samples were immediately saved in liquid nitrogen until use. We also obtained informed consents, which were provided by each participant, and our study was approved by the ethics committee of The Second Affiliated Hospital of Zhejiang Chinese Medical University (2018-KL-020-01). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

### *Cell culture*

Hep 3B cells (BNCC352197) were obtained from the BeNa Culture Collection (Beijing, China) and grown in MEM (Gibco) containing 10% fetal bovine serum (FBS; Gibco, cat. no. 26140079) with 1% Penicillin-Streptomycin Solution at 37 °C and 5% CO<sub>2</sub>.

### *Plasmid construction and transfection*

SAV1 and HERC4 overexpression plasmids were purchased from Genomeditech Company (Shanghai, China). HEP 3B cells (1×10<sup>5</sup> cells/well) were plated in 6-well plates and incubated for 8 h at 37 °C. Next, these cells were transfected with empty vector, SAV1-overexpressing plasmids and HERC4-overexpressing plasmids.

### *RNA extraction and quantitative real-time PCR (RT-qPCR) assay.*

HCC and para-carcinoma tissues were ground at a low temperature. TRIzol reagent (Invitrogen, USA) was used to extract total RNAs from the ground tissues and the treated HEP 3B cells according to the instruction. The obtained RNAs were quantitatively determined using a NanoDro2000c (Thermo Scientific). 1.0 µg RNA was used to synthesize cDNA using the reverse transcription kit (Takara, Japan). The expression level of each indicator was

**Table 1** The sequences of primers in this study

Gene	Sequence or target sequence
<i>GAPDH-F</i>	CACCCACTCCTCCACCTTTG
<i>GAPDH-R</i>	CCACCACCCTGTTGCTGTAG
<i>HERC4-F</i>	TGGAATCCCTTTCATGCAAGTT
<i>HERC4-R</i>	TCCTTGTTAGAGCAGCAGTAT
<i>SAV1-F</i>	ATGAGGCGTGAAAGCAACAG
<i>SAV1-R</i>	CCGCTGTGCTCATAGTATCTGTA

F, forward primer; R, reversed primer.

examined and quantified using Bestar™ qPCR MasterMix (DBI Bioscience) based on the specification provided by the supplier. GAPDH was used as an internal control. The sequences of primers are listed in *Table 1*.

#### **Western blotting analysis**

The ground tissues and the treated HEP 3B and Huh7 cells were combined with moderate ice-cold RIPA buffer (Santa Cruz) supplemented with the protease inhibitor cocktail (Sigma). After centrifugation (12,000 ×g for 30 min at 4 °C), the concentration of protein was quantitatively determined using the bicinchoninic acid (BCA) kit (Beyotime Biotechnology). A total of 30 µg protein was isolated by 10% SDS-PAGE and transferred onto PVDF membranes (Bio-Rad). After sealing with 5% skim milk, the membranes were treated with primary antibodies against anti-HERC4 (1:1,000; Abcam, ab221757), anti-SAV1 (1:1,000; Abcam, ab105105), and caspase 3 (1:500; Abcam, ab32042). Then horseradish peroxidase-conjugated secondary antibodies were used to treat the blots for 1 h. The blots were visualized using enhanced chemiluminescence (ECL; Bio-Rad, USA).

#### **Co-immunoprecipitation (Co-IP) assay**

HEP 3B and Huh7 cells were added with the cell lysis solution containing the protease inhibitor and incubated for 30 min on ice. After centrifugation (12,000 ×g for 30 min at 4 °C), an adequate amount of the protein was used as the input group, the remaining proteins were incubated with 2 µg of the corresponding antibodies (anti-HERC4) and anti-IgG (negative control) for 1 h at 4 °C. and the proteins were added to protein A-Agarose at 4 °C incubation overnight. After washing with lysates, the beads were

centrifuged (2,500 ×g for 2 min at 4 °C). After boiling denaturation with the loading buffer, the proteins were used for Western blot analysis.

#### **Immunofluorescence (IF) assay**

The treated HEP 3B and Huh7 cells were spread on slides and incubated for 8 h at 37 °C. The adhering cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) for 30 mins. After sealing with 10% normal goat serum for 30 mins, cells were incubated with the primary antibodies (anti-SAV1; 1 µg/mL, Abcam, ab105105) at 4 °C overnights. The cells were treated with fluorescent labeled goat anti-rabbit IgG (1:200) at room temperature for 1 h in the dark. After treatment with an anti-fluorescent quenching agent, they were observed and photographed under a fluorescent microscope.

#### **Edu staining**

The treated HEP 3B and Huh7 cells (2×10<sup>4</sup> cells/well) were put into 24-well plates and 300 µL Edu solution was added (50 µmol/L) for 1 h. After washing, cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) for 30 min and 2 mg/mL glycine was added for 5 min. After washing, cells were treated with 300 µL permeating agent including 0.5% Triton X-100 for 10 min and 300µL DAPI for 10 mins. The staining results were observed under a microscope.

#### **Colony formation assay**

HEP 3B and Huh7 cells were placed in 35-mm culture dishes at a concentration of 3000 cells/dish. After transfection with SAV1 or/and HERC4-overexpressing plasmids, cells were grown for two weeks at 37 °C with 5% CO<sub>2</sub>. The colonies were fixed in 4% paraformaldehyde for 10 min and stained with Giemsa solution for 10 mins. The number of colonies were counted under a microscope.

#### **Transwell assay**

For the migration assay, the transfected HEP 3B cells were counted, and a single cell suspension (5×10<sup>5</sup> cells/ml) was prepared using serum free medium after pancreatic enzyme digestion. 200 µL cell suspensions were inoculated into the upper Transwell chamber, and 600 µL medium containing 15%FBS was placed into the lower Transwell chamber. After incubation for 24 h at 37 °C, the migrated

cells were fixed using 4% paraformaldehyde (Sigma-Aldrich) for 10 min and dyed with 0.1% crystal violet for 20 min. After washing, the migrated cells were observed using a microscope. For the invasion assay, Matrigel was applied on the Transwell chamber and incubated for 30 min at 37 °C, while the other processes were the same as the migration assay.

#### *In vivo xenograft tumor-burdened model*

A total of 10 female Balb/c nude mice (weight: 14–17 g) were purchased from Zhejiang Chinese Medical University Laboratory Animal Research Center and then maintained in specific pathogen-free atmosphere. All mice have access to sterilized food and water. Mice were then injected subcutaneously with HEP3B cells (106 cells each mouse) after one week of adjustable feeding. 30 days later, all the mice were sacrificed and tumor tissues were collected for size measurement and low temperature preservation. Tumor volume was calculated using formula: tumor volume (mm<sup>3</sup>) = [tumor length × (tumor width)<sup>2</sup>]/2. All animals experiments were conducted according to institutional guideline and approved by Ethics Committee of The Second Affiliated Hospital of Zhejiang Chinese Medical University.

#### *Immunohistochemistry*

Tumor tissues were maintained in 4% paraformaldehyde for fixation followed by paraffin-embedding. Paraffin-embedded tissues serial sections (4 μm) were obtained, and then dewaxing and dehydration with xylene and hydrated respectively. Sections were incubated with primary antibody (1:200, PB9026, Boster, China) overnight at 4 °C after antigen retrieval and sealing. Afterwards, sections were incubated with horseradish peroxidase-conjugated secondary antibody (1:200, BM3895, Boster, China) at room temperature. Sections were developed with diaminobenzidine (DAB) and hematoxylin. Finally, sections sealed with neutral resin were observed under microscope.

#### *Statistical Analysis*

GraphPad Prism Software (Ver. Prism 7) was used for statistical analysis of data, all of which were expressed as mean ± SD. The Student *t*-test was used to calculate the difference between the two groups. The overall survival was obtained from Kaplan-Meier plots. *P*<0.05 was considered statistically significant.

## **Results**

### *HERC4 was highly expressed, while SAV1 was lowly expressed in HCC tissues*

To verify expression changes of HERC4 and SAV1, we extracted the total mRNA and protein from 15 pairs of HCC and para-carcinoma tissues. As shown in *Table 2*, the clinical feature of tumor size and TNM stage of patients presented significant difference with expression of SAV1. However, HERC4 expression displayed no significant difference with tumor size (*P*=0.13). This discrepancy might due to a small number of involved subjects (*Table 2*).

The results from the RT-qPCR assay showed that the level of HERC4 was markedly elevated in HCC tissue samples relative to para-carcinoma tissues (*P*<0.01, *Figure 1A*). Our results showed that SAV1 expression was significantly lower in HCC samples than that in para-carcinoma ones (*P*<0.01, *Figure 1B*). In addition, Western blot results also confirmed high expression of HERC4 and low expression of SAV1 in HCC tissues (*Figure 1C*). More importantly, data from Kaplan-Meier plots disclosed that HCC patients with high HERC4 expression exhibited a worse prognosis than those HCC patients with low HERC4, while HCC patients with high SAV1 expression had a longer survival trend than those HCC patients with low SAV1 expression (*Figure 1D*). Therefore, these data implied that HERC4 and SAV1 might make significant contributions during HCC tumorigenesis.

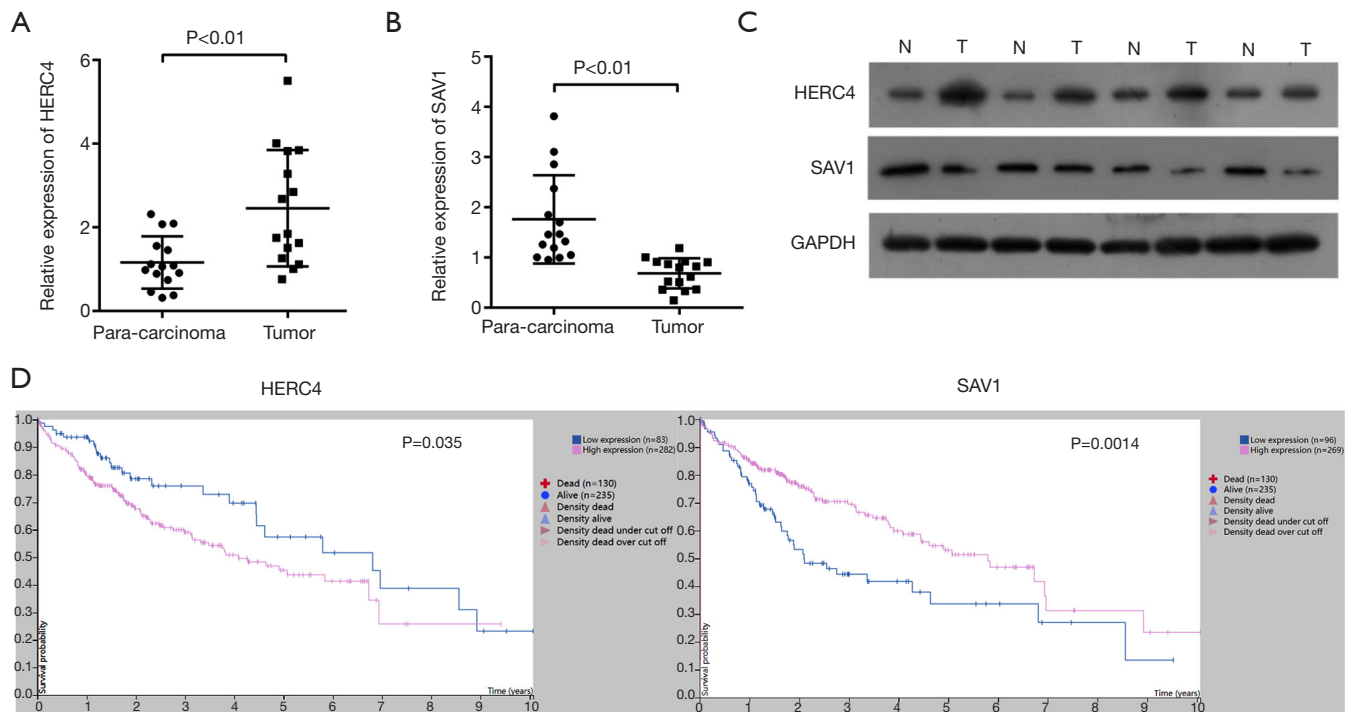
### *SAV1 significantly prevented HCC cell proliferation through interaction with HERC4*

Because of the opposite regulatory trends of HERC4 and SAV1 in HCC tissues, we further verified whether HERC4 was a direct target of SAV1 in HEP3B and Huh7 cells. Firstly, the Co-IP assay with anti-HERC4 antibodies was used to verify the interaction between HERC4 and SAV1. As shown in *Figure 2* and *Figure 3*, the results from the CO-IP assay proved the formation of a complex between HERC4 and SAV1 in HEP 3B cells (*Figure 2A*) and Huh7 cells (*Figure 3A*), suggesting that HERC4 can directly interact with SAV1 in HCC cells. In order to further verify the regulatory effect of SAV1 on HERC4 in HCC cells, HEP 3B cells were overexpressed with SAV1, and SAV1-overexpressing HEP 3B cells were then transfected with HERC4 overexpressing plasmids. Results showed that the level of SAV1 was prominently upregulated in HEP 3B and Huh7 cells after overexpression of SAV1, while

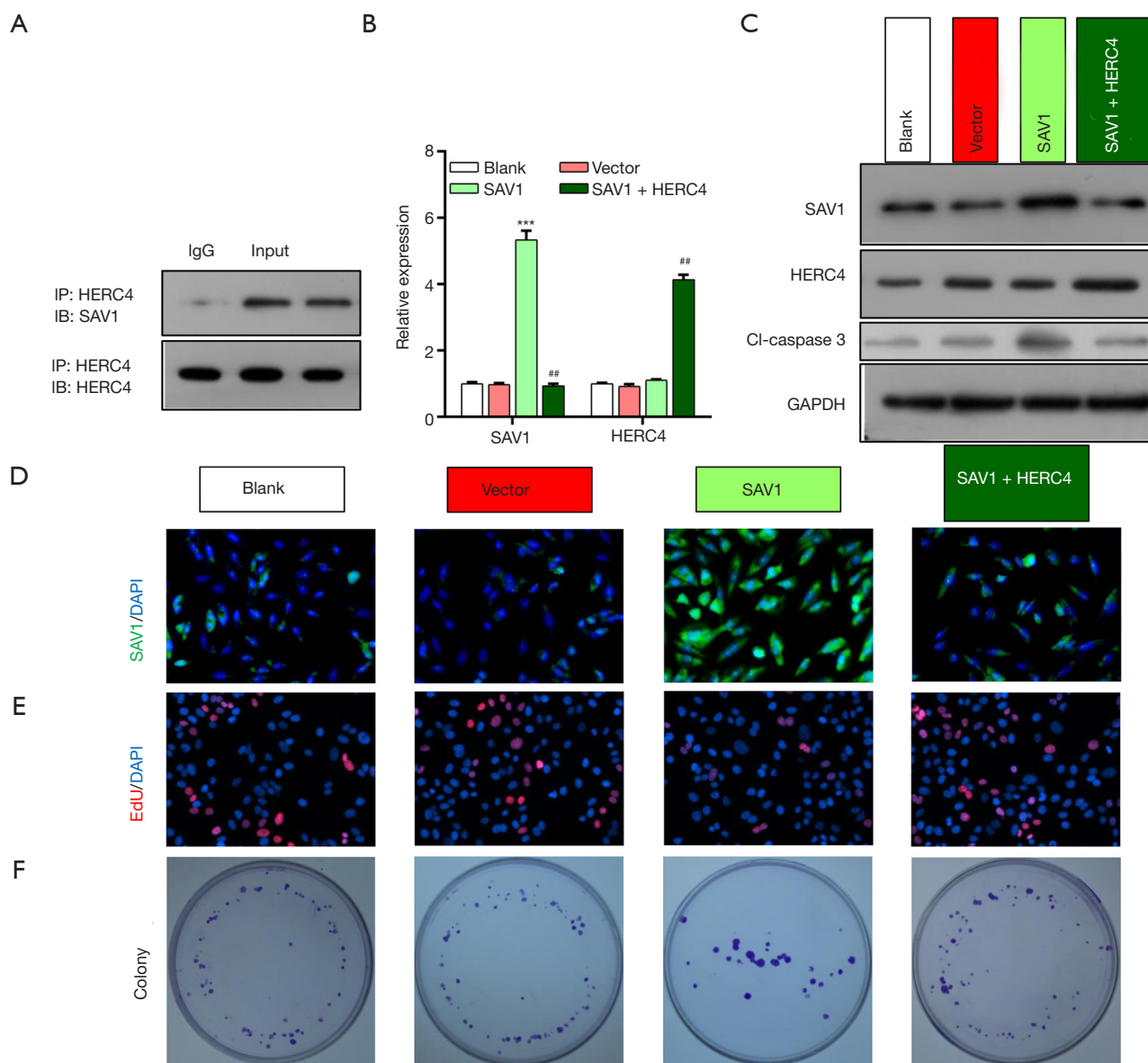
**Table 2** Correlation of SAV1 and HERC4 expression with clinicopathological features

Features	N	SAV1 expression			HERC4 expression		
		High	Low	P value	High	Low	P value
<b>Age</b>							
<53	8	2	6	0.31	5	3	0.31
≥53	7	5	2		2	5	
<b>Gender</b>							
Male	9	4	5	0.90	3	6	0.31
Female	6	3	3		4	2	
<b>Tumor size</b>							
<5 cm	8	6	2	0.04	2	6	0.13
≥5 cm	7	1	6		5	2	
<b>TNM stage</b>							
I + II	7	6	1	0.01	1	6	0.04
III + IV	8	4	7		6	2	

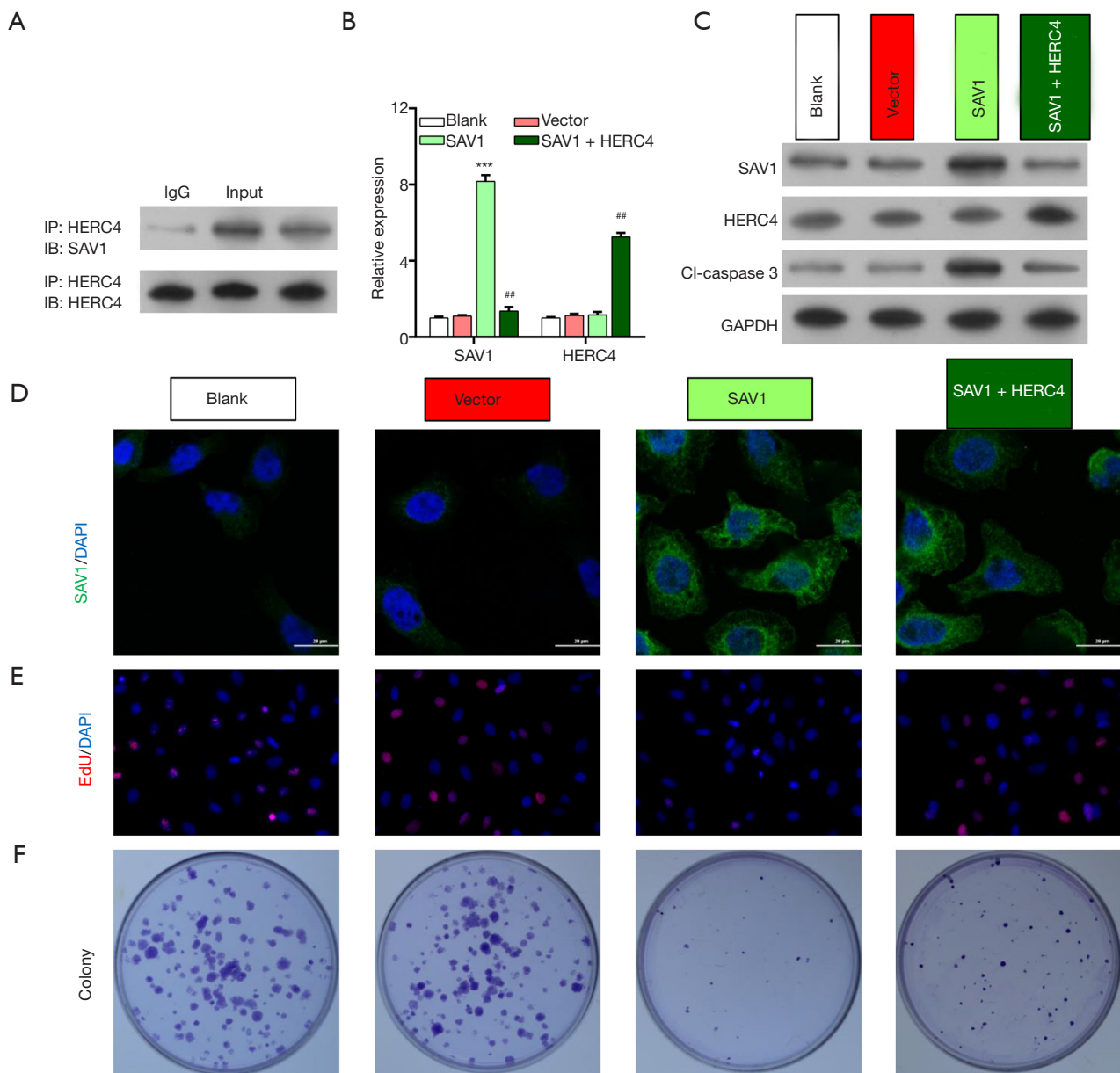
SAV1, Salvador 1.



**Figure 1** HERC4 was highly expressed, while SAV1 was lowly expressed in HCC tissues. The levels of HERC4 (A) and SAV1 (B) were identified by RT-qPCR assay in 15 pairs of HCC and para-carcinoma tissues,  $P < 0.01$ . (C) Western blot analysis of HERC4 and SAV1 in HCC and para-carcinoma tissues. (D) Kaplan-Meier plots exhibiting the overall survivals in HCC patients with high or low HERC4 and SAV1 expressions, respectively. N, non-tumor tissues; T, tumor tissues; SAV1, Salvador 1; HERC4, HECT And RLD Domain Containing E3 Ubiquitin Protein Ligase 4.



**Figure 2** SAV1 significantly prevented HCC cell proliferation through interaction with HERC4. (A) Direct binding of SAV1 and HERC4 was investigated by CO-IP Assay using anti-HERC4 antibodies. Input denotes positive control and IgG denotes negative control. The confirmations of HERC4 and SAV1 levels were by RT-qPCR (B) and Western blot assays (C) in HEP 3B cells after transfection with SAV1 or/and HERC4 plasmids, \*\*\*,  $P < 0.001$  vs. vector group; ##,  $P < 0.01$  vs. SAV1 group. (D) Expression and distribution of SAV1 was evaluated by IF assay, magnification, 100 $\times$ , scale bar = 100  $\mu$ m. (E) The impacts of SAV1 and HERC4 overexpression on HEP 3B cell proliferation were examined by Edu staining, 200 $\times$ . (F) The impacts of SAV1 and HERC4 overexpression on HEP 3B cell proliferation were examined by colony formation. DAPI, 4',6-diamidino-2-phenylindole; SAV1, Salvador 1; HERC4, HECT And RLD Domain Containing E3 Ubiquitin Protein Ligase 4; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EdU, 5-Ethynyl-2'-deoxyuridine. IP, immunoprecipitation.



**Figure 3** SAV1 significantly prevented HCC cell proliferation through interaction with HERC4 in Huh 7 cell line. (A) Direct binding of SAV1 and HERC4 was investigated by CO-IP Assay using anti-HERC4 antibody. Input denotes positive control and IgG denotes negative control. The confirmations of HERC4 and SAV1 levels were completed by RT-qPCR (B) and Western blot assays (C) in HEP 3B cells after transfection with SAV1 or/and HERC4 plasmids, **\*\*\***,  $P < 0.001$  vs. vector group; **\*\***,  $P < 0.01$  vs. SAV1 group. (D) The expression and distribution of SAV1 was evaluated by IF assay, magnification, 100 $\times$ , scale bar =100  $\mu$ m. (E) The impacts of SAV1 and HERC4 overexpression on HEP 3B cell proliferation were V (F) The impacts of SAV1 and HERC4 overexpression on HEP 3B cell proliferation were examined by colony formation. DAPI, 4',6-diamidino-2-phenylindole; SAV1, Salvador 1; HERC4, HECT And RLD Domain Containing E3 Ubiquitin Protein Ligase 4; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EdU, 5-Ethynyl-2'-deoxyuridine. IP, immunoprecipitation.

this upregulation was completely reversed by HERC4 overexpression. Also, it was found that overexpression of HERC4 could markedly improve the expression level of HERC4 in HCC cells ( $P < 0.01$ ,  $P < 0.001$ , *Figure 2B,C*, *Figure 3B,C*). In addition, the results of IF assay also demonstrated the inhibitory action of HERC4 on SAV1 (*Figure 2D*, *Figure 3D*). We further investigated the possible effects of HERC4 and SAV1 on HCC cell proliferation, and the results from Edu staining and colony formation assay showed that overexpression of SAV1 could inhibit HEP 3B and Huh7 cell proliferation, while this inhibiting effect could be almost completely reversed by HERC4 overexpression, indicating that HERC4-inhibition of SAV1 was involved in the proliferation of HCC cells (*Figure 2E,F*, *Figure 3E,F*). In consequence, we proposed that the interaction of HERC4 and SAV1 might contribute to the tumorigenesis of HCC.

#### ***Overexpression of SAV1 suppressed the migration and invasion of HCC cells by being regulated by HERC4***

To further verify whether HERC4 could regulate the role of SAV1 on the migration and invasion of HCC cells, we co-transfected the overexpressing plasmids of HERC4 and SAV1 into HEP 3B cells. Transwell results showed that the migration and invasion of HEP 3B cells could be significantly suppressed by overexpression of SAV1, while overexpression of HERC4 then dramatically reversed this suppression mediated by SAV1 ( $P < 0.05$ ,  $P < 0.01$ , *Figure 4*). As a consequence, it was found that HERC4 could participate in the migration (*Figure 4A,C*) and invasion (*Figure 4B,4D*) of HCC cells by downregulating SAV1 in HEP3B and Huh7 cells.

#### ***Overexpression of SAV1 inhibited tumor growth in nude mice***

To confirm the tumor-inhibitive effect of SAV1 in HCC, experiment *in vivo* involving nude mice with a tumor-burdened was performed. As shown in *Figure 5*, SAV1 overexpression significantly suppressed tumor growth (*Figure 5A,B,C*). Further investigation showed that SAV1 overexpression enhanced caspase 3 expression in tumor compared with NC (*Figure 5D*), together with weaker expression of Ki67 in tumor, as shown in *Figure 5E*.

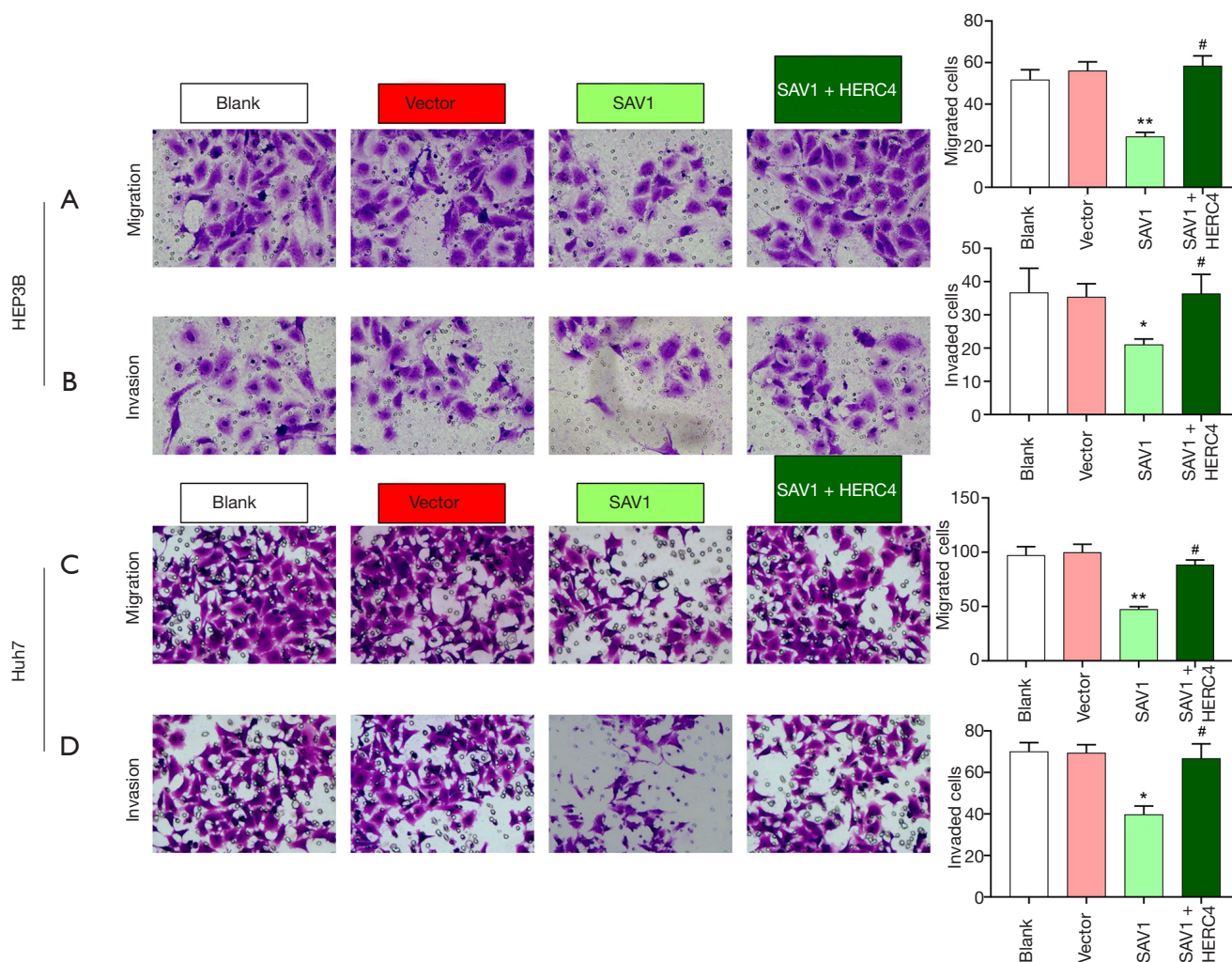
## **Discussion**

The Hippo pathway, a cascade enzyme chain reaction

composed of a series of protein kinases, is a newly elaborated cell signaling pathway (31). The Hippo pathway is highly genetically conserved, and mainly contains Mst1/2, SAV1, lats1/2, and Mob1 proteins (32). The Hippo pathway is considered as a tumor suppressor pathway, and the multiple core proteins of it are tumor suppressors (33). Studies have discovered that the inactivation of the Hippo pathway is connected with the occurrence and development of cancers (34,35). Among them, the WW and SARAH domains of SAV1 can interact with other proteins (36). In the Hippo pathway, SAV1 can act as a bridging protein between Mst1/2 and Lats1/2 (37). In addition, SAV1 can enhance Mst1 and Mst2 to induce apoptosis (38). Current research also suggested that SAV1 can prevent the development of colorectal cancer (39). *In vivo* experiments proved that the knockout of SAV1 can cause a liver volume increase and the formation of liver cancer. Therefore, SAV1 is thought to be a tumor-suppressor gene that can repress the proliferation of cancer cells. However, existing studies have shown little understanding of SAV1, and its biological function is not fully explained in HCC. In this report, we emphasized that the level of SAV1 was lowly expressed in HCC, and patients with high SAV1 had a good prognosis. It was found that overexpression of SAV1 significantly prevented the proliferation and colony formation capacity of HEP 3B cells. Also, we found that overexpression of SAV1 could dramatically result in the suppressions of migration and invasion of HEP 3B cells. Therefore, our data provide a strong hypothesis that SAV1 may be a potential suppressor gene for HCC.

The initiation of cancer is related to gene mutation, gene expression disorder, and protein regulation disorder (40). In HCC, mutations or expression disorders of key regulatory genes may result in the occurrence, development, and metastasis of cancers (41). Studies have proved that UPS is closely associated with the occurrence of cancer (42,43). Furthermore, its members can not only serve as potential cancer diagnostic markers or prognostic indicators, but also as potential molecular therapeutic targets. At present, E3 ubiquitin ligase in UPS is widely studied because of its specificity (43). Of note, HERC4, as a newly identified E3 ubiquitin ligase, has been shown to contribute to cancer progression and affect prognosis (44). For example, upregulation of HERC4 is positively related to the histological grade, TNM stage, and metastasis of lung cancer (20). HERC4 is associated with the progression of breast cancer (21), and contributes to the tumorigenesis of breast cancer (45). HERC4 has also been proved to



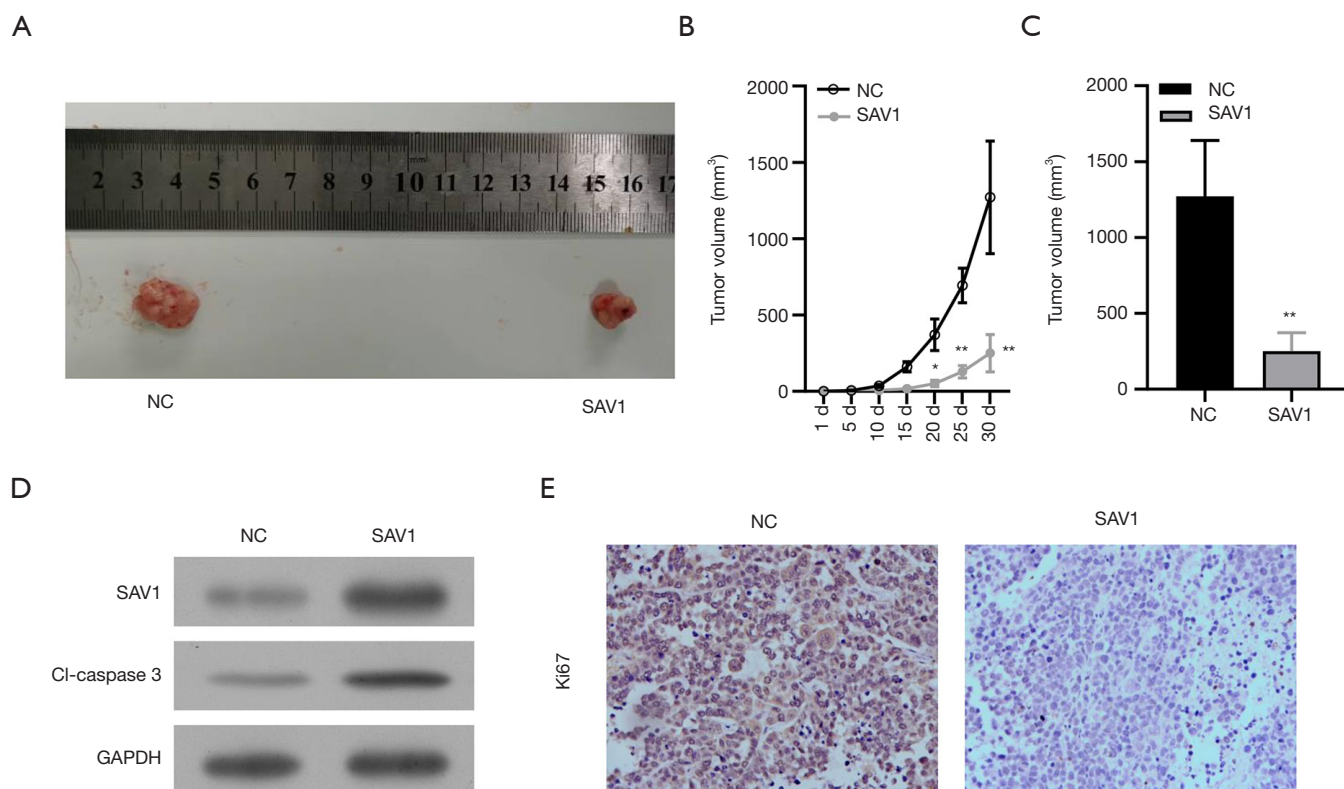


**Figure 4** Overexpression of SAV1 suppressed the migration and invasion of HCC cells by being regulated by HERC4 in HEP3B and Huh 7 cell line. Transwell assay was conducted in HEP 3B cells to assess the influences of SAV1 and HERC4 transfection on cell migration (A) and invasion (B). The migrated (C) and invaded cells (D) were counted according to the resulting graphs of Transwell assay, Cells were photographed at a magnification of 100× after stained with crystal violet. \*, P<0.05; \*\*, P<0.01 *vs.* vector group; #, P<0.05 *vs.* SAV1 group. SAV1, Salvador 1; HERC4, HECT And RLD Domain Containing E3 Ubiquitin Protein Ligase 4.

participate in the proliferation, apoptosis, and migration of cervical cancer (19). HERC4 can prevent the growth of myeloma xenografts by regulating c-Maf (46). In our study, the upregulation of HERC4 was validated in HCC tissue samples, and patients with high HERC4 exhibited a worse prognosis. In addition, our study is the first report demonstrating the targeted regulation between HERC4 and SAV1, SAV1 was identified as a target gene of HERC4, and it can be negatively regulated by HERC4 in HCC. Moreover, functional experiments have confirmed that HERC4 can reverse the inhibiting effects of SAV1 on HCC

cell proliferation, migration, and invasion. Therefore, the oncogenic role of HERC4 on HCC progress could be achieved by suppressing expression of SAV1.

In conclusion, by employing an overexpression of SAV1 in the HCC cell line, cell proliferation, migration, and invasion was suppressed. However, HERC4 overexpression rescued the inhibitory effect of SAV1 and cell proliferation, migration, and invasion were the enhanced. This study provided evidences that SAV1, an antioncogene of HCC, can be repressed by HERC4. This study suggested that HERC4 can be a potential target of HCC.



**Figure 5** Overexpression of SAV1 inhibited tumor growth in nude mice. (A,B,C) SAV1 overexpression suppressed tumor growth. (D) SAV1 overexpression enhanced expression of Caspase 3 in tumor. (E) SAV1 overexpression attenuated Ki67 expression in tumor. A DAB staining was performed to the slide and then photographed at magnification 100 $\times$ . \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  vs. NC group. NC, Negative control; d, days; SAV1, Salvador 1; HERC4, HECT And RLD Domain Containing E3 Ubiquitin Protein Ligase 4.

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## Footnote

**Data Sharing Statement:** Available at <http://dx.doi.org/10.21037/tcr-20-698>

**Conflicts of Interests:** All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr-20-698>). The authors have no conflicts of interest to declare.

**Ethical Statement:** The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki. The study was approved by The Second Affiliated Hospital of Zhejiang Chinese Medical University (2018-KL-020-01), and informed consent was taken from all the patients.

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