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Inhibition of Liver Carcinoma Cell Invasion and Metastasis by Knockdown of Cullin7 In Vitro and In Vivo

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Cullin7 is an E3 ubiquitin ligase. The Cullin7 protein family functions as a molecular scaffold to coordinate substrate ubiquitination in Skp, Cullin, and F-box-containing complex (SCF complex). Cullin7s control normal development and primary cellular processes and are characterized by a unique genomic network organization. Less is known about the involvement of Cullin7 with hepatocellular carcinoma (HCC). In this study, we found that Cullin7 showed a high expression in HCC tumor tissues, especially in metastatic HCC tumor tissues. Also, there was a negative correlation between Cullin7 expression and long survival. Silencing of Cullin7 in liver cancer cells can significantly reduce the migration, invasion, and metastatic abilities. Also, detection of epithelial–mesenchymal transition (EMT) marker expression showed that Cullin7 promotes epithelial–mesenchymal transformation of cancer cells. The results of this study helped to elucidate the oncogene functions of Cullin7 in liver cancers.

Key words: Cullin7; Hepatocellular carcinoma (HCC); Invasion; Metastasis

INTRODUCTION

Hepatocellular carcinoma (HCC) is a common malignant tumor and severely threatens health (1). Previous study showed that the epithelial–mesenchymal transition (EMT) confers the cancer cells to be strongly motivated and with invasive ability thus induces the distant metastases in liver cancer. Exploring the molecular mechanism of the occurrence of EMT can greatly benefit our understanding of tumor progression and metastasis in liver cancer.

The ubiquitin/26S proteome pathway is a major post-transcriptional regulatory process that allows cells to lower the concentration of a target protein in a timely fashion. This process required three enzymes: E1, E2, and E3. Of the cellular E3s, the SCF complexes are the largest family composed by a cullin family molecule containing a conserved cullin domain, and its RING finger partner ROC1/Rbx1/Hrt1 (2). Cullins act as the molecular scaffolds to coordinate substrate ubiquitination. In prokaryotes, cullin1, Rbx1, and Skp1 subunits comprise the core ligase activity, while F-box proteins bind both with Skp1 proteins and the substrate proteins through variable protein interaction domains (3). Cullin7 is a non-typical cullin protein composed of nearly 1,700 amino acids. It acts as a component of SCF complex, interacting with Skp1, F-box protein Fbw8, and ROC1 (4,5). Unlike CUL1, Cullin7 does not bind the Skp1 adaptor alone but selectively interacts with the Skp1-Fbw8 heterodimer (4). Cullin7 controls normal development, primary cellular processes, and are characterized by a unique genomic network organization (6). Less is known about the involvement of Cullin7 with liver cancerogenesis.

The intrahepatic and extrahepatic metastasis is the main reason for the poor prognosis in primary liver cancer. EMT is a process that is characterized by specific morphological and phenotypic alterations in epithelial cells during embryonic development (7). Previous study showed that EMT confers the cancer cells to be strongly motivated and with invasive ability, thus induces the distant metastases (8). During EMT, the polarity and the adhesion to the surrounding cells of epithelial cells and matrix were reduced and became similar in morphology to fibroblasts, thus acquiring an enhanced migratory ability (9). The occurrence of EMT is accompanied by a different expression of specific epithelial and mesenchymal molecular markers. Downregulation of epithelial cell markers such as E-cadherin, α-catenin, β-catenin, and

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γ-catenin indicated the transformation process from epithelial to mesenchymal, whereas the expression upregulation of mesenchymal tissue markers such as N-cadherin, α-smooth muscle actin, vimentin, and fibronectin protein is upregulated (10). EMT is thought to be closely associated with tumor progression and resistance to chemotherapy (8). Thus, EMT has attracted increased attentions from academics, clinicians, and pharmaceutical researchers in liver cancer.

In this investigation, we found that Cullin7 showed a high expression in HCC tumor tissues, especially in metastatic HCC tumor tissues. Also, there was a negative correlation between Cullin7 expression and long survival. Silencing of Cullin7 in liver cancer cells can significantly reduce the migration, invasion, and metastatic abilities. Also, detection of EMT marker expression showed that Cullin7 promotes epithelial–mesenchymal transformation of cancer cells. In conclusion, our findings define Cullin7 as a promotor of EMT and metastasis in HCC that predicts poor clinical outcomes.

MATERIALS AND METHODS

Chemicals and Antibodies

Lipofectamine 2000 transfection and TRIzol LS reagents were purchased from Invitrogen (Grand Island, NY, USA). Antibodies against Cullin7 were purchased from Abcam (Cambridge, MA, USA). E-cadherin, N-cadherin, vimentin, and β-actin antibodies were from Cell Signaling technology (Danvers, MA, USA). Anti-α-catenin antibody was from BD (Franklin Lakes, NJ, USA). Unless otherwise noted, all other chemicals were from Sigma-Aldrich (St. Louis, MO, USA).

Cell Lines and Cell Culture

Liver cancer cell lines HepG2, Huh7, SNU423, MHCC97H, Sk-Hep1, SNU886, and HCCLM3 were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Chinese Academy of Sciences. Liver cancer cell lines were routinely cultured as previously described (9). Cell lines were maintained at 37°C in an atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium or RPMI-1640 supplemented with 10% fetal bovine serum.

Patients and Specimens

Thirty-four tumor and para-cancerous tissues, which were used for qRT-PCR and Western blot analysis, were randomly collected from HCC patients who underwent curative resection with informed consent between 2011 and 2014 at the Department of Laparoscopic Surgery, Linyi People's Hospital. Study protocols were approved by the Hospital Ethics Committee of Linyi People's Hospital, and written informed consent was obtained from patients based on the Declaration of Helsinki.

Immunohistochemical Analysis

The tissues were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight and subsequently embedded in paraffin wax. Sections cut at a thickness of 4 μm were stained with hematoxylin and eosin for histological analysis. Immunohistochemical analysis was performed for different markers in these tissues as described previously (11). The proportion of stained cells was semiquantitatively determined following published protocols (12).

Establishment of Cullin7 Knockdown and Cullin7 Stable Expression Cell Lines

pBabe retroviral construct containing human Cullin7 cDNA and pSuper with shRNA against human Cullin7 were prepared as described previously (10). The generation of retrovirus supernatants and transfection of cancer cells were conducted as described previously. Infected cells were selected by adding 2 μg/ml puromycin to the culture medium for 48 h and then maintained in complete medium with 0.5 μg/ml puromycin. Empty retroviral-infected stable cell lines were also produced by the above protocols. The expression of Cullin7 was confirmed by qRT-PCR and Western blotting analysis.

Western Blot

Standard methods were used for Western blot. Cell lysates were prepared by extraction with lysis buffer. Proteins (10 μg) were separated by SDS-PAGE under reducing conditions and blotted onto a polyvinylidene difluoride membrane. Membranes were probed with specific antibodies. Blots were washed and probed with respective secondary peroxidase-conjugated antibodies and the bands visualized by chemoluminescence.

qRT-PCR

Total RNA was extracted using TRIzol reagent, and cDNA was synthesized using SuperScript II Reverse Transcriptase. qRT-PCR and data collection were performed with an ABI PRISM 7900HT sequence detection system.

Cell Invasion and Motility Assay

Invasion assay was performed using Matrigel (BD)-coated Transwell inserts (Costar, Manassas, VA, USA) containing polycarbonate filters with 8-μm pores as detailed previously. According to the manufacturer's recommendations, the inserts were coated with 50 μl of 1 mg/ml Matrigel matrix. Cells (2×10^5) in 200 μl of serum-free medium were plated in the upper chamber, whereas 600 μl of medium with 10% fetal bovine serum was added to the lower well. After 24 h of incubation, cells that migrated to the lower surface of the membrane were fixed and stained. For each membrane, five random fields were counted at 10× magnification. Motility assays

were similar to Matrigel invasion assay except that the Transwell insert was not coated with Matrigel.

In Vivo Tumor Growth and Metastasis

For metastatic assays in vivo, cells were resuspended in PBS at a concentration of 1×10^7 cells ml^{-1}. Cell suspension (0.1 ml) was injected into tail veins of nude mice. All of the mice were killed by CO_2 60 days after inoculation.

Statistical Analysis

Data was described as the mean ± SD. Comparisons between different groups were undertaken using the Student's two-tailed *t*-test. The limit of statistical significance was $p < 0.05$. Statistical analysis was done with SPSS/Win11.0 software (SPSS, Inc., Chicago, IL, USA).

RESULTS

Cullin7 Is Highly Expressed in HCC Tissues

The mRNA levels of Cullin7 were detected in 34 pairs of tumor and adjacent tissues. In most of these tissues, Cullin7 expressed higher than that in adjacent tissues in transcriptional level (Fig. 1A and B). Analyzing the expression of Cullin7 mRNA in adjacent tissues, nonmetastatic tumor tissues and metastatic tumor tissues revealed that in metastatic tumor tissues, the percent of Cullin7 high expression was the highest (Fig. 1C). Western blot was employed to detect the protein level of Cullin7 in tumor and adjacent tissues. In all the 10 pairs, the Cullin7 protein showed higher expression in tumor tissues than that in adjacent tissues (Fig. 2A and B). Similarly, compared with adjacent tissues and nonmetastatic tumor tissues, the metastatic tumor tissues possess the highest Cullin7 protein level (Fig. 2C).

Cullin7 Is Highly Expressed in Distant Metastatic Tumor Tissues

Immunohistochemical was used to analyze the expression level of Cullin7 in more HCC tissues. Compared with normal tissues, Cullin7 showed significant high expression in tumor tissues, especially in

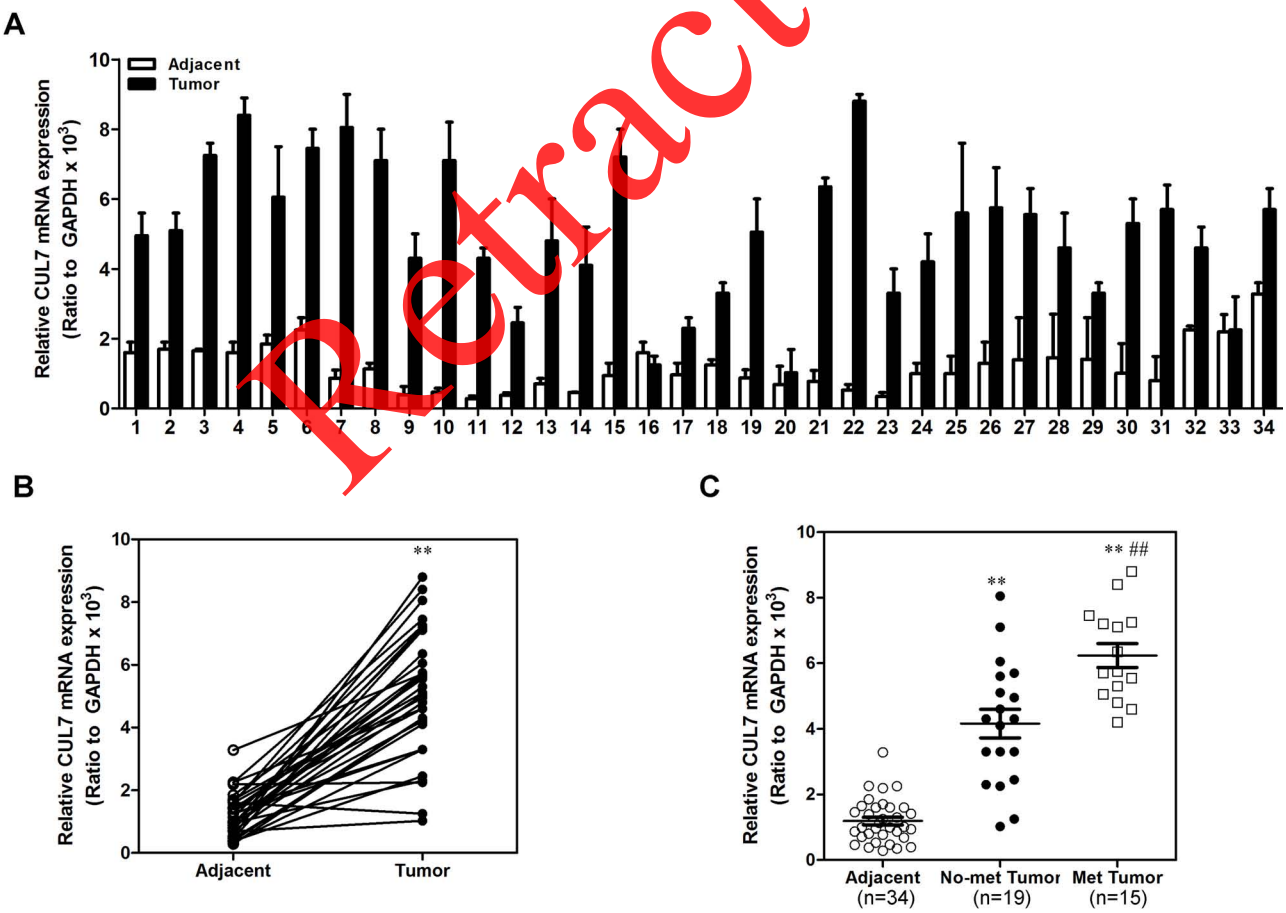


Figure 1. Expression of Cullin7 mRNA was upregulated in HCC tissues. (A) Cullin7 mRNA expression was analyzed by quantitative RT-PCR in HCC and adjacent tissues. (B) Comparison of the expression levels of Cullin7 mRNA in HCC and adjacent tissues. (C) Comparison of the expression levels of Cullin7 mRNA in nonmetastatic and metastatic HCC tissues. $^{**}p < 0.01$ is based on the Student *t*-test.

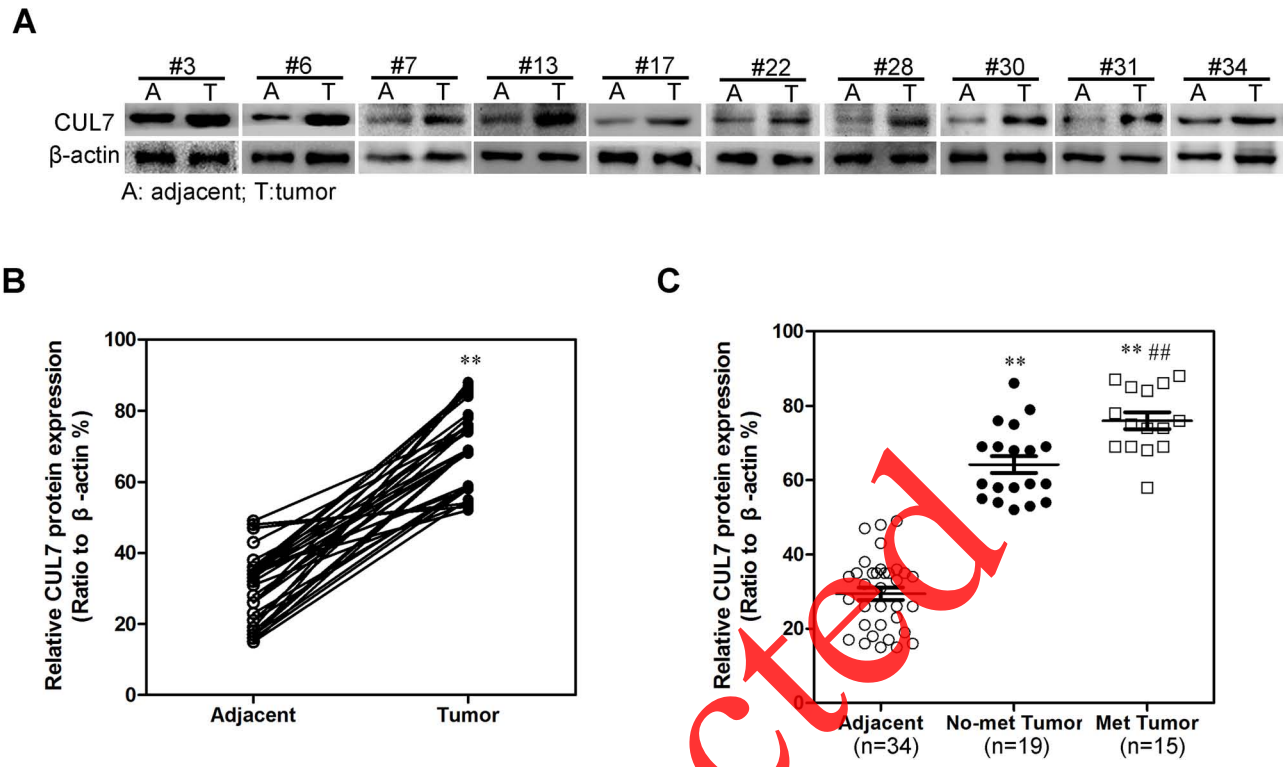


Figure 2. Expression of Cullin7 protein was upregulated in HCC tissues. (A) Cullin7 protein expression was analyzed by Western blotting in HCC and adjacent tissues. (B) Comparison of the expression levels of Cullin7 protein in HCC and adjacent tissues. (C) Comparison of the expression levels of Cullin7 protein in nonmetastatic and metastatic HCC tissues. $^{**}p<0.01$ is based on the Student *t*-test.

distant metastasis (Fig. 3A and B). The survival curves in Figure 3C indicated that the patients with higher Cullin7 level in liver cancer tissues had shorter living length than those with lower Cullin7 expression level, suggesting there was a negative correlation between Cullin7 and HCC survival.

Knockdown of Cullin7 Inhibits Migration and Invasion Capacity of Liver Cancer Cells In Vitro

Cullin7 showed significant high expression in invasive cancer cells than that in noninvasive cancer cells in both mRNA (Fig. 4A) and protein levels (Fig. 4C). Silencing and stable overexpression of Cullin7 in liver cancer cells were retrovirally established using SNU886 and HepG2 cell lines, respectively, according to the basic expression level in these cell line. The constructed plasmids were designated as SNU886-shCUL7 #1, #2, #3, and HepG2-CUL7. The levels of Cullin7 in these cell lines were verified on protein and mRNA levels (Figs. 5A, B and 6A, B).

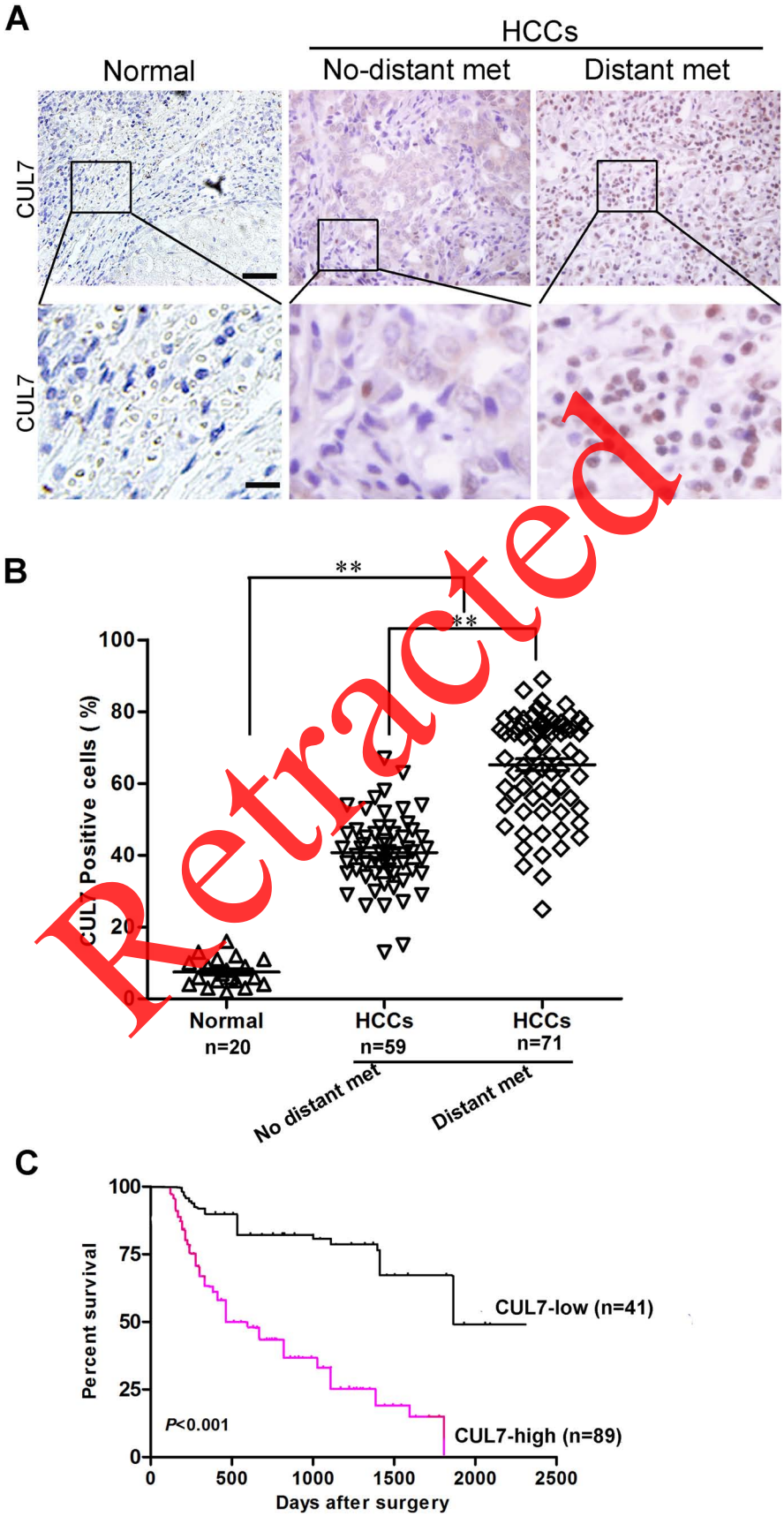
Transwell assay and Matrigel assay were carried out to evaluate the effects of Cullin7 on migration and invasion ability in liver cells. As shown in Figure 5C, silencing Cullin7 can significantly reduce the numbers of cells migrating through the membrane to the bottom of the aperture. Statistical analysis showed that only about half of the cells with Cullin7 silencing migrated through the membrane (Fig. 5C). Similarly, silencing Cullin7can also significantly decrease the number of invaded liver cancer cells (Fig. 5D). On the contrary, high expression of Cullin7 led to an increase in the number of liver cancer cells migrating through the membrane (Fig. 6C and D). These results revealed that Cullin7 promotes migration and invasion of liver cancer cells.

Knockdown of Cullin7 Inhibits Metastatic Capacity of Liver Cancer Cells In Vivo

Plasmid SNU886-shCUL7 #2 and its corresponding control cells were injected into nude mice through the tail

FACING PAGE

Figure 3. Negative correlations between Cullin7 and long survival in liver cancers. (A) Expression of Cullin7 protein was measured by IHC in HCC and normal liver tissues. Representative images of Cullin7 expression in normal liver tissues and HCC tissues are shown. (B) Cullin7-positive cells were calculated in normal liver tissues, nondistant metastasis, and distant metastasis HCC tissues. (C) Survival analysis of patients. (D) $p<0.0001$ based on the Kaplan–Meier survival analysis. $^{**}p<0.01$ is based on the Student *t*-test.


A
HCCs
Normal
No-distant met
Distant met
CUL7
CUL7
B
**
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CUL7 Positive cells (%)
100
80
60
40
20
0
Normal
n=20
HCCs
n=59
HCCs
n=71
No distant met
Distant met
C
Percent survival
100
75
50
25
0
P<0.001
CUL7-low (n=41)
CUL7-high (n=89)
0
500
1000
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2000
2500
Days after surgery

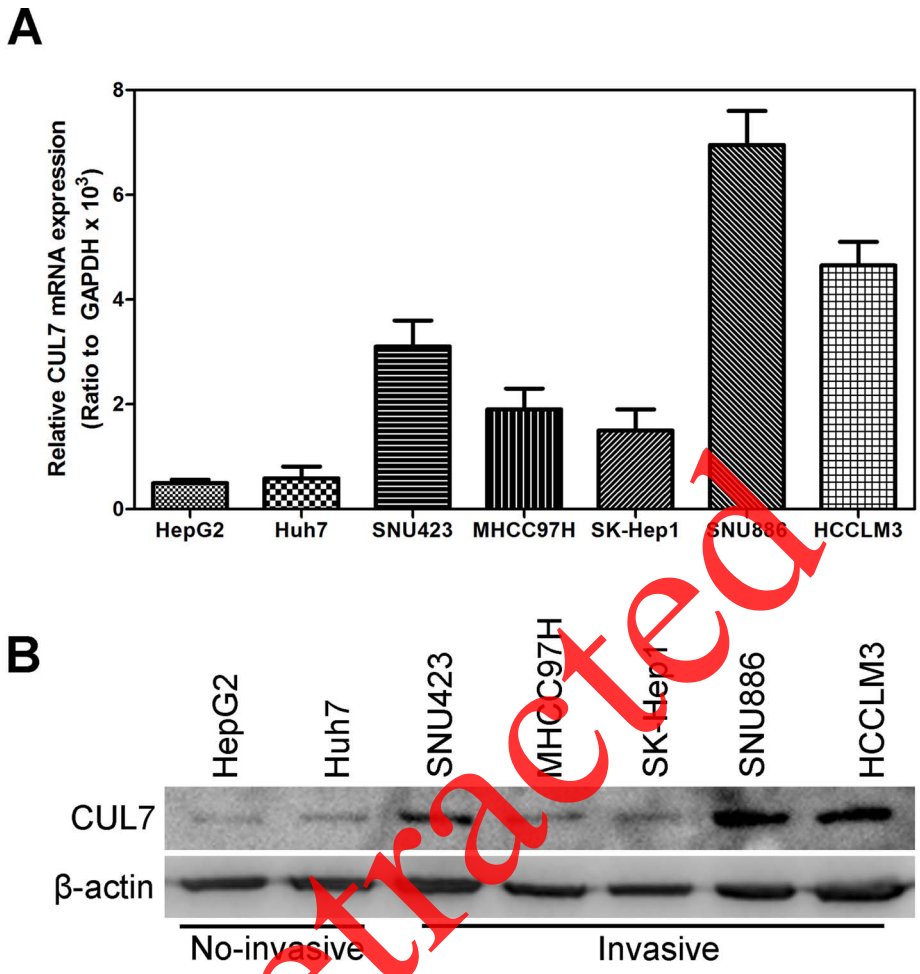


Figure 4. Expression level of Cullin7 was measured in HCC cell lines. Expression level of Cullin7 was measured by qRT-PCR (A) and Western blotting (B) in noninvasive HCC cell lines (HepG2 and Huh7) and invasive HCC cell lines (SNU423, SK-Hep1, SNU886, MHCC97H, and HCCLM3).

vein to detect the function of Cullin7 in distant metastasis in vivo. Silencing Cullin7 significantly decreased the number of mice with distant metastasis (Fig. 7A). In addition, less metastatic foci in lung (Fig. 7C) were counted in each mouse injected with liver cancer cells silencing Cullin7.

Cullin7 Regulates the Expression of EMT Markers in HCC Cells

Expression level of protein markers of EMT was assessed to evaluate the relationship between Cullin7 and EMT. Western blot showed that in the Cullin7-silenced cell lines, epithelial cell markers (E-cadherin and α-catenin) were upregulated, and mesenchymal cell markers (N-cadherin, fibronection and vimentin) were downregulated (Fig. 8A). Also, in the Cullin7 overexpression cell lines, the mesenchymal cell markers were increased, and the epithelial cell markers decreased significantly (Fig. 8C). qRT-PCR assay showed the trend of mRNA level of EMT markers was consistent with the results mentioned above (Fig. 8B and D).

DISCUSSION

Hepatocellular carcinoma (HCC) is one of the most common and generally incurable malignancies, which represent the third leading cause of cancer-related deaths worldwide (13). The high mortality is due to late-stage detection of this cancer when most of the therapies available are not effective (14). The disease is progressive and death usually occurs within 10 months of initial diagnosis. Most HCC-related deaths are due to advanced metastatic disease, resulting from lymphatic, blood, or contiguous local spread, highlighting the need for a better understanding of this disease pathogenesis (15). Mounting evidence shows that in epithelial cancers, including HCC, induction of EMT is a major event that provides mobility to cancer cells in order to generate metastases (16).

EMT is a process that cells undergo a morphological switch from the epithelial phenotype to mesenchymal phenotype. In this process, epithelial cells not only lose defined cell–cell/cell–substratum contacts and their

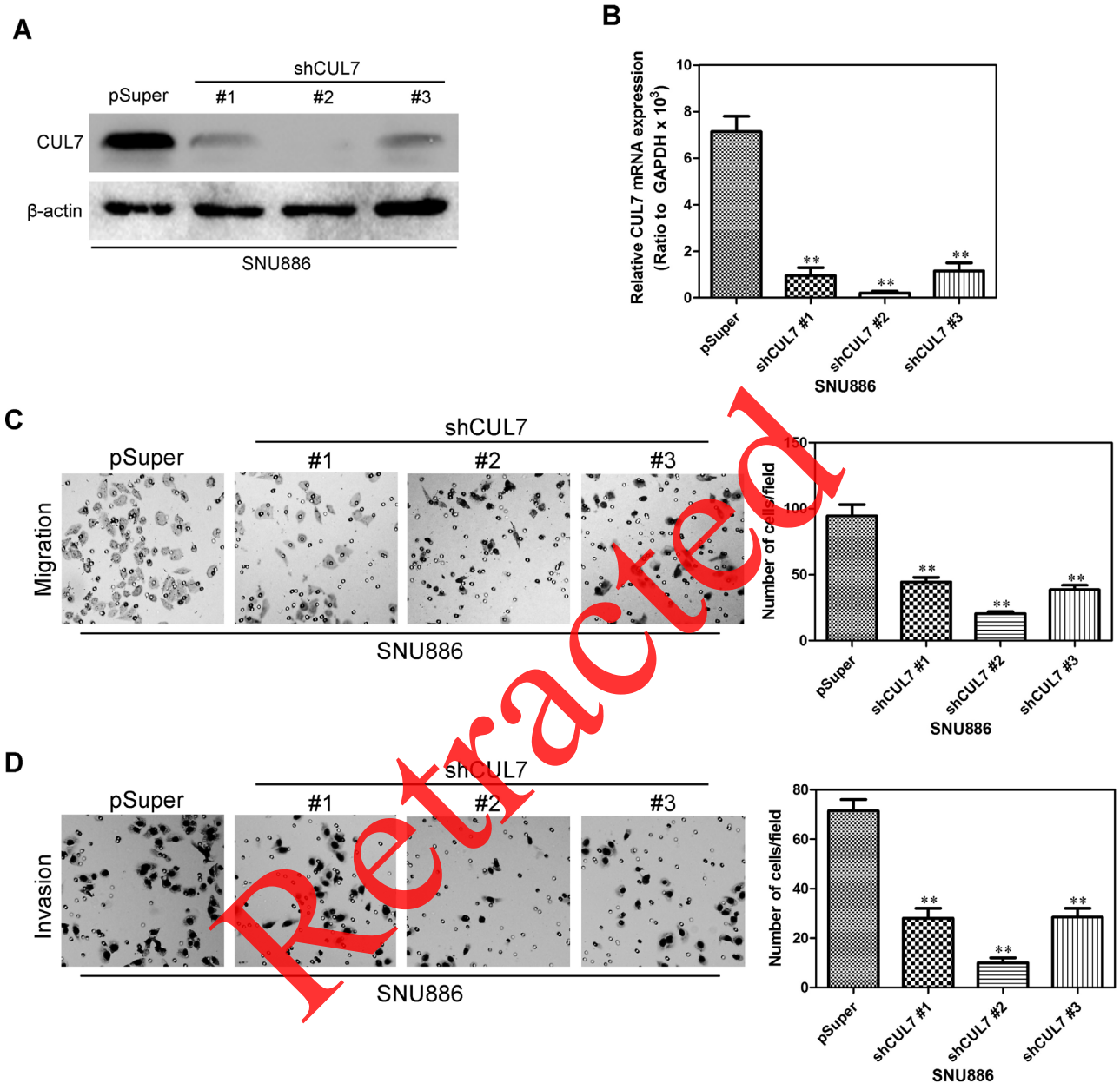


Figure 5. Silencing Cullin7 inhibits HCC cell migration and invasion in vitro. The levels of Cullin7 were verified by Western blotting (A) and qRT-CPR (B) in SNU886-shCUL7s and their control cells. (C) SNU886-shCUL7s and their control cells were subjected to Transwell migration assay. (D) SNU886-shCUL7s and their control cells were subjected to Matrigel invasion assay. $^{**}p<0.01$ is based on the Student *t*-test.

structural polarity, but also they become spindle shaped (17). On the molecular level, EMT is such defined that it is the loss of cell–cell adhesion molecules (E-cadherin and α-catenin), downregulation of epithelial differentiation markers, and transcriptional induction of mesenchymal markers such as N-cadherin and vimentin (17). It has been shown that EMT is associated with cancer progression and cancer-related death. Moreover, EMT has been recognized to play pivotal roles in several diverse processes during embryonic development, chronic inflammation and fibrosis, as well as tumor progression (18). Numerous observations support the concept that the EMT process plays a role in the progression of tumors, including HCCs (19).

In this study, the clinical significance of Cullin7 in HCC and the mechanistic role of Cullin7 in inhibiting HCC cell metastasis were first delineated to our knowledge. We found that Cullin7 down expression in HCC cells inhibited

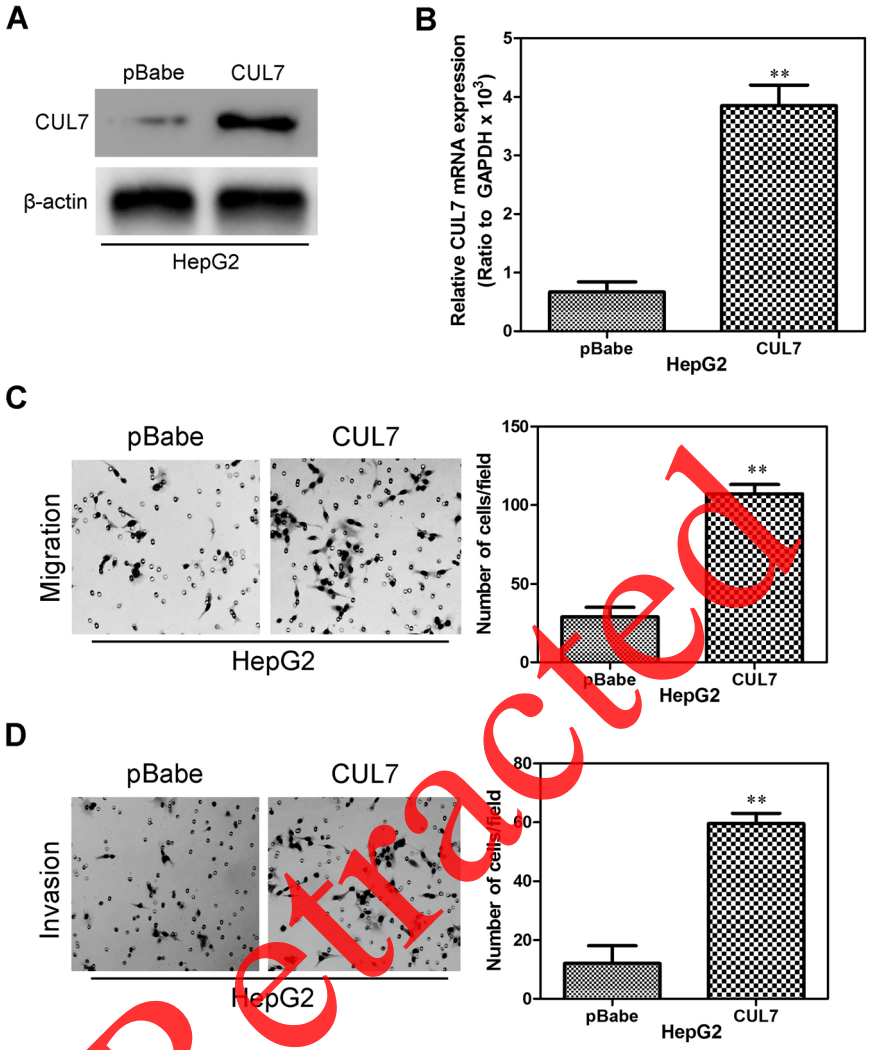


Figure 6. Ectopic Cullin7 expression promotes HCC cell migration and invasion in vitro. The levels of Cullin7 were verified by Western blotting (A) and qRT-CPR (B) in HepG2-CUL7 and its control cells. (C) HepG2-CUL7 and its control cells were subjected to Transwell migration assay. (D) HepG2-CUL7 and its control cells were subjected to Matrigel invasion assays. $^{**}p<0.01$ is based on the Student *t*-test.

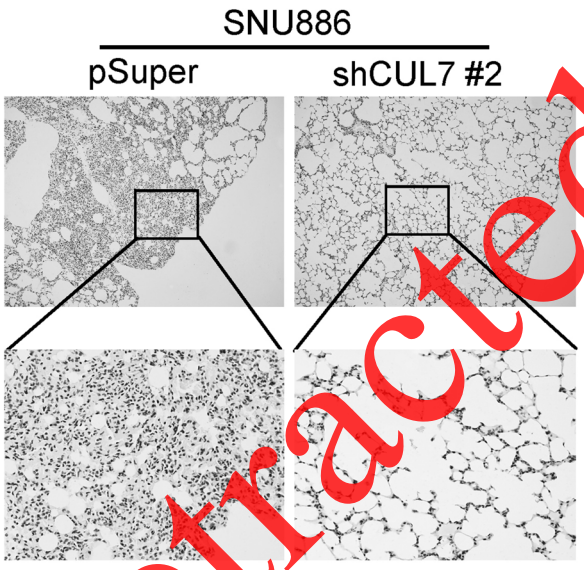
EMT, migration, and invasion in vitro and enhanced metastatic capacity in vivo. In contrast, ectopic Cullin7 expression reversed these events in other HCC cells. It was also shown that a mechanistic link exists between Cullin7 and EMT. Considering all these results, we were prompted to propose a model for Cullin7 regulation of EMT and metastasis in HCC cells.

Cullin7 was originally discovered as a 185-kDa protein (p185) associated with the large T antigen of simian virus 40 (SV40) (6). The C terminus of Cullin7 harbors a BH3 domain, which presumably promotes apoptosis. Together with Skp1, Fbx29, and ROC1, Cullin7 forms the SCF-ROC1 E3 ligase complex (SCF7) (6). Furthermore, Cullin7 was shown to form an E3 ligase with Cul1 and the F-box protein FBX29, which confers substrate specificity (20). Association with the SCF7 complex is required for cellular transformation by SV40 large T antigen. Cullin7 is highly homologous to PARC (PARkin-like, cytoplasmic, p53-binding protein), which negatively regulates p53 by cytoplasmic sequestration (20). Cullin7 has been shown to promote tumor progression through regulatory pathways involved in growth, differentiation, and apoptosis (21,22). Recently, research has demonstrated that Cullin7 can heterodimerize with other TRIM proteins and has E3 ubiquitin ligase activity (23). All of these data strongly support a role for Cullin7 as an oncogene. However, the exact mechanisms of Cullin7's involvement in HCC remain unclear. Our study points to a novel function of Cullin7 in HCC metastasis through inhibiting essential characteristics of metastatic disease in HCCs: EMT. All

A

	Number of mice with lung metastasis
SNU886 pSuper	9/10
SNU886 shCUL7 #2	2/10

B



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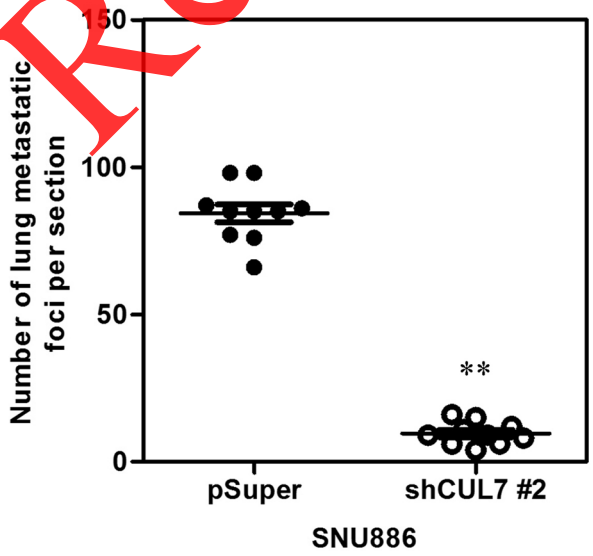

Figure 7. Silencing Cullin7 inhibits HCC cell distant metastasis in vivo. (A) The total numbers of mice with distant metastasis at 60 days after injection of SNU886-shCUL7 #2 and its control cells. (B) The representing pictures of lung H&E stain of individual mouse with injection of SNU886-shCUL7 #2 and its control cells. (C) The numbers of metastatic foci per section in the lung of individual mouse with the injection of SNU886-shCUL7 #2 and its control cells. $^{**}p<0.01$ is based on the Student *t*-test.

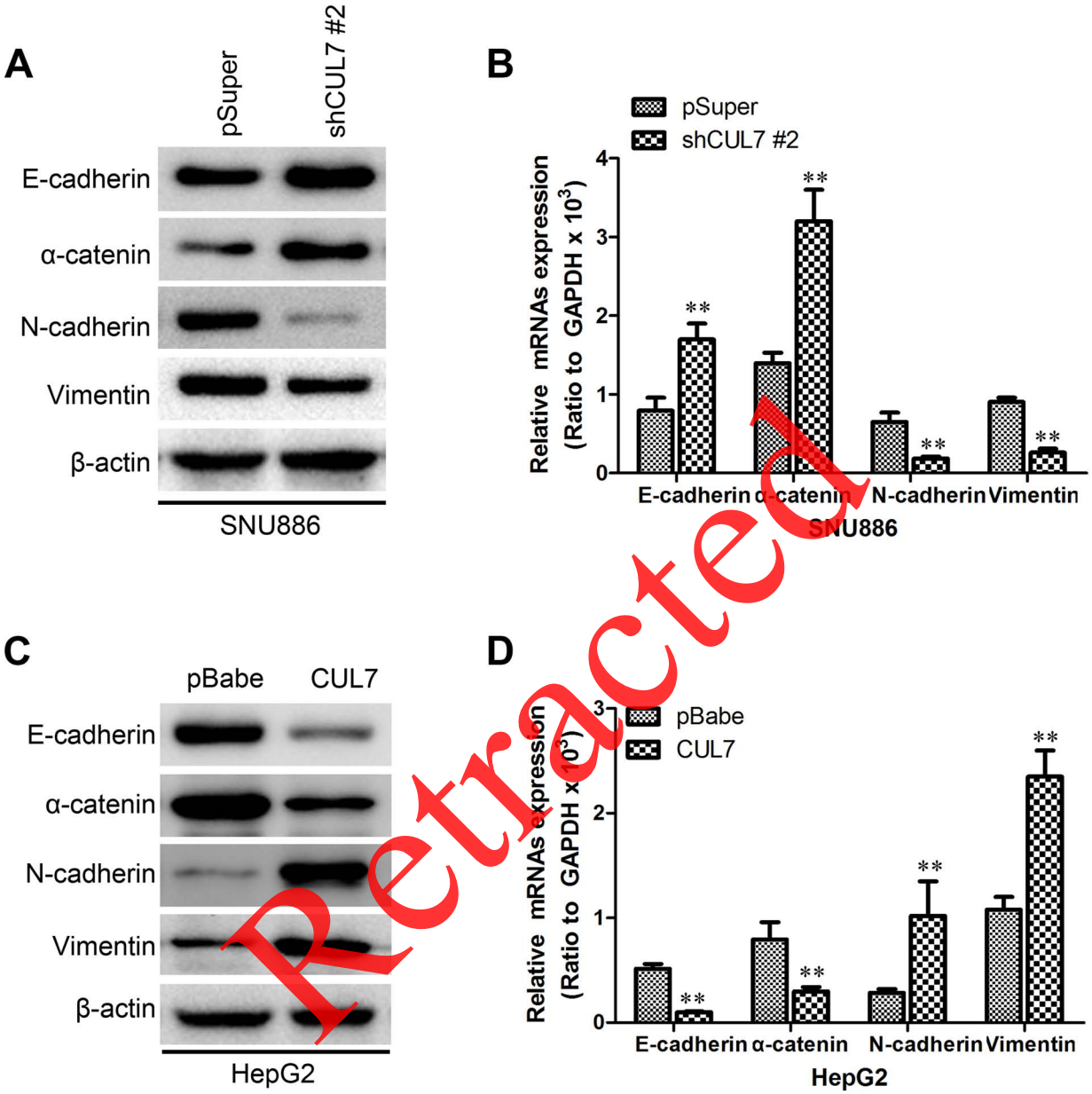

Figure 8. Cullin7 regulates the expression of EMT markers in HCC cells. Expression of epithelial and mesenchymal marker was analyzed by Western blotting (A) and qRT-CPR (B) in SNU886-shCUL7 #2 and its control cells. Expression of epithelial and mesenchymal marker was analyzed by Western blotting (C) and qRT-CPR (D) in HepG2-CUL7 and its control cells. $^{**}p<0.01$ is based on the Student *t*-test.

of these characteristics that are induced by shCullin7 in vitro culminated to decreased numbers of distant metastases in vivo. These empirical findings provide a mechanistic framework to explain the clinical observations that HCC patients with high levels of Cullin7 in tissue samples have more chance of distant metastasis.

Metastasis and EMT are essential for HCC cells to disseminate from adjacent tissues and seed new tumors in distant sites (24). Our results demonstrated that Cullin7 regulated these two essential characteristics of metastatic disease, and Cullin7-induced processes are reversible with the suppression of Cullin7 expression, providing us an optimal therapeutic option to manipulate Cullin7 levels in clinical HCC practice.

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