Supplementary information

RACK1 promotes self-renewal and chemoresistance of cancer stem cells in human hepatocellular carcinoma through stabilizing Nanog

Junxia Cao[1,*], Min Zhao[1,2,*], Jian Liu[1,2], Xueying Zhang[1], Yujun Pei[1], Jingyang Wang[1], Xiao Yang[3], Beifen Shen[1,2], Jiyan Zhang[1,2]

1. Institute of Basic Medical Sciences, 27 Taiping Road, Beijing 100850, China
2. Beijing Institute of Brain Sciences, 27 Taiping Road, Beijing 100850, China
3. Genetic Laboratory of Development and Diseases, State Key Laboratory of Proteomics, Institute of Biotechnology, 20 Dongdajie, Beijing, China

Supplementary Figures

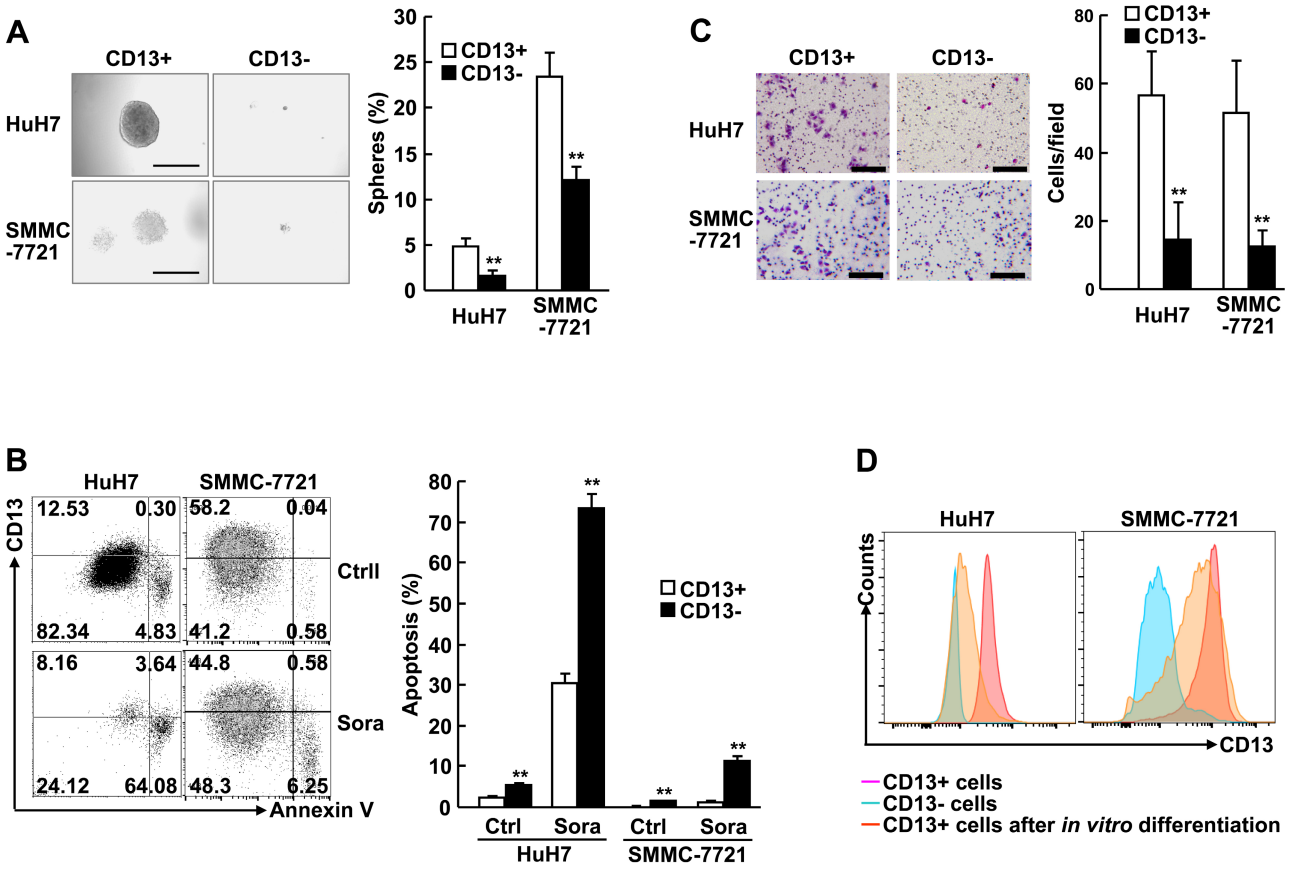

Figure S1. CD13+ subpopulations in HuH7 and SMMC7721 cells show characteristics of CSCs.

(**A**) CD13+ and CD13- subpopulations in HuH7 and SMMC7721 cells were sorted and subjected to sphere formation assays. *Left*, representative data; Scale bar: 250 μm. *Right*, mean±s.d. (n=5); *P<0.05, **P<0.01. (**B**) HuH7 and SMMC7721 cells were treated with or without 50μM sorafenib for 24h, apoptosis of CD13+ and CD13- subpopulations were then analyzed with anti-CD13-PE and Annexin V-FITC staining. *Left*, representative data; *Right*, mean±s.d. (n=3). Ctrl, control; Sora, Sorafenib. (**C**) CD13+ and CD13- subpopulations in HuH7 and SMMC7721 cells were sorted and

subjected to migration assays. *Left*, representative data; Scale bar: 200 μm. *Right*, mean±s.d. (*n*=3). (**D**) *In vitro* differentiation of CD13+ cells. CD13+ subpopulations in HuH7 and SMMC7721 cells were sorted and cultured in conventional conditions. CD13 expression was determined by flow cytometry after 10 days.

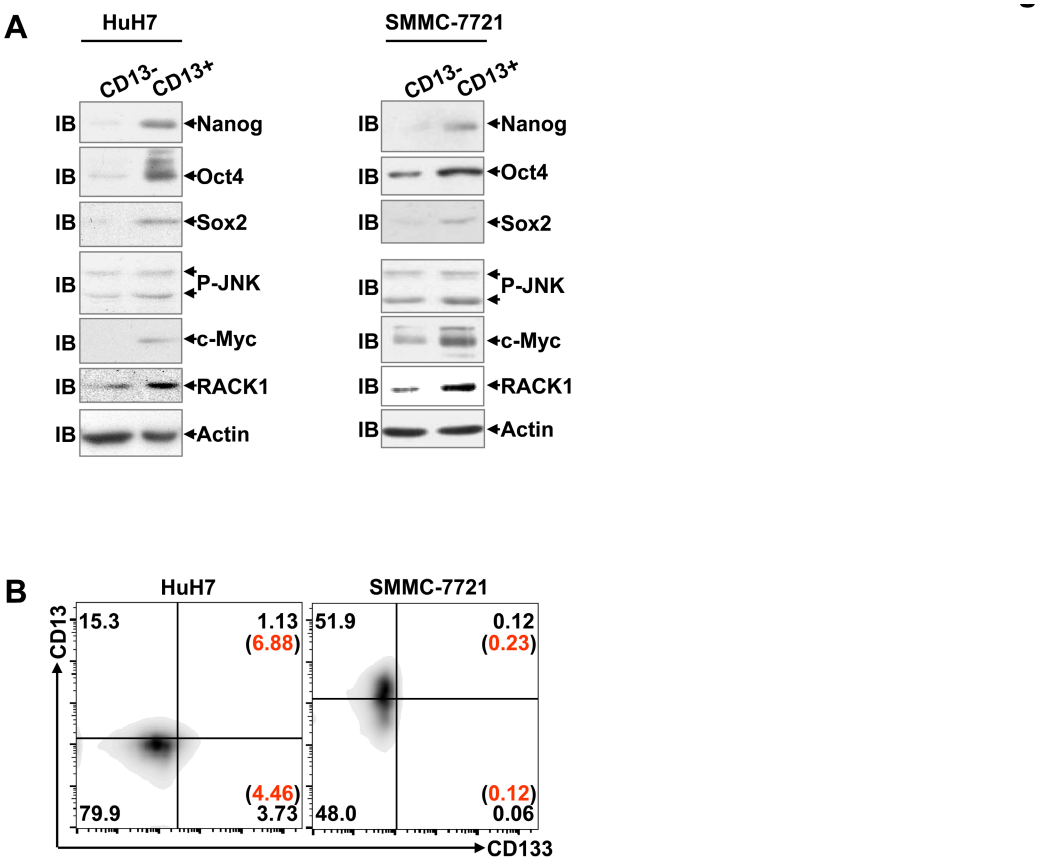

Figure S2. Correlation of CD13 with ESC-specific pluripotency transcription factors (A), RACK1-related signaling (A), and another CSC marker CD133 (B) in HuH7 and SMMC7721 cells.

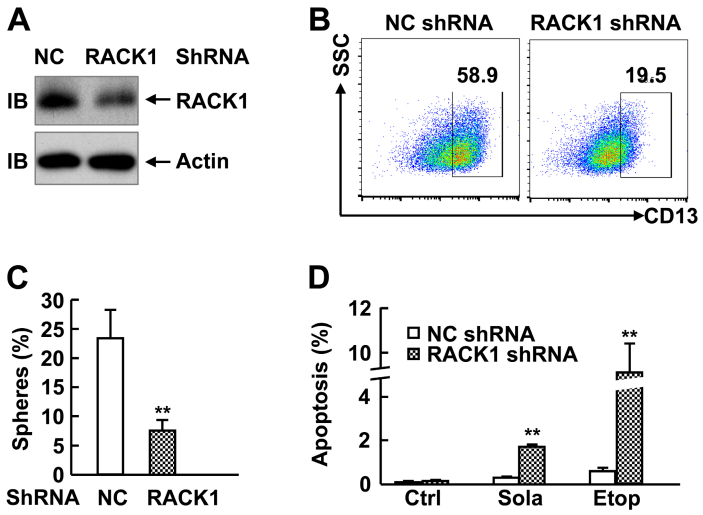

Figure S3. Effects of RACK1 knockdown on CD13+ SMMC-7721 cells. SMMC-7721 cells were infected with lentivirus expressing non-targeting control shRNA or RACK1 shRNA. 96 h later, cells were subjected to the following assays: (**A**) Immunoblotting analysis of RACK1 expression in total cells. (**B**) Flow cytometric analysis of CD13 expression in total cells. (**C**) Sphere formation efficiency of sorted CD13+ subpopulation. mean±s.d. (*n*=3). (**D**) Etoposide (Etop, 100μM, 48h)- or sorafenib (Sora, 50μM, 24h)-induced apoptosis of CD13+ subpopulation. mean±s.d. (*n*=3).

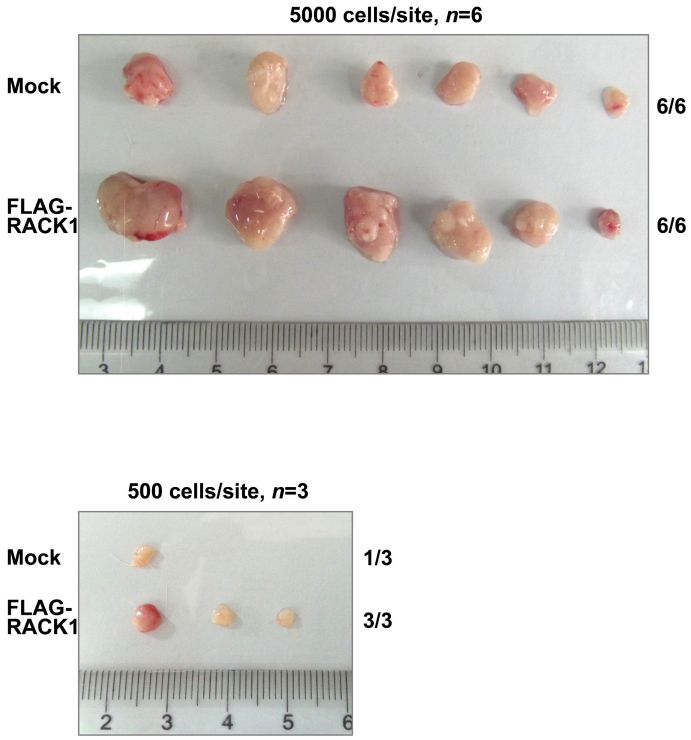

Figure S4. Tumor formation of CD13+ HuH7 cells upon RACK1 over-expression.

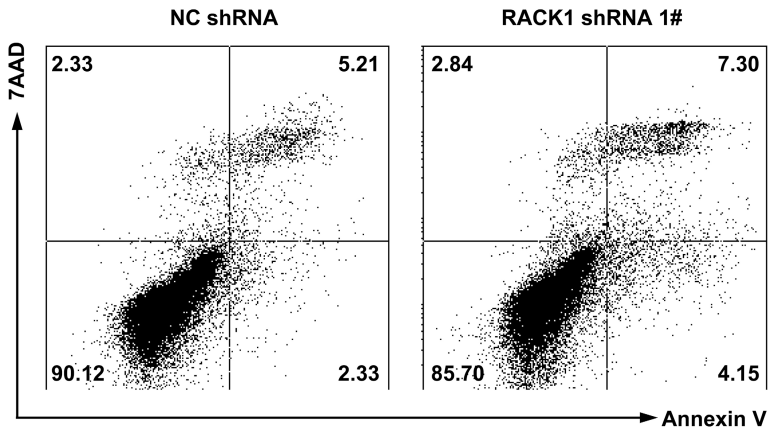

Figure S5. The effect of RACK1 knockdown on murine ESC survival.

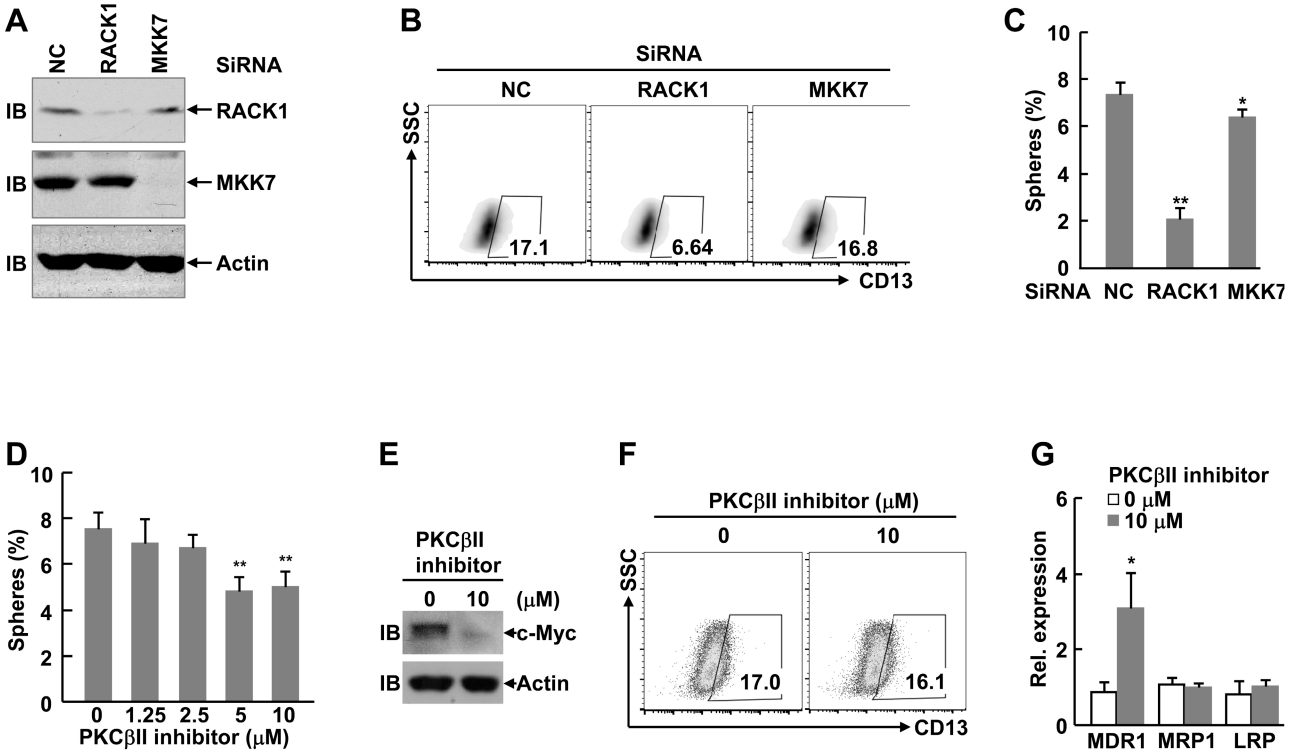

Figure S6. The roles of MKK7 and PKCβII in the stemness of HuH7 cells. (**A-C**) 48 h after HuH7 cells were transfected with the indicated siRNAs, cells were subjected to the following assays: (**A**) Immunoblotting analysis of RACK1 and MKK7 expression in total cells. (**B**) Flow cytometric analysis of CD13 expression in total cells. (**C**) Sphere formation assays of sorted CD13+ subpopulation. mean±s.d. (n=3); *P<0.05, **P<0.01. **(D)** Sphere formation assays of sorted CD13+ subpopulation in the presence of different doses of selective PKCβII inhibitor CGP53353. mean±s.d. (n=3). (**E-G**) 48 h after HuH7 cells were transfected with or without 10 μM CGP53353, cells were subjected to the following assays: (**E**) Immunoblotting analysis of c-Myc expression in total cells. (**F**) Flow cytometric analysis of CD13 expression in total cells. (**G**) qRT-PCR analysis of sorted CD13+ subpopulation for the expression of the indicated drug-resistant genes. mean±s.d. (n=3).

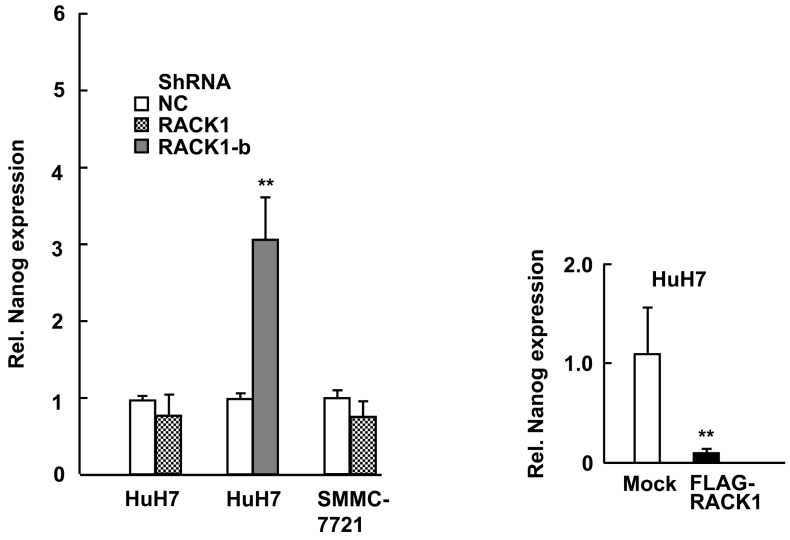

Figure S7. qRT-PCR analysis of the mRNA levels of Nanog in HuH7 and SMMC-7721 cells upon RACK1 knockdown or over-expression. mean±s.d. (*n*=3).

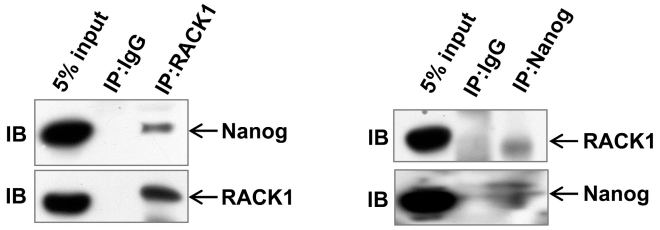

Figure S8. The physiological interaction between RACK1 and Nanog in murine ESCs. Immunoblotting analysis of the interaction between endogenous Nanog and endogenous RACK1 in murine ESCs after immunoprecipitation with an anti-RACK1 antibody (*Left*, control antibody: rabbit IgG) or an anti-Nanog antibody (*Right*, control antibody: rabbit IgG).

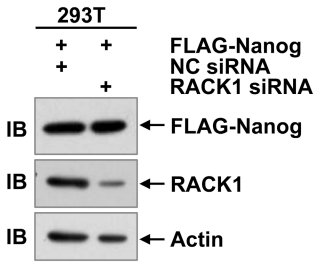

Figure S9. The effect of RACK1 knockdown on ectopic FLAG-Nanog expression in 293T cells.

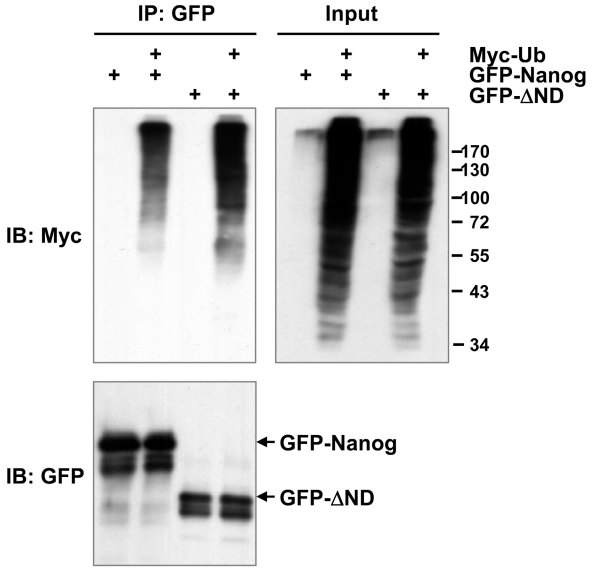

Figure S10. Effects of deletion of RACK1-binding domain on Nanog ubiquitination in human HCC cells. HuH7 cells were transfected with mammalian expression vectors as indicated. The ubiquitination of exogenous Nanog was analyzed by immunoblotting after immunoprecipitation with an anti-GFP antibody. Cells were treated with 20 μM MG132 for 6 h before cell lysates were harvested.

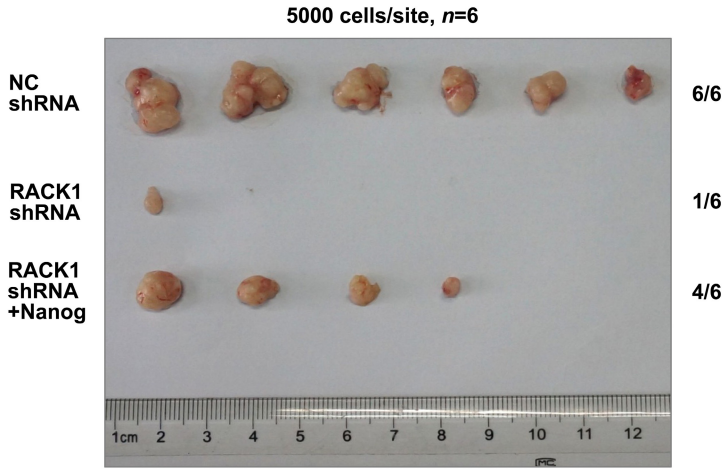

Figure S11. Tumor formation of CD13+ HuH7 cells with or without ectopic Nanog expression upon RACK1 knockdown.

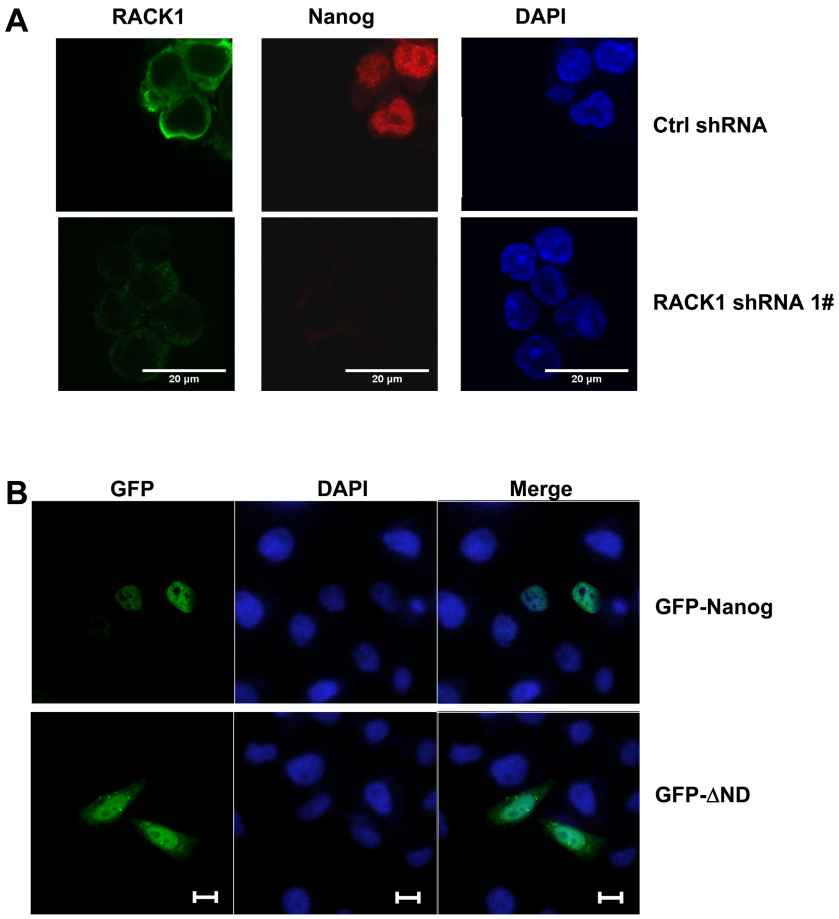

Figure S12. The role of RACK1 in the nuclear translocation of Nanog. (**A**) 96 h after murine ESCs were infected with lentivirus expressing non-targeting control shRNA or RACK1 shRNA 1#, cells were subjected to indirect immunofluorescence analysis with antibodies against Nanog and RACK1, then counterstained with DAPI followed by confocal microscopy (scale bar, 20 μm). (**B**) HuH7 cells were transfected with mammalian expression vectors as indicated. The subcellular localization of GFP-tagged proteins was observed via confocal microscopy (scale bar, 10 μm). Nuclei were counterstained for DNA by DAPI.

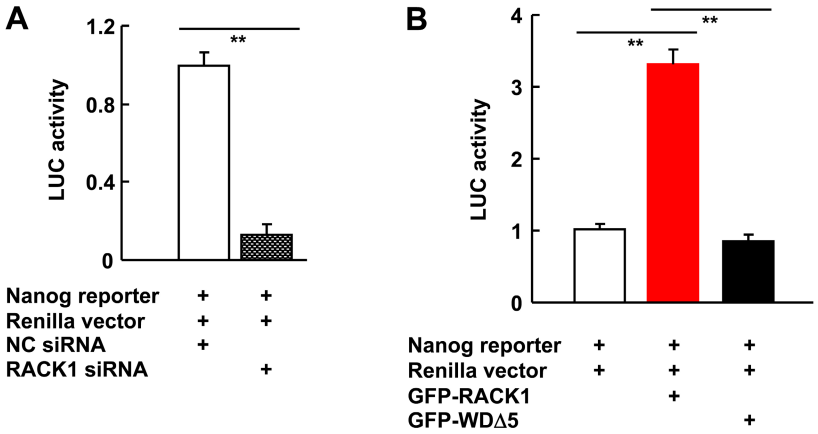

Figure S13. The role of RACK1 in the transactivation of Nanog. mean±s.d. (*n*=3).

Supplementary Tables

Table S1. Summary of clinicopathologic variables

Characteristic	Number of Patients
Patients	136
Sex	
male	113
female	23
Age (years)	18-98, median=50.6
Tumor size (cm, available for 121 samples)	0.6-20, median=5.8
Differentiation	
1	16
2	68
3	52
Clinical stage	
I	1
II	53
III	80
IV	2

Table S2. Comparison of RACK1 expression in tumor tissues with that in peritumoral liver tissues

		RACK1 score			
	n	1-4	5-8	9-12	*P* value
Peritumor	16	13	3	0	<0.0001
Tumor	136	24	51	61	

Table S3. Correlation of RACK1 expression in HCC tissues with clinicopathological features of 136 patients

		RACK1 score			
Feature	n	1-4	5-8	9-12	*P* value
Age					
≤ 60 year	107	20	38	49	0.9358
> 60 year	29	4	13	12	
Sex					
Male	113	22	39	52	0.9849
Female	23	2	12	9	
Tumor size					
≤ 5 cm	68	15	32	21	0.0177
> 5 cm	53	8	16	29	
Differentiation					
1	16	5	6	5	
2	68	13	22	33	0.3291
3	52	6	23	23	
Clinical stage					
I-II	54	14	26	14	0.0004
III-IV	82	10	25	47	

Table S4. Comparison of Nanog expression in tumor tissues with that in peritumoral liver tissues

		Nanog score				
	n	0	1-4	5-8	9-12	*P* value
Peritumor	16	15	1	0	0	<0.0001
Tumor	136	47	58	11	20	

Table S5. Correlation of Nanog expression in HCC tissues with clinicopathological features of 136 patients

		Nanog score				
Feature	n	0	1-4	5-8	9-12	*P* value
Age						
≤ 60 year	107	36	45	9	17	0.4748
> 60 year	29	11	13	2	3	
Sex						
Male	113	37	50	9	17	0.4515
Female	23	10	8	2	3	
Tumor size						
≤ 5 cm	68	23	32	5	8	0.7331
> 5 cm	53	20	23	4	6	
Differentiation						
1	16	11	5	0	0	
2	68	22	32	8	6	0.0018
3	52	14	21	3	14	
Clinical stage						
I-II	54	19	20	5	10	0.6131
III-IV	82	28	38	6	10	