PDLIM5 inhibits STUB1-mediated degradation of SMAD3 and promotes the migration and invasion of lung cancer cells

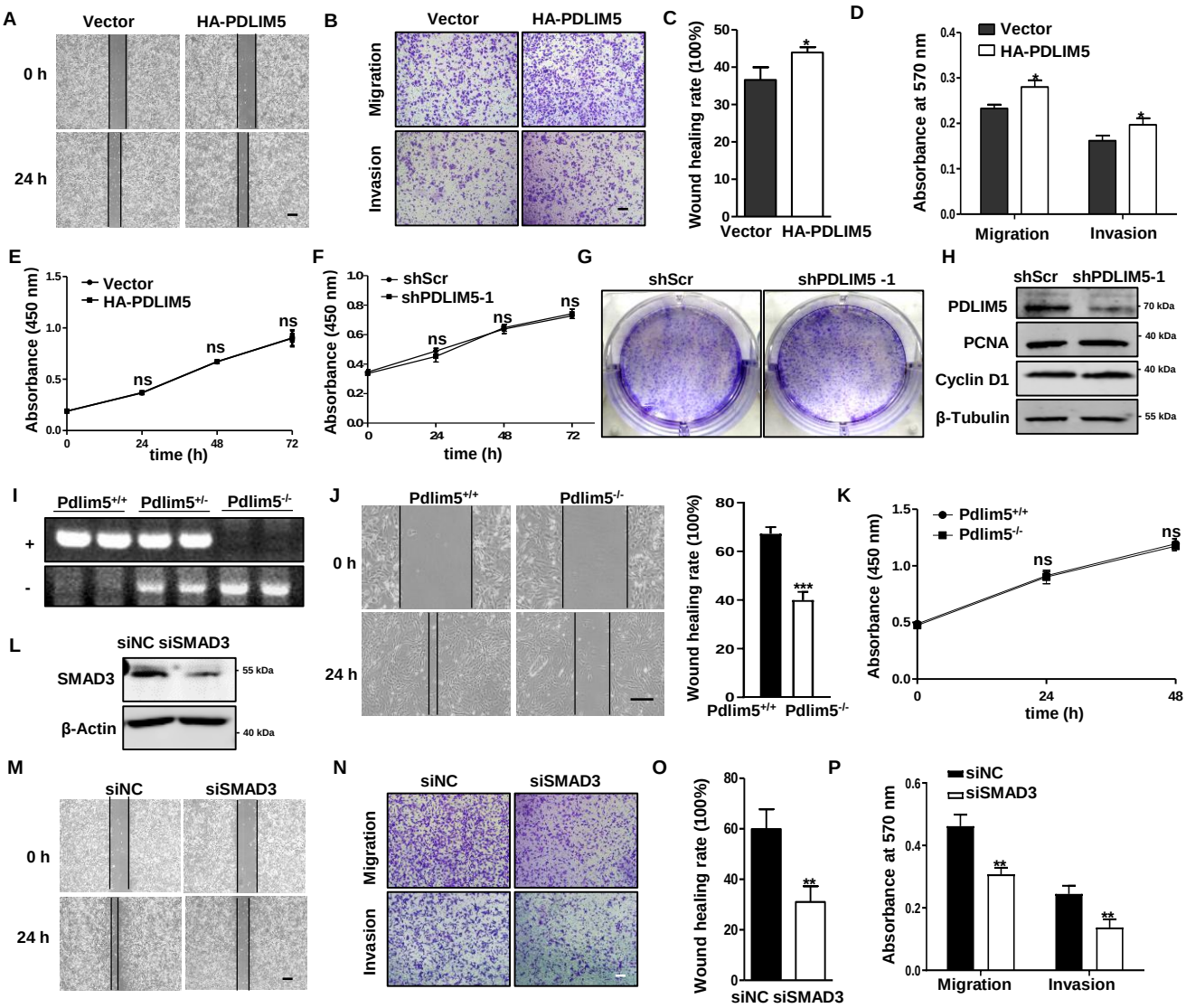

Figure S1. PDLIM5 and SMAD3 promotes cell migration and invasion and PDLIM5 is not necessary for cell proliferation. (A) Representative images of the wound-healing assay in *PDLIM5* overexpressed PC9 cells. Images were captured at 0 and 24 h after scratching. Scale bar: 200 μm. (C) The wound-healing rate was analyzed by the Image J software (n = 3). (B) Representative images of transwell migration and transwell invasion assay for *PDLIM5* overexpressed PC9 cells. Scale bar: 200 μm. (D) The migration and invasion index were quantified (n=3). (E, F) CCK8 assay was performed in *PDLIM5* overexpressed PC9 cells (E) and *PDLIM5* knockdown A549 cells (F) at indicated time (0 h, 24 h, 48 h, 72 h) (n = 3). (G) Representative images of clone formation assay of *PDLIM5* knockdown A549 cells. (H) Western blot analysis of PCNA and Cyclind D1 in *PDLIM5* knockdown A549 cells. β-Tubulin was used as a loading control. (I) PCR results showed *Pdlim5* wildtype and *Pdlim5* knockout allele. (J) Representative images of the wound-healing assay in *Pdlim5* wildtype ($Pdlim5^{+/+}$) and *Pdlim5* knockout ($Pdlim5^{-/-}$) mouse embryonic fibroblasts. Images were captured at 0 and 24 h after scratching. Scale bar: 200 μm. The wound-healing rate was analyzed by the Image J software (n = 3). (K) CCK8 assay was performed in *Pdlim5* wildtype ($Pdlim5^{+/+}$) and *Pdlim5* knockout ($Pdlim5^{-/-}$) mouse embryonic fibroblasts at indicated time (0 h, 24 h, 48 h) (n = 3). (L) Western blot analysis of *SMAD3* knockdown efficiency in A549 cells. (M) Representative images of the wound-healing assay for *SMAD3* knockdown A549 cells. Images were captured at 0 and 24 h after scratching. Scale bar: 200 μm. (O) The wound-healing rate was analyzed by the Image J software (n = 3). (N) Representative images of transwell migration and transwell invasion assay for *SMAD3* knockdown A549 cells. Scale bar: 200 μm. (P) The migration and invasion index were quantified (n=3). Data were shown as mean ± SD. Analysis was performed using two-tailed Student's t-test for C, D, J, O, P and one way ANOVA with Tukey post hoc test for E, F, K. * $p < 0.05$, ** $p < 0.01$.

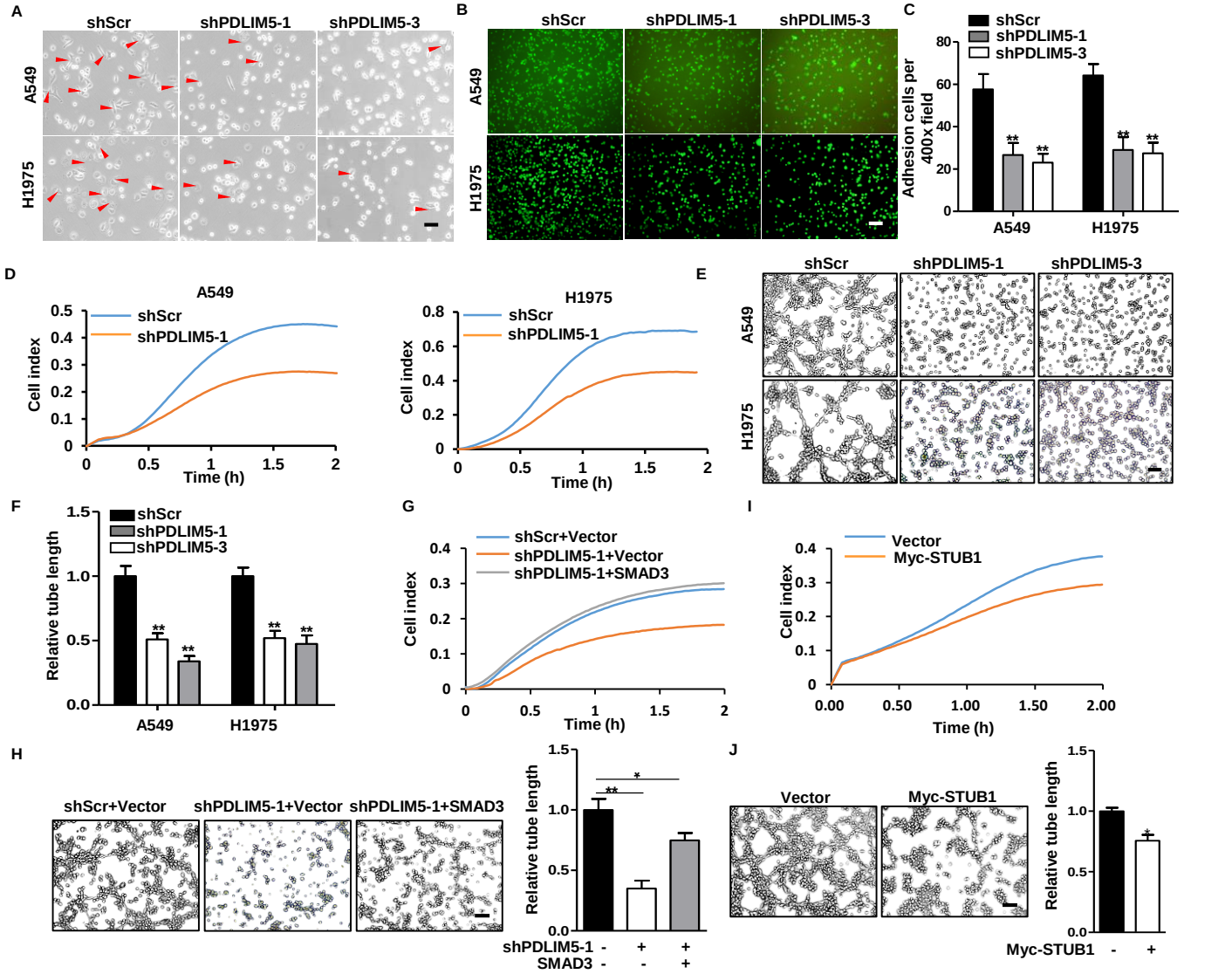

Figure S2. *PDLIM5* knockdown and *STUB1* overexpression impair cell adhesion and vasculogenic mimicry, which were rescued by *SMAD3* overexpression. (A) Spreading of *PDLIM5* knockdown A549 and H1975 cells after re-plating for 3 h. Arrows indicated spreading cells. Scale bar: 50 μm. (B) Representative images of *PDLIM5* knockdown A549 and H1975 cells adhered to endothelial cells. Scale bar: 100 μm. (C) the adhered cells were quantified (n = 4). (D) Adhesion of *PDLIM5* knockdown A549 and H1975 cells were recorded by a RTCA. (E) Representative images of the vasculogenic mimicry assay for *PDLIM5* knockdown A549 and H1975 cells. Scale bar: 100 μm. (F) The tube length was analyzed by the Image J software (n = 4). (G) Adhesion of *PDLIM5* knockdown A549 cells with or without *SMAD3* overexpression were recorded by a RTCA. (H) Representative images of the vasculogenic mimicry assay of *PDLIM5* knockdown A549 cells, with or without *SMAD3* overexpression. Scale bar: 100 μm. The tube length was analyzed by the Image J software (n = 4). (I) Adhesion of *STUB1* overexpressed A549 cells recorded by a RTCA. (J) Representative images of vasculogenic mimicry assay with *STUB1* overexpression in A549 cells. Scale bar: 100 μm. The tube length was analyzed by using the Image J software (n=3). Data were shown as the mean ± SD. Analysis was performed using two-tailed Student's t-test for C, F, J and one way ANOVA with Tukey post hoc test for H. * $p < 0.05$, ** $p < 0.01$

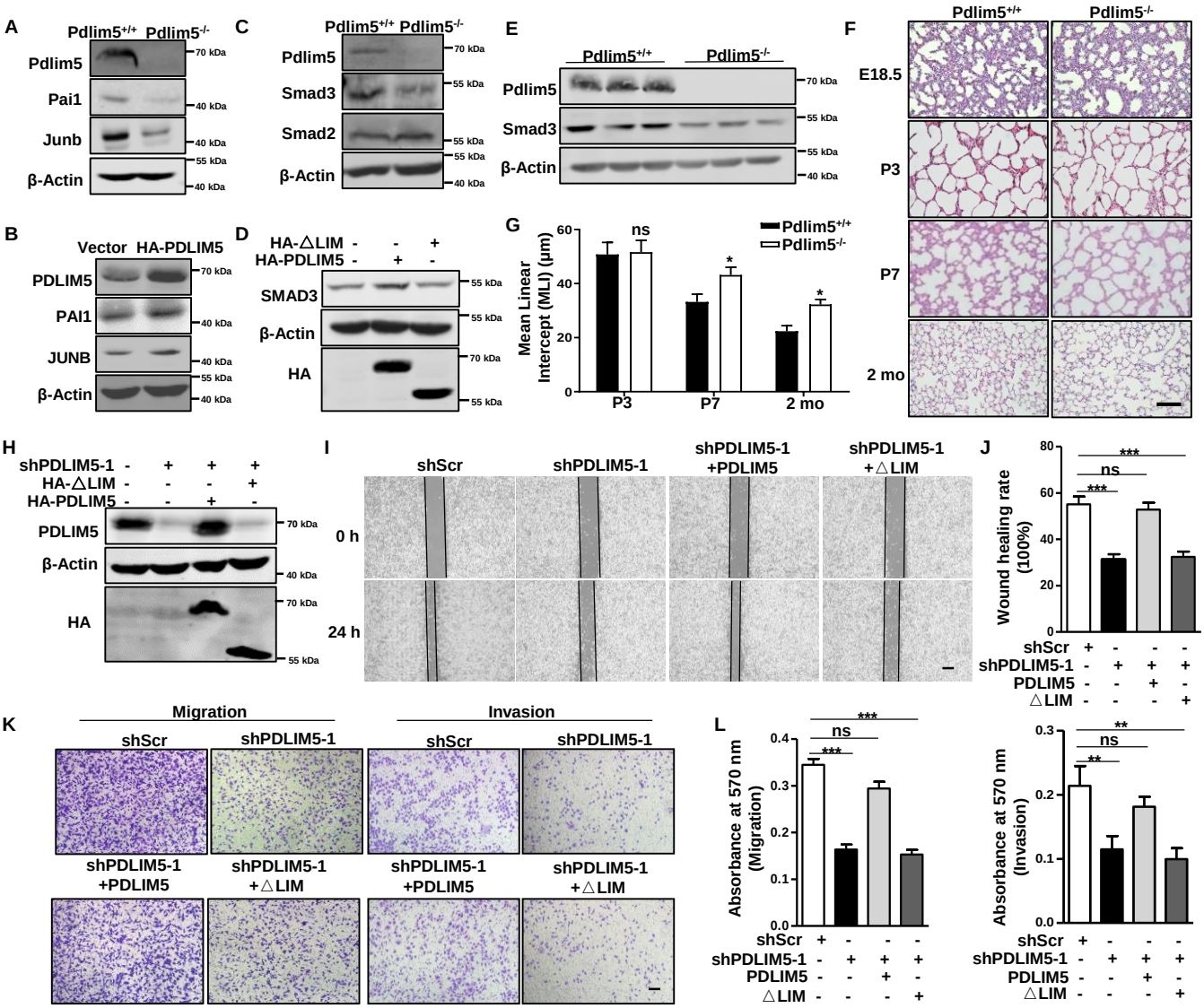

Figure S3. SMAD3 mediates PDLIM5 function. (A) Western blot analysis of Pai1 and Junb in *Pdlim5* wildtype (*Pdlim5*$^{+/+}$) and *Pdlim5* knockout (*Pdlim5*$^{-/-}$) mouse embryonic fibroblasts. β-Actin was used as a loading control. (B) Western blot analysis of PAI1 and JUNB in *PDLIM5* overexpressed PC9 cells. β-Actin was used as a loading control. (C) Western blot analysis of Smad2 and Smad3 in *Pdlim5* wildtype (*Pdlim5*$^{+/+}$) and *Pdlim5* knockout (*Pdlim5*$^{-/-}$) mouse embryonic fibroblasts. β-Actin was used as a loading control. (D) Western blot analysis of SMAD3 in PDLIM5 full-length and LIM-domain deletion mutant overexpressed PC9 cells. β-Actin was used as a loading control. (E) Western blot analysis of Pdlim5 and Smad3 in the left lungs of *Pdlim5* wildtype (*Pdlim5*$^{+/+}$) and *Pdlim5* knockout (*Pdlim5*$^{-/-}$) mice. β-Actin was used as a loading control (n = 3). (F) Hematoxylin and eosin staining for lung sections from *Pdlim5* wildtype (*Pdlim5*$^{+/+}$) and *Pdlim5* knockout (*Pdlim5*$^{-/-}$) mice. The lung was collected from E18.5 embryos and postnatal 3 days, 7 days, and 2 months. Each group contained 3 or 4 embryos or mice. Scale bar: 50 μm. (G) Quantitative analyses of the pulmonary alveolar sizes through measuring mean linear intercept (MLI). (H) Western blot analysis of PDLIM5 levels in *PDLIM5* knockdown cells, with *PDLIM5* full-length or LIM-domain deletion mutant overexpression. β-Actin was used as a loading control. (I) Representative images of the wound healing assay of *PDLIM5* knockdown A549 cells with PDLIM5 full-length and LIM-domain deletion mutant overexpression. Images were captured at 0 and 24 h after scratching. Scale bar: 200 μm. (J) The wound-healing rate was analyzed by the Image J software (n = 3). (K) Representative images of the transwell migration and invasion assay of *PDLIM5* knockdown A549 cells with PDLIM5 full-length and LIM-domain deletion mutant overexpression. Scale bar: 200 μm. (L) The migration and invasion index were quantified (n = 3). Data were shown as mean ± SD. Analysis was performed using one way ANOVA with Tukey post hoc test for J, L. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

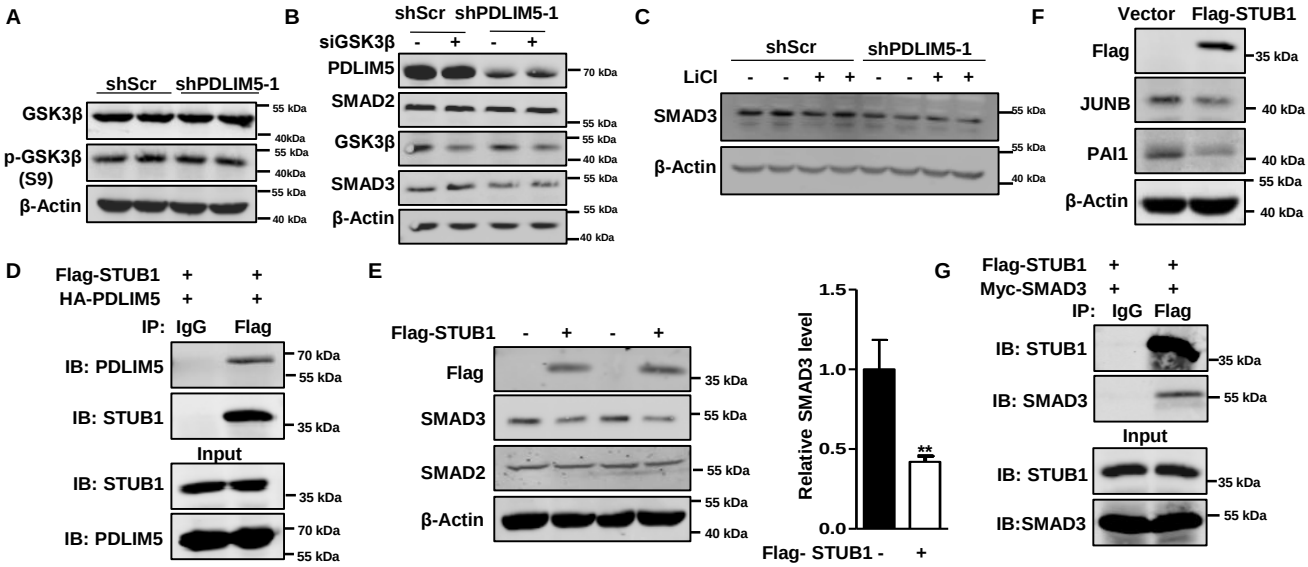

Figure S4. GSK3β is not responsible for *PDLIM5*-knockdown induced SMAD3 degradation, and *STUB1* overexpression inhibits TGFβ signaling. (A) Western blot analysis of GSK3β and phosphorylated GSK3β (p-GSK3β) in *PDLIM5* knockdown A549 cells. β-Actin was used as a loading control. (B) Western blot analysis of SMAD3 in *PDLIM5* knockdown A549 cells, with or without *GSK3β* knockdown. β-Actin was used as a loading control. (C) Western blot analysis of SMAD3 in *PDLIM5* knockdown A549 cells, with or without LiCl (inhibitor for GSK3β) treatment. β-Actin was used as a loading control. (D) Co-immunoprecipitation analysis of the interaction between PDLIM5 and STUB1 in HEK293T cells. (E) Western blot analysis of SMAD3 and SMAD2 in A549 cells transfected with Flag-STUB1 expressing vector. SMAD3 levels were quantified and normalized to the control (n = 3). β-Actin was used as a loading control. (F) Western blot analysis for PAI1 and JUNB in *STUB1* overexpressed A549 cells. β-Actin was used as a loading control. (G) Co-immunoprecipitation analysis of the interaction between STUB1 and SMAD3 in HEK293T cells. Data were shown as mean ± SD. Analysis was performed using two-tailed Student's t-test for E . * $p < 0.05$.

Supplementary Table S1. The target sequence of shRNA and siRNA

shPDLIM5-1	CCGGTGTTAGGTAGTTATGAGTAAACTCGAGTTTACTCATAACTACCTAACATTTTTTG
shPDLIM5-2	CCGGCGCCCATTGTAACCAGGTCATCTCGAGATGACCTGGTTACAATGGGCGTTTTT
shPDLIM5-3	CCGGTGTTAGGTAGTTATGAGTAAACTCGAGTTTACTCATAACTACCTAACATTTTTG
shPdlim5 (Mouse)	CCGGCTGCATGTTAGTGCCAATCTTCTCGAGAAGATTGGCACTAACATGCAGTTTTTG
siGSK3β	5′-GAUCUGUCUUGAAGGAGAATT-3′
siSTUB1	5′-CCCAAGTTCTGCTGTTGGACTCT-3′

Supplementary Table S2. Antibody

Antibody	**Cat number**	**Dilution**	**Source**	**Company**
Anti-PDLIM5	ab196559	WB: 1:1000; IF/IHC/IP: 1:100	Rabbit	Abcam
Anti-Flag	AF519	WB: 1:1000; IP: 1:100	Mouse	Beyotime
Anti-Flag	#14793	WB: 1:1000; IP: 1:100	Rabbit	Cell Signaling
Anti-Myc	TA150121	WB: 1:1000; IP: 1:100	Mouse	ORIGENE
Anti-HA	E022010	WB: 1:1000; IP: 1:100	Mouse	EarthOx
Anti-PCNA	#13110	WB: 1:1000	Rabbit	Cell Signaling
Anti-Cyclin D1	#2922	WB: 1:1000	Rabbit	Cell Signaling
Anti-PAI	13801-1-AP	WB: 1:1000	Rabbit	proteintech
Anti-JunB	10486-1-AP	WB: 1:1000	Rabbit	proteintech
Anti-Vimentin	#5741	WB: 1:1000	Rabbit	Cell Signaling
Anti-E-Cadherin	#3195	WB: 1:1000	Rabbit	Cell Signaling
Anti-p-SMAD2	#18338	WB: 1:1000	Rabbit	Cell Signaling
Anti-p-SMAD3	#9520	WB: 1:1000	Rabbit	Cell Signaling
Anti-SMAD2	#5339	WB: 1:1000	Rabbit	Cell Signaling
Anti-SMAD3	#9523	WB: 1:1000	Rabbit	Cell Signaling
Anti-Snail1	A5544	WB: 1:1000	Rabbit	Abclonal
Anti-SMAD4	#46535	WB: 1:1000	Rabbit	Cell Signaling
Anti-SMAD7	sc-11392	WB: 1:1000	Rabbit	Santa Cruz
Anti-TGFβR1	sc-398	WB: 1:1000	Rabbit	Santa Cruz
Anti-GSK3β	22104-1-AP	WB: 1:1000	Rabbit	proteintech
Anti-p-GSK3β	ab107166	WB: 1:1000	Rabbit	Abcam
Anti-SMAD3	sc-101154	IF:1:100	Mouse	Santa Cruz
Anti-STUB1	55430-1-AP	WB: 1:1000	Rabbit	proteintech
Anti-HA	db5297	WB: 1:1000	Rabbit	Diagbio
Anti-β-Actin	M1210-2	WB: 1:5000	Mouse	HuaBio
800CW Goat anti-Rabbit (WB)	925-32211	1:5000	Goat	LI-COR
680RD Goat anti-Mouse (WB)	925-68070	1:5000	Goat	LI-COR
Goat anti-Rabbit, Alexa Fluor546 (IF)	A-11035	1:200	Goat	ThermoFisher
Goat anti-Mouse, Alexa Fluor 488 (IF)	A-11001	1:200	Goat	ThermoFisher

Supplementary Table S3. The primers used for RT-PCR analysis

Primer Name	Primer Sequence 5’-3’
CDH1-F	TGGGCCAGGAAATCACATCCTACA
CDH1-R	TTGGCAGTGTCTCTCCAAATCCGA
CDH2-F	TGTGGGAATCCGACGAATGGATGA
CDH2-R	TGGAGCCACTGCCTTCATAGTCAA
Vim-F	AGAACCTGCAGGAGGCAGAAGAAT
Vim-R	TTCCATTTCACGCATCTGGCGTTC
PAI1-F	GGCTGACTTCACGAGTCTTTCA
PAI1-R	ATGCGGGCTGAGACTATGACA
SMA-F	ACCCGCCCAGAAACTAGACACAAT
SMA-R	TCGCCCACGTAGGAATCTTTCTGA
Zeb1-F	ATACCTGTGAATGGGCGACCAAGA
Zeb1-R	ACTGCCTGGTGATGCTGAAAGAGA
Snail1-F	ACTGCAACAAGGAATACCTCAG
Snail1-R	GCACTGGTACTTCTTGACATCTG
FN-F	GGCTGAAGACACAAGGAAATAAG
FN-R	CATTTGAGTTGCCACCGTAAG
PDLIM5-F	TCCTTGGAGAAGTCATCAATGC
PDLIM5-R	CACCATCCTCCAAGTGAAAAAC
SMAD2-F	CGTCCATCTTGCCATTCACG
SMAD2-R	CTCAAGCTCATCTAATCGTCCTG
SMAD3-F	TGGACGCAGGTTCTCCAAAC
SMAD3-R	CCGGCTCGCAGTAGGTAAC
SMAD5-F	GGCCGGATTTGCAGAGTCAT
SMAD5-R	AGAATCTGGAAACGTGGCATTT
18S-F	GTAACCCGTTGAACCCCATT
18S-R	CCATCCAATCGGTAGTAGCG