SUPPLEMENTAL INFORMATION

DLC1 Inhibits TGFβ-induced PTHLH Production and Breast Cancer Osteolytic Metastasis

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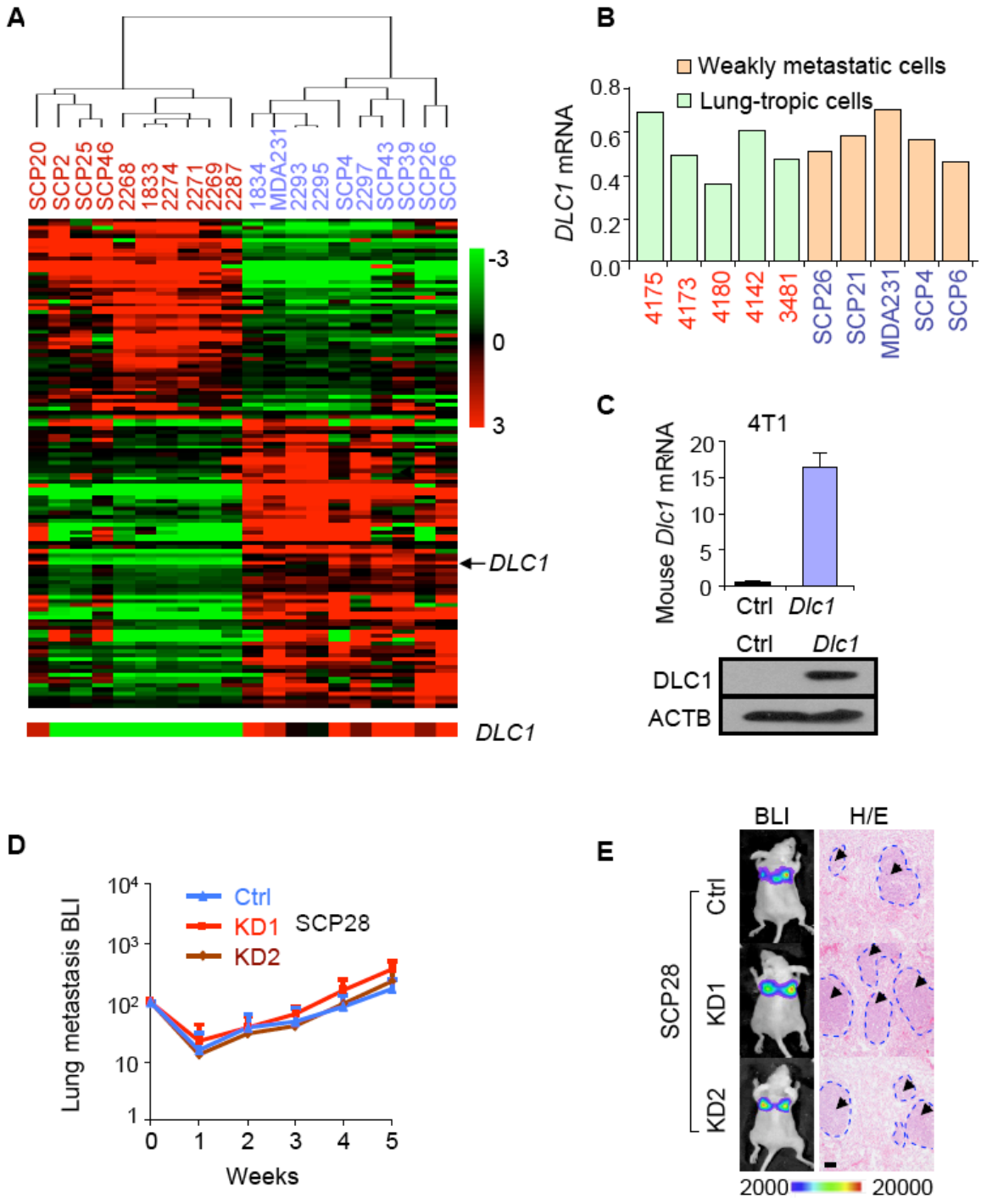
Supplemental Information contains Supplemental Data (including 9 figures and 2 tables), Supplemental Experimental Procedures and Supplemental References.

SUPPLEMENTAL FIGURES AND LEGENDS



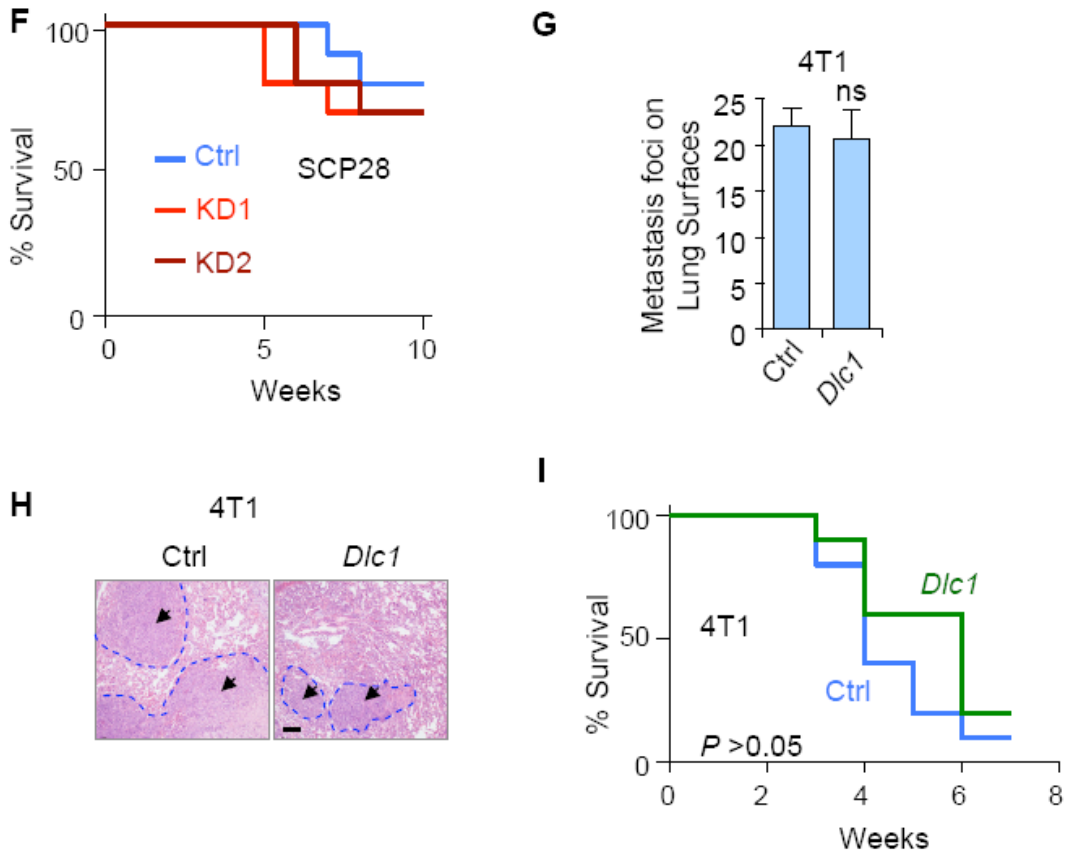
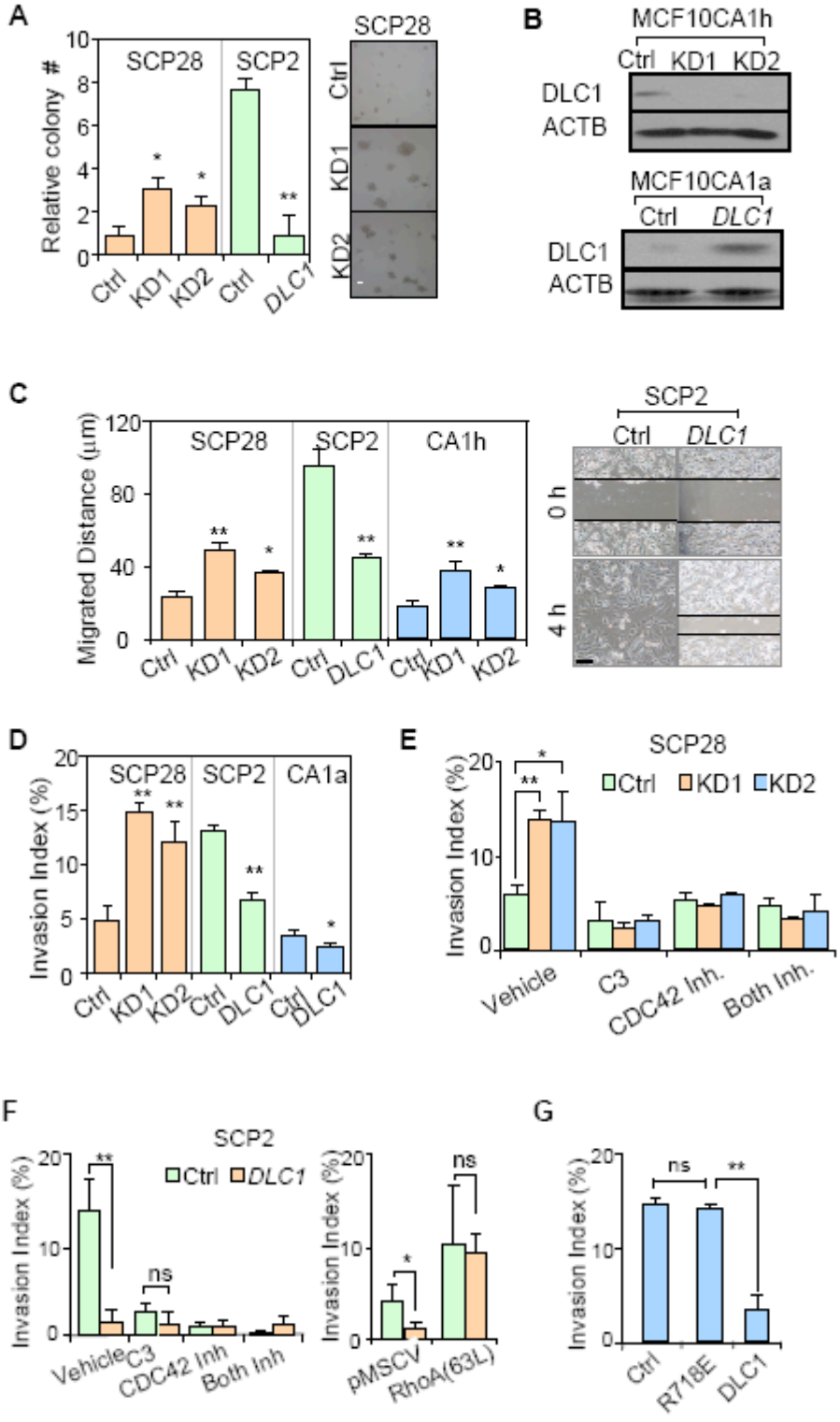


Figure S1. *DLC1* expression and its metastasis function in breast cancer cell lines. (A) Expression heatmap of the bone-metastasis signature genes (1) in MDA231 derivative cell lines organized by unsupervised clustering analysis. Green and red fonts denote cell lines with weak and strong bone-metastasis capabilities, respectively. **(B)** DLC1 expression in MDA231 derivative cells with different lung metastatic capabilities. **(C)** *Dlc1* OE in 4T1. **(D)** BLI quantitation of lung metastasis from SCP28 cells with *DLC1* KD (n=10). **(E)** Representative BLI and H/E images of SCP28 lung metastasis. **(F)** Animal survival analysis of *DLC1* KD. **(G)** Metastasis foci on the lung surface of Balb/c mice injected with 4T1 cells of *Dlc1* OE (n=5). **(H)** H/E images of lung metastasis by 4T1 cells with *Dlc1* OE. **(I)** Animal survival analysis of *Dlc1* OE. Scale bar, 50 μm.


A
Relative colony #
SCP28
SCP2
Ctrl
KD1
KD2
DLC1
B
MCF10CA1h
DLC1
ACTB
MCF10CA1a
C
Migrated Distance (μm)
CA1h
0 h
4 h
D
Invasion Index (%)
CA1a
E
Vehicle
C3
CDC42 Inh.
Both Inh.
F
pMSCV
RhoA(63L)
G
R718E
ns

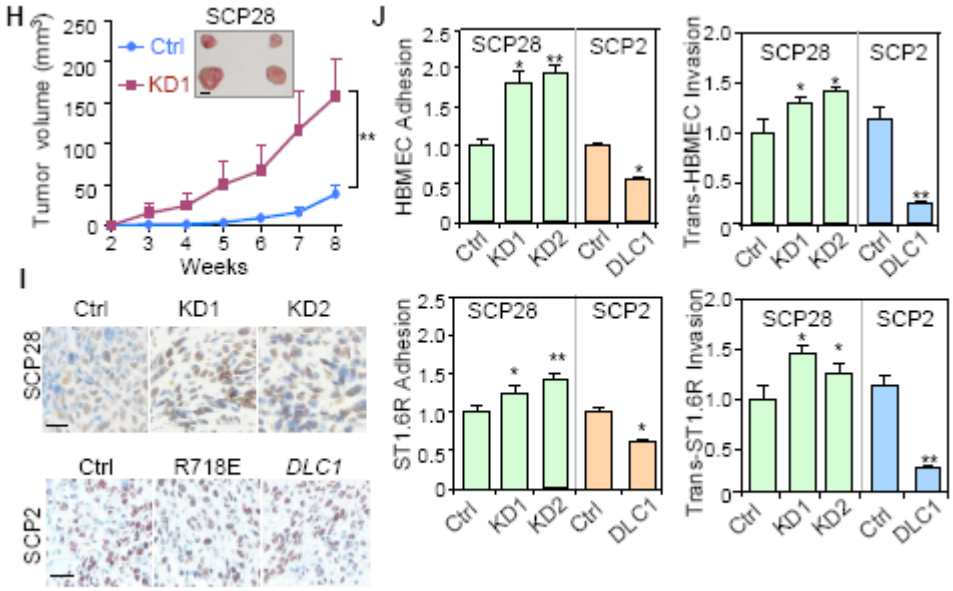


Figure S2. DLC1-Rho signaling regulates the intrinsic malignancies of breast cancer cells. (A) Soft-agar colony formation of cancer cells with *DLC1* KD and OE. Shown at the right are representative images (n=3). (**B)** *DLC1* KD and OE in MCF10CA1h and MCF10CA1a cells. **(C)** Wound-healing assays (n=3). **(D)** Transwell invasion assays (n=3). (**E**) Transwell invasion assays of SCP28 treated with inhibitors of small GTPases (n=3). (**F**) Transwell invasion assays of SCP2 cells with *DLC1* or RhoA(63L) OE, or treated with GTPase inhibitors (n=3). (**G**) Transwell invasion assays of SCP2 with wild type and mutant *DLC1* OE (n=3). (**H**) Primary tumor growth rates of SCP28 cells injected into mammary fat pads of nude mice (n=5). **(I)** Ki67 proliferation analysis of the SCP2 and SCP28 cells in bone metastasis tumors. (**J**) The abilities of cancer cells to adhere to (left) and invade through (right) the monolayer of bone endothelial (HBMEC-60) and lung endothelial (ST1.6R) cells. Data shown are relative to controls (n=3). Scale bar, 20 μm. (A,C,I); 4 mm (H). * $P < 0.05$; ** $P < 0.01$.

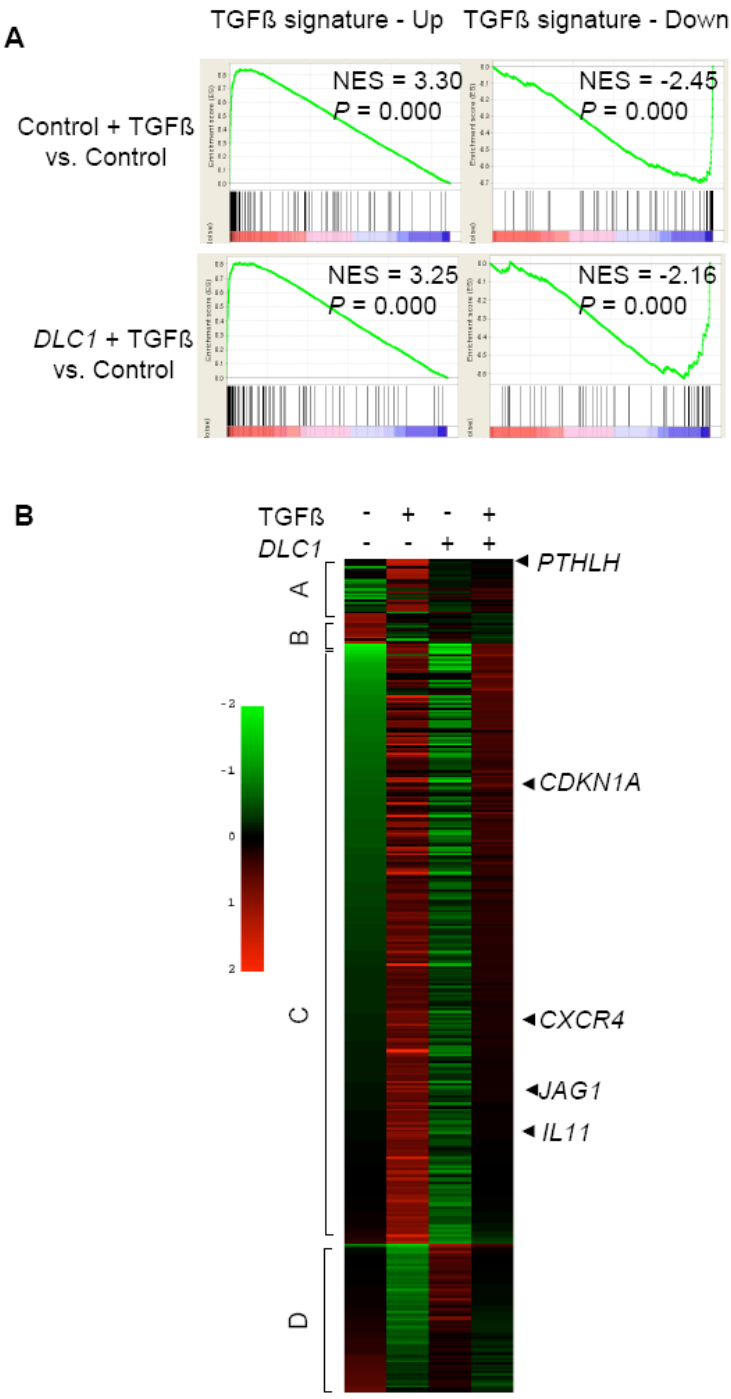


Figure S3. Gene expression analyses of control SCP2 and *DLC1* OE cells with or without TGFβ treatment. (A) GSEA analyses of a TGFβ-responsive signature (2). **(B)** Expression heatmap of the genes regulated by TGFβ in SCP2 cells. Group A, up-regulated by TGFß and reversed by DLC1; Group B, down-regulated by TGFß and reversed by DLC1; Group C, up-regulated by TGFß, but not reversed by DLC1; Group D, down-regulated by TGFß, but not reversed by DLC1.

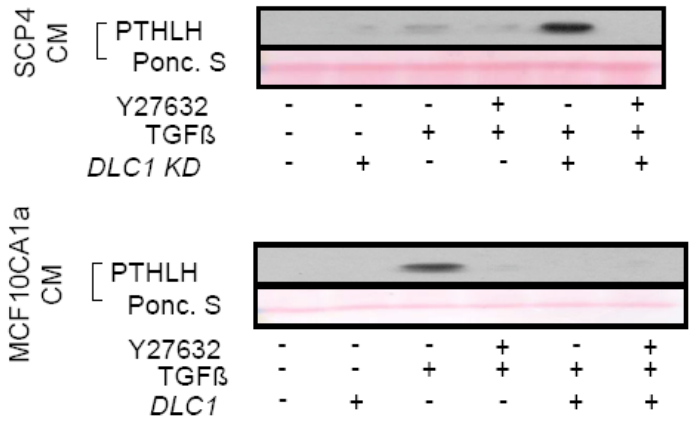


Figure S4. DLC1 regulation of TGFβ-induced PTHLH production in SCP4 (top) and MCF10CA1a (bottom).

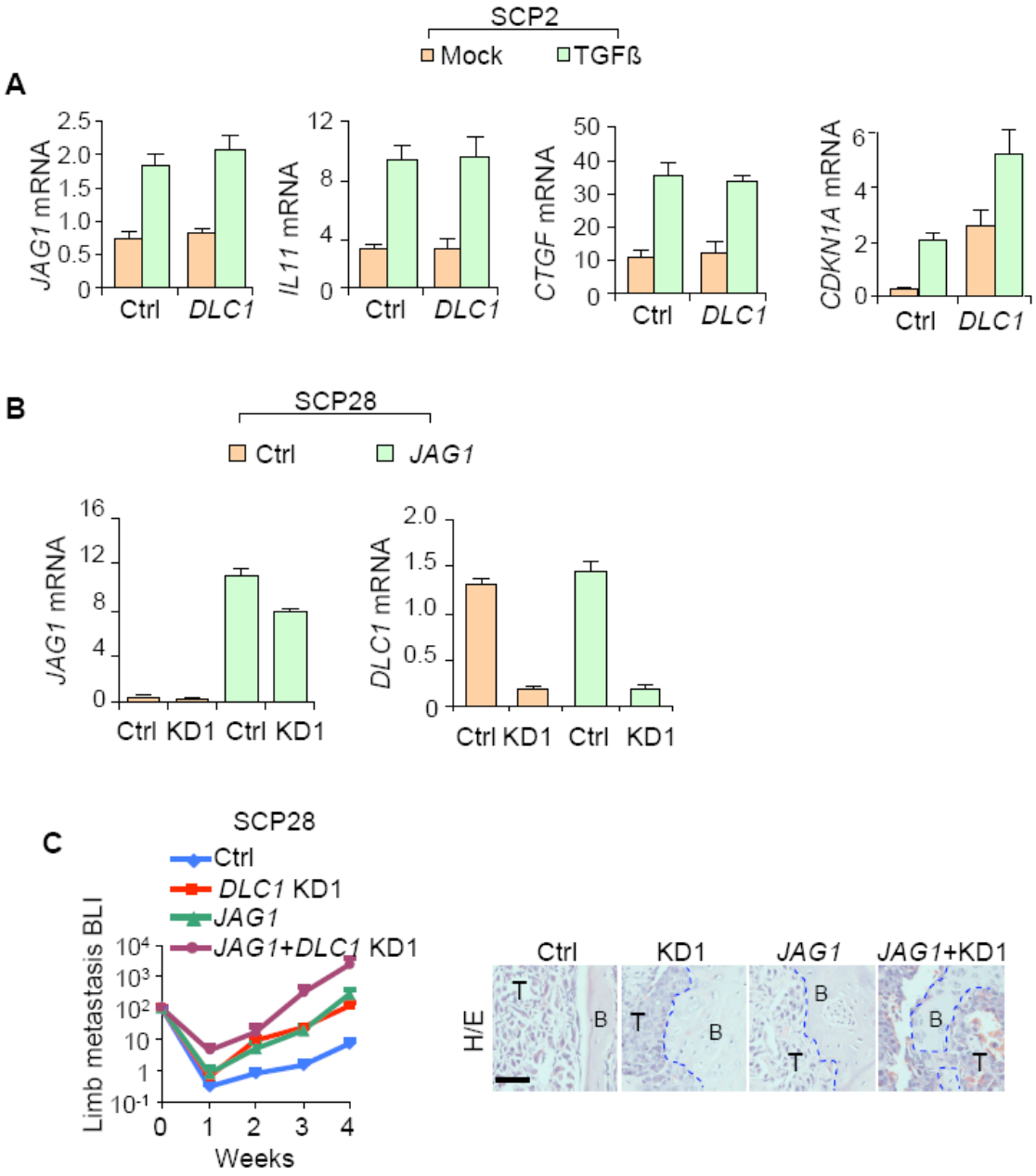


Figure S5. The relationship of DLC1 with the JAG1/Notch pathway. (A) DLC1 did not regulate TGFβ induction of other target genes including *JAG1*, *IL11, CTGF,* and *CDKN1A*. Shown are qPCR results (n=3). **(B)** qPCR analyses of *JAG1* and *DLC1* mRNA levels in SCP28 with *JAG1* OE, *DLC1* KD or both. The data showed that *DLC1* does not regulate the expression of *JAG1*, or vice versa (n=3). **(C)** In vivo bone metastasis of SCP28 cells with *JAG1* OE, *DLC1* KD or both. Shown are BLI quantitation (n=10) and H/E images. Scale bar, 50 μm.

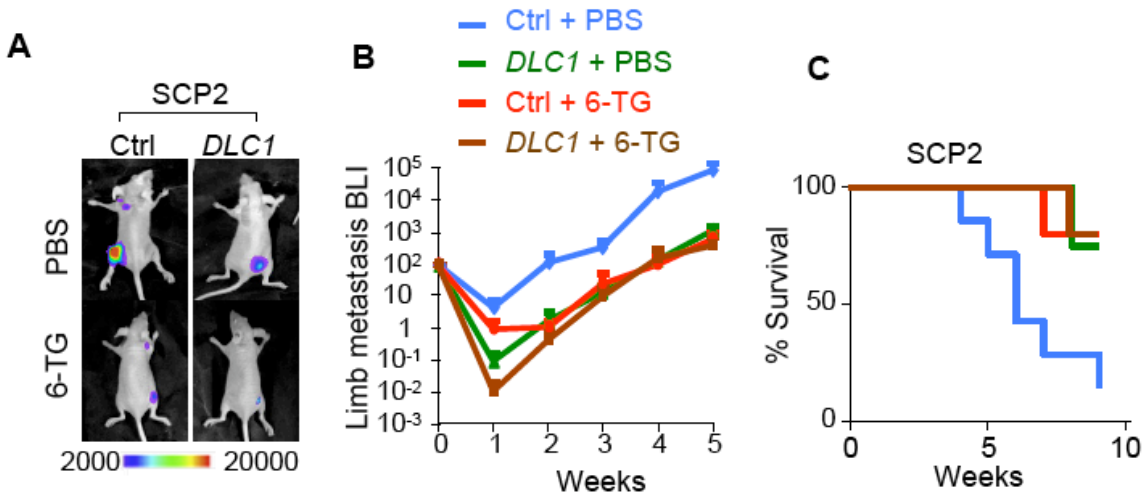


Figure S6. The effect of DLC1 *OE* in SCP2 cells on bone metastasis when the mice were treated with 6-TG. Shown are BLI images **(A)**, BLI quantitation **(B)** and animal survival **(C)** of the mice (n=10).

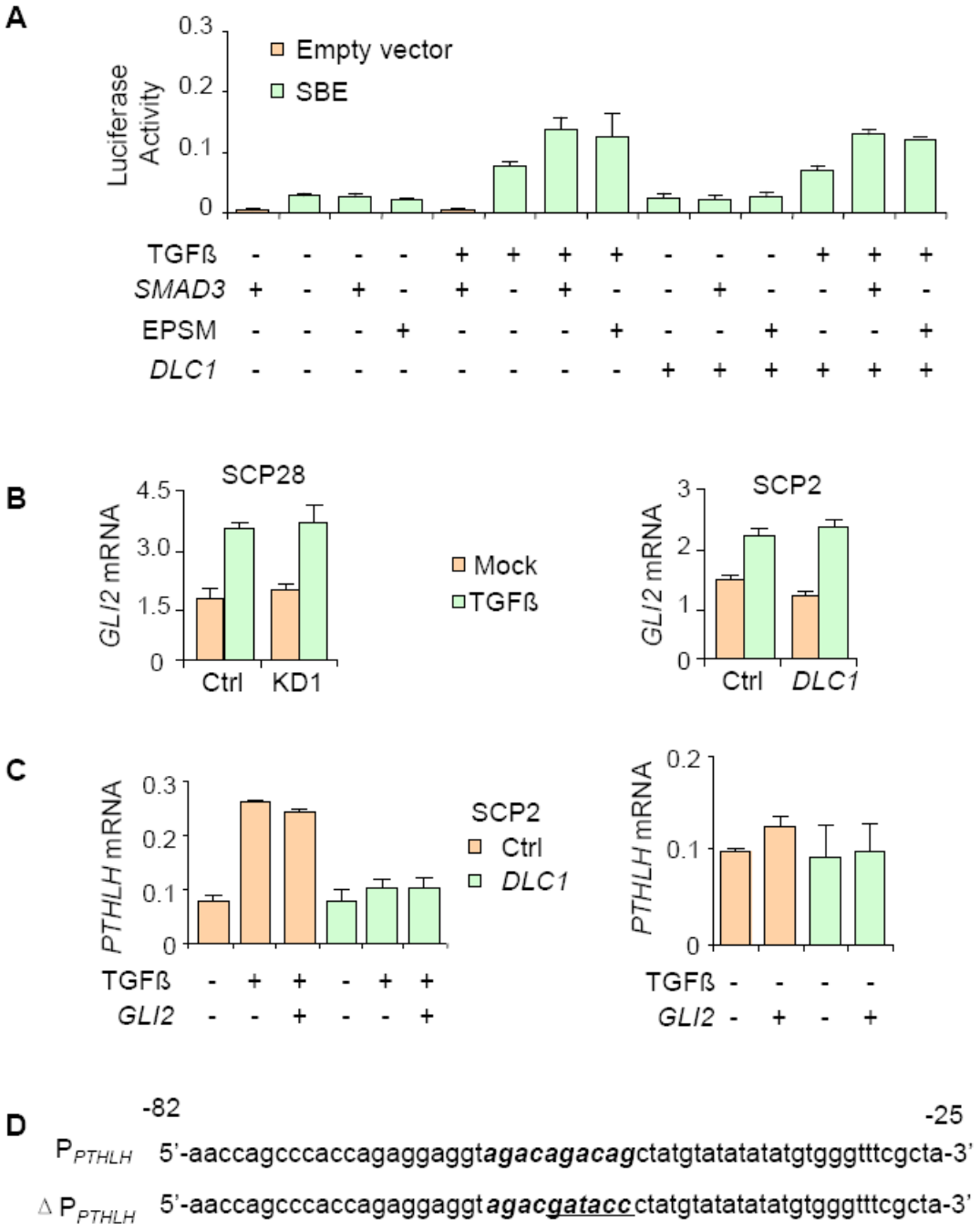


Figure S7. *PTHLH* is a direct target of SMADs. (A) SMAD-binding site (SBE) luciferase reporter assays for TGFβ treatment, wild type and EPSM mutant *SMAD3*, and *DLC1* OE (n=3). **(B)** *GLI2* expression levels after *DLC1* KD/OE and TGFβ treatment (n=3). **(C)** *PTHLH* expression levels after *DLC1* OE, TGFβ treatment and *GLI2* OE (n=3). **(D)** *PTHLH* promoter sequences. Bold area indicates the SMAD binding site, and underline indicates the mutated nucleotides in the promoter mutant.

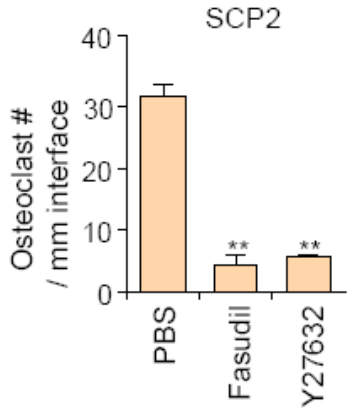
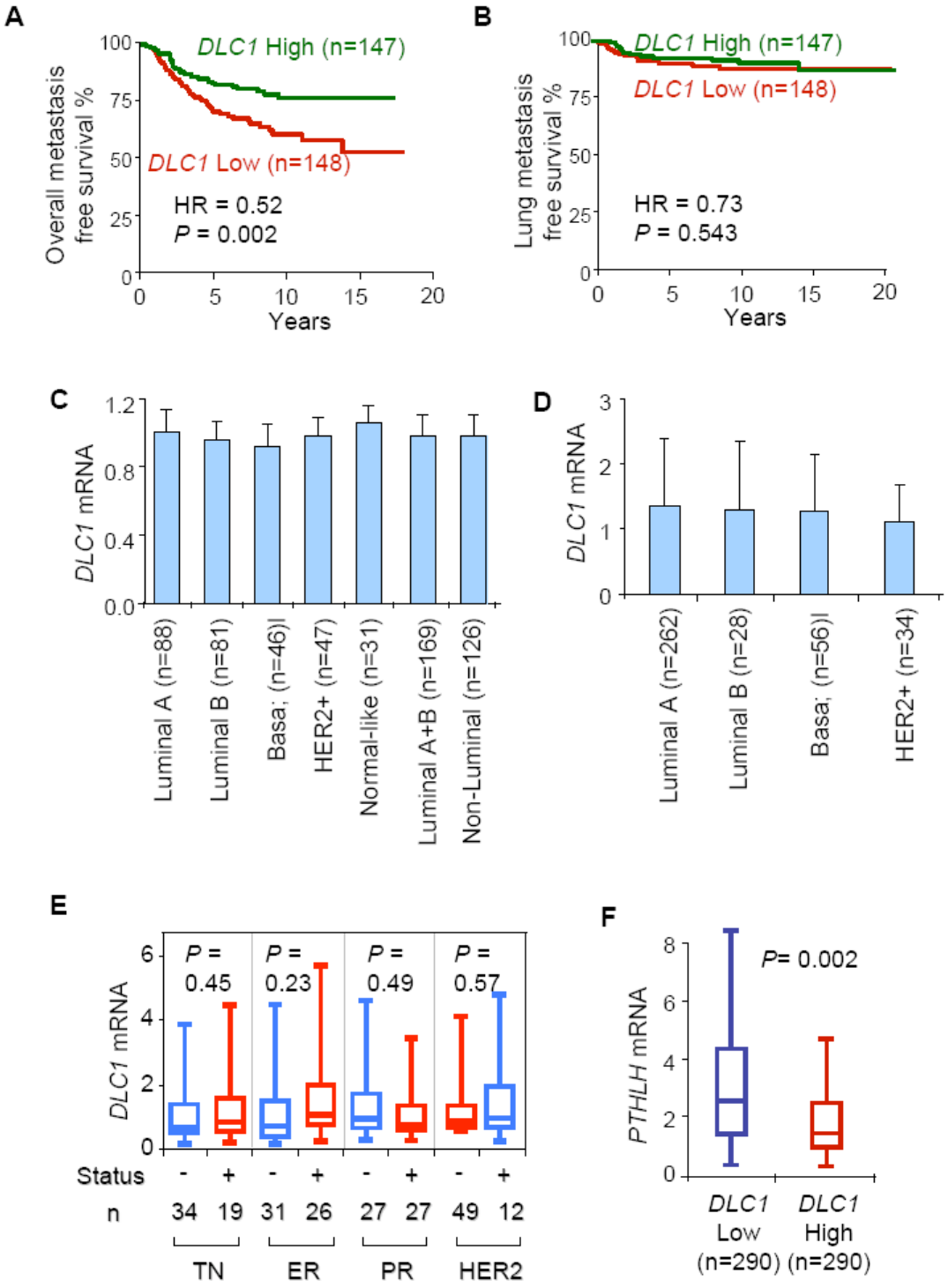


Figure S8. ROCK inhibitors Fasudil and Y27632 inhibited osteoclast maturation in the bone metastases by SCP2.


A
Overall metastasis free survival %
100
75
50
25
0
DLC1 High (n=147)
DLC1 Low (n=148)
HR = 0.52
P = 0.002
0
5
10
15
20
Years
B
Lung metastasis free survival %
100
75
50
25
0
DLC1 High (n=147)
DLC1 Low (n=148)
HR = 0.73
P = 0.543
0
5
10
15
20
Years
C
DLC1 mRNA
1.2
0.8
0.4
0.0
Luminal A (n=88)
Luminal B (n=81)
Basa; (n=46)I
HER2+ (n=47)
Normal-like (n=31)
Luminal A+B (n=169)
Non-Luminal (n=126)
D
DLC1 mRNA
3
2
1
0
Luminal A (n=262)
Luminal B (n=28)
Basa; (n=56)I
HER2+ (n=34)
E
DLC1 mRNA
6
4
2
0
P = 0.45
P = 0.23
P = 0.49
P = 0.57
Status - + - + - + - +
n 34 19 31 26 27 27 49 12
TN
ER
PR
HER2
F
PTHLH mRNA
8
6
4
2
0
P= 0.002
DLC1 Low (n=290)
DLC1 High (n=290)

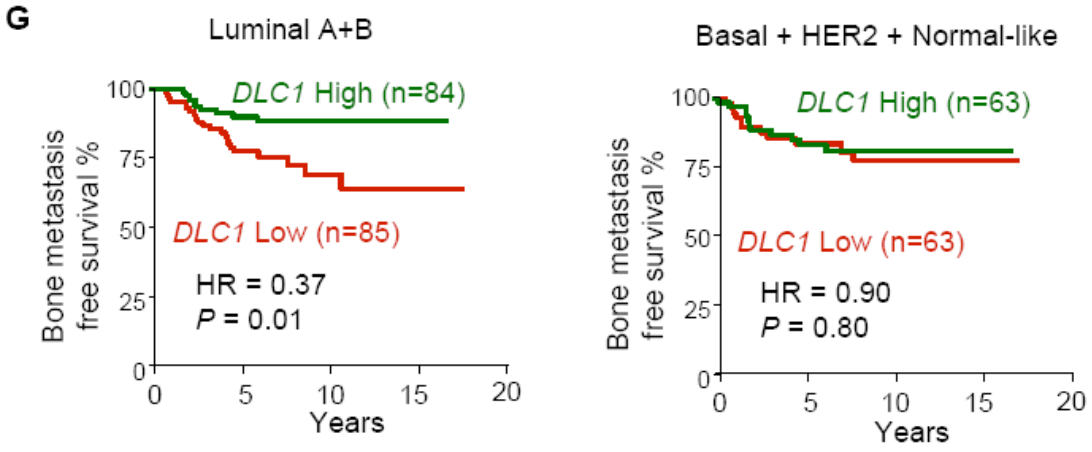


Figure S9. Correlation of *DLC1* expression with *PTHLH* and clinical parameters. (A) Overall metastasis analyses of the NKI tumors with different *DLC1* expression. **(B)** Lung metastasis analyses of the NKI tumors with different *DLC1* expression. **(C)** The expression of *DLC1* in the NKI tumors of different molecular subtypes. **(D)** The expression of *DLC1* in the TCGA breast tumors of different molecular subtypes. **(E)** The expression of *DLC1* in the Qilu tumors with different ER, PR, HER2 expression or triple negative (TN) status. **(F)** Correlation of *DLC1* and *PTHLH* expression in the TCGA tumors. **(G)** The association of *DLC1* expression to bone metastasis in the NKI tumors of luminal type or non-luminal type.

SUPPLEMENTAL TABLES

Table S1. The expression of genes that are upregulated by TGFß but reversed by *DLC1* in SCP2.

Probe	Gene Symbol	Expression				TGFß response		
		Vector	Vector+ TGFß	DLC1	DLC1+ TGFß	In Vector*	In DLC1*	Response Ratio[#]
211756_at	PTHLH	1.86	7.29	1.64	1.70	3.92	1.03	0.26
207601_at	SULT1B1	0.23	1.04	0.17	0.21	4.57	1.29	0.28
238460_at	FAM83A	1.49	4.97	1.18	1.21	3.33	1.03	0.31
201416_at	SOX4	2.77	11.70	8.29	11.04	4.22	1.33	0.32
243993_at	AA436887	0.38	1.34	0.32	0.37	3.55	1.14	0.32
238741_at	FAM83A	0.92	3.61	0.85	1.10	3.95	1.30	0.33
226844_at	MOBKL2B	0.33	1.10	0.27	0.34	3.35	1.28	0.38
222146_s_at	TCF4	0.69	1.90	0.55	0.58	2.75	1.06	0.39
239586_at	FAM83A	1.80	6.03	1.35	1.85	3.34	1.37	0.41
201417_at	SOX4	6.84	25.16	18.34	27.60	3.68	1.50	0.41
213668_s_at	SOX4	0.56	1.82	1.20	1.60	3.23	1.33	0.41
205387_s_at	CGB	0.45	1.39	0.64	0.83	3.13	1.30	0.42
225867_at	VASN	0.91	2.93	1.26	1.73	3.23	1.38	0.43
235976_at	SLITRK6	0.40	1.07	1.97	2.28	2.65	1.16	0.44
212099_at	RHOB	0.65	2.40	0.88	1.45	3.69	1.65	0.45
232481_s_at	SLITRK6	1.21	3.49	5.80	7.63	2.88	1.32	0.46
211844_s_at	NRP2	0.54	1.53	0.30	0.40	2.85	1.33	0.47
232176_at	SLITRK6	1.31	3.36	6.14	7.38	2.57	1.20	0.47
205801_s_at	RASGRP3	0.67	1.64	0.66	0.77	2.45	1.17	0.48
201170_s_at	BHLHE40	5.85	21.03	11.50	19.79	3.59	1.72	0.48
203753_at	TCF4	1.60	4.38	1.33	1.80	2.74	1.35	0.49
238688_at	TPM1	0.46	1.70	0.48	0.88	3.73	1.84	0.49
238712_at	BF801735	0.54	2.07	0.56	1.05	3.82	1.89	0.49
224650_at	MAL2	20.19	5.79	29.59	17.08	0.29	0.58	2.01
226189_at	ITGB8	7.53	3.03	2.45	2.00	0.40	0.81	2.02

209392_at	ENPP2	1.30	0.51	0.50	0.40	0.39	0.80	2.06
222150_s_at	PION	3.01	1.48	1.00	1.01	0.49	1.01	2.07
221541_at	CRISPLD2	1.83	0.75	0.55	0.48	0.41	0.88	2.16
1555310_a_at	PAK6	1.60	0.60	0.94	0.78	0.38	0.82	2.18
211742_s_at	EVI2B	7.94	1.89	5.50	2.90	0.24	0.53	2.21
206569_at	IL24	1.54	0.50	0.75	0.55	0.33	0.73	2.23
213142_x_at	PION	5.94	2.86	1.81	1.96	0.48	1.08	2.25
210143_at	ANXA10	2.83	0.92	1.39	1.09	0.32	0.78	2.41
235921_at	AI761207	11.12	2.76	5.44	3.29	0.25	0.60	2.43
222717_at	SDPR	4.15	0.96	1.07	0.65	0.23	0.61	2.63
212558_at	SPRY1	3.61	0.66	4.26	2.28	0.18	0.53	2.91
206022_at	NDP	1.14	0.37	0.22	0.24	0.32	1.10	3.42

* TGFß response in *DLC1*-overepressing or control cells was calculated as the gene expression level in TGFß-treated cells divided by that in untreated cells.

TGFß response ratio was calculated as the TGFß response in *DLC1*-overepressing cells divided by that in control cells.

Table S2. Clinical information of the Qilu cohort.

ID	Age	follow up time (months)	met	ER	PR	HER2	TN	DLC1	PTHLH
2	54	18.3	1	1	0	0	0	0.46	0.50
5	71	91.6	0	0	0	1	0	0.47	0.50
6	58	47.3	1	0	0	0	1	0.32	0.59
7	56	46.9	0	0	0	1	0	0.13	1.66
11	53	44.4	0	1	1	1	0	0.45	0.52
12	53	41.2	0	0	0	0	1	0.27	0.40
13	50	120.8	0	1	1	0	0	0.55	0.06
14	64	60.4	1	0	0	0	1	2.53	0.02
17	45	6.4	0	0	0	0	1	0.99	0.25
19	37	48.0	0	0	0	0	1	0.49	0.47
23	45	21.4	0	0		0		1.74	0.08
25	45	21.4	0	0	1	0	0	0.12	1.96
28	72	106.6	0	0	0	1	0	1.78	0.02
29	43	41.7	0	1	1	1	0	0.51	0.27
30	67	125.2	0			0		0.53	0.08
31	59	87.5	0	1	1	0	0	0.28	0.77
32	42	82.0	0	1	1	0	0	0.20	1.29
35	47	43.4	0	0	0	0	1	0.67	0.29
36	64	12.1	1	1		0		0.52	0.26
39	47	15.4	1			0		0.76	0.29
42	59	53.4	0	1	1	0	0	0.77	0.14
43	37	37.9	0	1	1	0	0	1.00	0.07
44	49	78.2	0	0	0	0	1	1.73	0.91
47	59	128.2	0	1	1	0	0	0.54	0.06
48	66	39.3	0	1		1		6.23	0.03
50	56	46.9	0	1	1	0	0	1.43	0.19
53	69	41.9	1	0	1	0	0	0.10	0.91
54	38	22.8	0	1	1	0	0	0.53	0.08
57	57	95.3	0	1	1	0	0	1.40	0.19
59	41	52.8	0	0	0	0	1	0.32	0.61
63	63	29.6	1	0	0	0	1	0.13	1.36
66	49	48.1	0	0	0	0	1	1.53	0.05
72	48	7.4	1	0	0	0	1	0.02	1.42
77	48	26.0	0	0	1	1	0	0.10	2.11
78	58	12.1	1	0	0	0	1	0.52	0.07
79	40	41.7	0	0	0	0	1	0.20	1.24
80	45	122.5	0	1	1	0	0	2.66	0.02
81	46	58.7	1			0		0.01	0.51

82	55	5.5	0	1	1		0	1.21	0.20
84	66	49.8	0	0	0	1	0	0.56	0.33
86	46	87.5	0	0	0	0	1	0.25	0.78
87	55	109.0	0			0		0.91	0.06
87	45	55.3	0	0	1	0	0	1.29	0.02
89	72	110.7	0	0	0	0	1	1.45	0.05
91	45	21.4	0	1	1		0	0.15	0.56
92	57	31.9	0	0	0	1	0	1.78	0.03
94	71	91.6	0	1	1	0	0	1.27	0.21
95	40	41.7	0	0	0	0	1	0.34	0.99
96	51	123.8	0	0	0	0	1	0.44	0.52
98	40	25.9	1	1	1	0	0	0.37	0.52
102	57	46.0	0	1	1	0	0	0.15	1.34
105	37	71.9	0	1	1	0	0	0.56	0.04
106	69	21.1	1			0		0.28	0.05
108	50	92.5	0			1		0.29	0.67
110	64	134.1	0	1	1	1	0	0.76	0.30
113	45	12.1	1	0	0	0	1	0.49	0.39
114	57	106.6	0	1	1	1	0	0.21	1.14
121	52	14.9	1	0	0			0.10	2.64
122	66	51.6	0	1	1	0	0	0.78	0.12
123	59	103.3	0	1	0	0	0	4.93	0.02
124	36	33.1	1	1	1	0	0	0.62	0.29
125		41.7	0	0	1	0	0	1.11	0.26
126	43	112.8	0			0		0.83	0.11
132		50.6	0	0	0	0	1	0.49	0.34
N1*								4.97	
N2								1.99	
N3								6.01	
N4								4.61	
N5								2.30	
N6								3.43	
N7								1.57	
N8								3.35	
N9								0.50	

* N1-N9 are paracancerous normal tissues.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell lines

4T1 and MCF10 cell line series were obtained from Dr. Fred Miller (3, 4). The human lung endothelial cell line ST1.6R was from Dr. C.J. Kirkpatrick (5). MDA-MB-231 derivatives, 4T1, C2C12 and RAW264.7 were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) with 10% fetal bovine serum (FBS, HyClone), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen). MCF10 series were cultured in DMEM/F12 medium supplemented with 5% horse serum, 10 μg/mL insulin (Roche), 100 ng/mL cholera toxin (Merck), 0.5 μg/mL hydrocortisone (Merck), 20 ng/mL epidermal growth factor (Invitrogen), 100 U/mL penicillin and 100 μg/mL streptomycin. ST1.6R were maintained in basal medium 199 (Invitrogen) with 20% FBS and 50 μg/mL endothelial growth factor supplements (Sigma). The human bone marrow endothelial cell line HBMEC-60 was maintained in basal medium 200 (Invitrogen) with 20% FBS and low serum growth supplements (Invitrogen). For bioluminescent imaging, cell lines were stably transfected with a fusion protein construct encoding the firefly luciferase.

Primers

The mouse *DLC1* cDNA was amplified using primers 5'-ACCTTAATGTGTAGAGACGAGCCGG-3' (sense) and 5'-TGCTTCATTCGCGTCGGTCA-3' (anti-sense). The primers for human *DLC1* mRNA qPCR were 5'-CCGTGCTTGATGTGCAGAAAG-3' (sense) and 5'-ACCAGTTGCCCGTAGCCAAT-3' (antisense). The primers for mouse *Dlc1* mRNA qPCR were 5'-CGGACACCATGATCCTAACA-3' (sense) and 5'-ATACTGGGGGAAACCAGTCA-3' (antisense). The primers for human *PTHLH* mRNA qPCR were 5'- ACAGTTGGAGTAGCCGGTTG-3' (sense) and 5'- CCCTTGTCATGGAGGAGCTG-3' (antisense). The primers for human *IL11* mRNA qPCR were 5'- AGCGGACAGGGAAGGGTTA-3' (sense) and 5'-CTGTATCTGGCCACAGGCTC-3' (antisense). The primers for human *CTGF* mRNA qPCR were 5'- CCTGCAGGCTAGAGAAGCAG-3' (sense) and 5'-TGGAGATTTTGGGAGTACGG-3' (antisense). The primers for human *JAG1* mRNA qPCR were 5'-ATGGGCCCCGAATGTAACAG-3' (sense) and 5'-ATCACAGTACAGGCCTTGCC-3' (antisense). The primers for human *Smad4* mRNA qPCR were 5'- CCATCCAGCATCCACCAAGT-3' (sense) and

5’- GGCCCTGATGCTATCTGCAA-3’ (antisense). The primers for human *CDKN1A* mRNA qPCR were 5’- AGTCAGTTCCTTGTGGAGCC-3’ (sense) and 5’- CATTAGCGCATCACAGTCGC-3’ (antisense). The primers for human *GLI2* mRNA qPCR were 5’- CACGCTCTCCATGATCTCTG-3’ (sense) and 5’- CCCCTCTCCTTAAGGTGCTC-3’ (antisense).The ChIP-qPCR primers for human *PTHLH* promoter were 5’-CCCACCAGAGGAGGTAGACA-3’ (sense) and 5’- CCTTTCGTTCCAGAGCCACT-3’ (antisense). The ChIP-qPCR primers for human *IL11* promoter were 5’- TTGAGCCTGAGTGTCTGCTC-3’ (sense) and 5’- AGGGCACGGAAGGAAAAGTT-3’ (antisense). The primers for *DLC1* gene copy number qPCR were 5’-CAGCCAGCGCTCCTTTAGGTGT-3’ (sense) and 5’-ACAGAGCCGTTGTTACAGAGGGT-3’ (antisense). The primers for mouse *Rankl* mRNA (6), for mouse *Opg* mRNA qPCR (7), and for *DLC1* promoter bisulfite sequencing (8) were previously described.

Colony formation assay

5×10^3 or 10^4 cells were suspended in 0.5 mL of 0.3% low melting point agar (Invitrogen) in DMEM containing 10% FBS and this suspension was overlaid on pre-solidified 0.6% agar in the same medium in 24-well plates. Normal growth medium was gently layered over the cultures every 4 days for 14 days. The colonies were stained with 0.1% crystal violet for 1 h at room temperature and counted under an inverted microscope.

Migration assay

Cells were seeded on 6-well plates. When the cells reached 100% confluence, they were starved in DMEM containing 0.5% FBS. The cell monolayer was scraped with a pipette tip from the center of each well and marked at the injury line. Photomicrographs of the wounds were taken at 0, 4, and 30 h.

Transwell invasion assay

5×10^4 or 10^5 serum-starved cancer cells were resuspended in serum-free medium and seeded in the inserts (Costar) containing 8 μm pores with 3 mg/mL matrigel (Becton, Dickinson and Company). These inserts were placed in wells that contained medium with 10% FBS for 12 or 24 h after seeding. Then the medium was aspirated, and 350 μL of trypsin was added into the wells to trypsinize the cells that had passed through the pores, followed by serum neutralization of the trypsin. The trypsinized cells were centrifuged for 4

min at 1000 rpm, resuspended in 100 μL phosphate-buffered saline (PBS), and counted using a hemacytometer.

Endothelial adhesion assay

The endothelium cells were grown to confluence in 24-well plates, treated at 37°C with 10 ng/mL TNFa for 6 h and then washed twice with PBS. Then 2 × 10^5 of firefly luciferase-labeled cancer cells were added to the endothelial monolayer and incubated for 1 h. Non-adherent cancer cells were removed from the plate by gentle washing twice with PBS, and the monolayer-associated cancer cells was quantitated by luciferase reporter assay using a pre-established standard curve of bioluminescent intensities of 10^2, 10^3, 10^4, 10^5, 10^6 cancer cells.

Trans-endothelial migration assay

The endothelium cells were grown to confluence on transwell polycarbonate filters (8 μm) and washed with PBS before seeding of 5 × 10^4 cancer cells on top of the endothelial layers. These transwell inserts were placed in wells containing culture medium with 10 ng/mL EGF for 24 h. Cancer cells passed through the pores was collected and counted according to the above protocol of transwell invasion assays.

Luciferase assay

The cells were transfected by the target reporter plasmids and a Renilla luciferase control plasmid with Lipofectamine™ 2000 (Invitrogen). After 24 h, media was removed and 50 μL lysis buffer was added into each well. The 24-well plate was rocked for 1 h at room temperature and then two aliquots of 10 μL lysate per well was mixed with 30μL luciferin substrate solution and 30 μL renilla luciferase substrate solution, respectively. The luminescence intensities were measured using a Lumat LB 9507 luminometer (Berthold).

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