Supplementary Materials

Effects of DLC1 Deficiency on Endothelial Cell Contact Growth Inhibition and Angiosarcoma Progression

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Supplementary Methods

Cell culture. Human Umbilical Vein Endothelial Cells (HUVEC) pooled from 10 donors (Lifeline Cell Technology, Frederick, MD; FC-0044) were maintained in M199 (Corning, NY; 10-060-CV) supplemented with 20% newborn calf serum (Sigma-Aldrich, St. Louis, MO; N4637), 5% human serum (Sigma, 4522), 25 µg/ml Heparin (Sigma, H3149), 50 µg/ml Ascorbic Acid (Sigma, A4034), 15 µg/ml endothelial cell growth factor (Sigma, E9640) and 1% Penicillin/Streptomycin (Sigma, P4333). HUVEC were used between passage 4 and 7, unless specified. The Human Dermal Microvascular Endothelial Cells (HMVEC-d) from Lonza (Allendale, NJ; CC-2543; used between passage 2 and 5) and the SV40-transformed Human Microvascular Endothelial Cell Line (HMEC-1) from ATCC (Manassas, VA; CRL-3243) were maintained in EGM2 medium (Lonza, CC-3202). Human primary fibroblasts from colon (CCD-18Co) from ATCC (CRL-1459) were maintained in Eagle's minimum essential medium (Gibco/Thermo Fisher Scientific, Gaithersburg, MD, 12440-053) with 10% FCS (Sigma, F2442), and used between passage 10 and 12. The angiosarcoma cell line ISOS-1 (a kind gift from Dr. H. Kato), the HUVEC-epithelial A594 hybrid cell EA.hy926 and HEK 293T/17 lines (ATCC, CRL-2922 and CRL-11268, respectively) were maintained in DMEM (Corning, Vienna, VA, 10-017-CV) supplemented with 10% FCS (Sigma, F2442). All cell used tested negative for mycoplasma contamination; the cell lines were not authenticated after purchase from ATCC/other vendors; the ISOS-1 line was provided by the originator.

Cell proliferation. Cell proliferation was measured by ^{3}H-thymidine incorporation. Briefly, 0.5 µCi ^{3}H-thymidine (Perkin Elmer, Waltham, MA) was added to cells (in 0.1 ml of culture medium) and incubated for 16 h. Cells were detached from the plates by freezing/thawing,

harvested onto glass fiber filters and ^{3}H-thymidine incorporation was measured using a liquid scintillation counter (1450-Microbeta, Perkin Elmer).

Cell number and population doublings. Cell cultures were photographed every three hours using IncuCyte (Essen BioScience, Ann Arbor, MI) with or without prior (2 hours) addition of Calcein-AM (Invitrogen, Carlsbad, CA; C3100MP) and images were quantified using Fiji to calculate the cell number. Alternatively, cell numbers were estimated by DNA quantification using Hoechst staining against a standard curve. Briefly, wells were washed twice with 0.2 ml PBS, air-dried and stored at -80 °C until measurement. After thawing, addition of distilled water (0.1 ml/well), freezing (-80 °C, 1 h) and thawing, 0.1 ml of a TNE 2X solution (20 mM Tris, 2 M NaCl, 1 mM EDTA) containing 0.002 mg/ml Hoechst 33342 (Invitrogen, H-3570) was added, and the plate was read (excitation at 360 nm and emission at 460 nm). Viable cells were counted using a dual fluorescence automated cell counter (Luna Logos Biosystems, Annandale, VA). Population doublings were calculated from viable cell counting as log (C_t/C_o)/log (2), where C_o is the number of cells seeded and C_t is the number of cells harvested.

Cell cycle. Cells were harvested, suspended in cold 70% EtOH (1 ml, added drop wise to cell pellet while gently vortexing) and fixed (30 min on ice). After washing (twice with PBS), cells were treated with Ribonuclease A (Invitrogen, 12091), stained with propidium iodide and analyzed using a BD FACSCanto.

RhoGTP pull-down. After seeding (60% confluence, 150-mm plates), incubation overnight (37 °C in complete M199 medium) and 1 h in M199 medium with 1% BSA, DLC1-silenced and pLKO-transduced cells were treated with thrombin (final concentration 1 U/ml) for 14 min.

GTP-bound Rho was measured with Rho activation assay kit (EMD Millipore, 17-294) following manufacturer's instructions (using ice-cold lysis buffer containing magnesium and Halt inhibitors, Thermofisher 78442, 0.030 mg of the Rho assay reagent slurry and 1 mg cell lysate). Rho was detected by western blotting using Rho antibody (clone 55; EMD Millipore, 05-778).

Inhibitors testing. Tanespimycin/17-AAG (Selleck Chemicals S1141; 200 nM) was added to HUVEC (2 x 10^4 cells/well in complete culture medium). The medium was replaced every other day until harvest. C3 Transferase from C. botulinum conjugated to a cell penetrating moiety (Cytoskeleton, Denver, CO; CT04) was added (0.001 mg/ml final concentration) to confluent HUVEC that had been incubated overnight in complete M199 medium, washed and then incubated for 1 h in M199. Incubation with the C3 toxin was for 2 h (moderate phenotype) or 6 h (strong phenotype).

Cell density and DLC1 expression. Exponentially growing cells (HUVEC, HMVEC-d, HMEC-1, CCD-18Co) seeded at low (8 x 10^4 cells/10-cm plate) or high (2.0 x 10^6 cells/10-cm plate) density were incubated for 16 h before lysis with TNTG lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10 % Glycerol with freshly added 1 mM PMSF, 1X Halt Protease and Phosphatase inhibitor (Thermo Fisher Scientific, Waltham, MA)).

Lentivirus production. Lentiviral particles were prepared using a third-generation system, concentrated by ultracentrifugation, and titrated with Lenti-X qRT-PCR Titration Kit (Clontech, Mountain View, CA; 631235) or by assessing their transduction efficiency. Non-Mammalian shRNA Control Plasmid DNA (SHC002) and DLC1-silencing vectors were purchased from

Sigma (Mission shRNA against the coding sequence: TRCN0000047823, -24, -25, -26, -27 and the untranslated region (UTR): TRCN0000423034). WT-DLC1 and GAP-dead R718A-DLC1 lentiviruses were derived from previously described pEGFP-DLC1 plasmids (8) and cloned into the pLenti CMV puro DEST plasmid (Addgene: 17452) via pENTR4 plasmid (Addgene: 17424). The plasmids were sequenced using ReadyMade EGFP-C (CATggTCCTgCTggAgTTCgTg) and EGFP-N (CgTCgCCgTCCAgCTCgACCAg) primers from IDT (Coralville, IA).

NFκB reporter and reporter assay. pLKO- or shDLC1-transduced HUVEC were further transduced (MOI = 10) with a lentiviral vector in which expression of the firefly luciferase reporter gene is driven by NF-κB activation and dTomato is constitutively expressed by the ubiquitin C (UBC) promoter, allowing normalization and tracking of transduced cells (Addgene plasmid #49335). Alternatively, HUVEC were transduced with the reporter and then transduced with pLKO, shControl, shDLC1 or shUTR followed by GFP control or WT-DLC1-GFP. Luciferase activity was measured using the Luciferase Assay System with Reporter Lysis buffer, per manufacturer's instructions (Promega, Madison, WI; E4030). Light emission was measured in a PolarStar Optima reader (BMG Labtech, Ortenberg, Germany) for a period of twelve seconds and averaged for the plateau readings.

RNA extraction, microarray and Q-PCR. RNA was purified (RNeasy Mini Kit; Qiagen, Hilden, Germany), cDNA was synthesized using random primers (QuantiTect Reverse Transcription Kit; Qiagen) and PCR was run using FastStart Universal SYBR Green Master (Roche, Basel, Switzerland) in an ABI 7900HT thermocycler (Applied Biosystems, Foster City, CA). Primers (listed in **Supplementary Table 1**) were either designed using Primer3 (53-55) or obtained from the existing literature.

We used 100 ng of high quality RNA (RIN >7) to compare gene expression profiles between pLKO and DLC1-silenced HUVEC using Affimetrix human microarray Hu Gene ST 2.0 array. Microarray raw data are deposited in ArrayExpress (E-MTAB-5263). Differentially expressed genes were identified with ANOVA analysis using Partek Genomics Suite (St. Louis, MO). Genes with significant expression difference were analyzed for pathways enrichment using Ingenuity Pathway Analysis (Qiagen).

Western blotting and protein array. Primary antibodies and dilutions used are listed in **Supplementary Table 2**. Validation of DCL1 (**Supplementary Figure 4A-C**) and TNFAIP3/A20 (**Supplementary Figure 5**) immunoblotting is shown. Secondary antibodies were from GE (Pittsburgh, PA; Sheep a-mouse-HRP, NA931V; Donkey a-rabbit-HRP, NA934V) or Santa Cruz (Donkey a-goat-anti-HRP, sc-2020). ECL reaction was performed with ECL Prime (GE; Actin, Clusterin, GAPDH) or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher, 34094; BIM, DLC1, TNFAIP3). Images were acquired using a LAS 4000 imager device (GE). The proteome profiler human apoptosis array kit (R&D Systems, Minneapolis, MN; ARY009) was used for assessment of apoptosis-related proteins following the manufacturer's instructions; spots were quantified using Fiji.

Immunostaining. Anonymized tissue samples were obtained from patients under institutionally-approved protocols with written informed consent (**Supplementary Table 3**). Tissue sections of formalin-fixed, paraffin-embedded human tissue specimens were heated (1 h, 60°C) and deparaffinized. Antigen retrieval was carried out in Tris-EDTA buffer (10 mM Tris Base, 1 mM EDTA solution, 0.05% Tween 20, pH 9.0) by boiling for 20 min in a microwave, followed by washing in running tap water for 10 min. Slides were washed twice in TBS, washed once in TBS

containing 0.2 % Triton X-100 (10 min), blocked (30 min each at room temperature in background buster; Fc-Blocker, Innovex, Richmond, CA; and 10% Donkey Serum, 1% BSA in TBS 0.2% Triton X-100) and incubated with primary antibody (overnight, 4 °C, in blocking buffer) at the appropriated dilution (**Supplementary Table 2**). Secondary antibody (Donkey anti-Rabbit A488, A-21206; Fab-anti-Mouse 546, A-11018, all from Invitrogen) was added (1:500 dilution) in blocking buffer (45 min, room temperature in the dark). The slides were finally incubated with Hoechst 33342 (Thermo, H3570, 0.001 mg/ml in TBS, 20 min), washed, air-dried and mounted. Mouse tumor sections were fixed in 4% PFA, embedded in OCT with cryoprotection, sectioned, and stained as above, using Unitrieve (Innovex, NB325, 30 min, 68 °C) as the antigen retrieval step. Cells cultured in vitro were washed in PBS, fixed in 4% PFA, treated with Unitrieve for antigen retrieval, and stained as above. Validation of immunostaining for DLC1 (**Supplementary Figure 4A-C**), TNFAIP3/A20 (**Supplementary Figure 5A**) and clusterin (**Supplementary Figure 5B**) is shown.

Quantitative analysis of immunostaining. Images were acquired using a 710 NLO confocal (Carl Zeiss, Oberkochen, Germany) at 63X. For each slide, 22 fields were acquired (11 from a region identified as normal after H&E, CD31and Ki67 examination; and 11 from a region identified as angiosarcoma). Each image was processed using Fiji to quantify the fluorescence intensity of DLC1, TNFAIP3 or CLU in CD31 positive areas. The results are presented as boxplots.

Tumor treatment experiments. All mouse experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the Center for Cancer Research, NCI, Bethesda, MD, USA and were conducted in compliance with approved protocols. BALB/c mice (8-weeks

old; Charles River Laboratories) were inoculated s.c. with 10 x 10^6 ISOS-1 cells from culture. When the first tumor reached an estimated volume (V) of 1000 mm^3 (determined by caliper measurements of the longest (D) and shortest (d) perpendicular diameters, and estimated as $V=1/2 \times D \times d^2$), the mice were euthanized and tumors removed. After ISOS-1 tumor disaggregation, using the tumor dissociation kit following the manufacturer's instructions (Miltenyi, 130-095-929), tumor-derived cells were inoculated s.c. into 40 BALB/C mice (8-10 weeks old; $1x10^6$/mouse). One week after inoculation, the mice were randomized into groups (n = 10/group). Each group of mice received i.p. injections (0.100 ml for a 20 g mouse) of either Vehicle (DMSO:Water:TBS-0.1%-Tween20, 10:2.5:1), Fasudil (50 mg/kg), Tanespimycin/17-AAG (80 mg/kg) or the combination of Fasudil (50 mg/kg) and 17-AAG (80 mg/kg) three times per week, for 15 days. The mice were euthanized one day after the last dose; tumors were excised and weighed; and samples were taken for protein and RNA extraction, or fixed in 4% PFA for immunostaining.

Supplementary Table 1. Primers used in quantitative PCR

Target (h/m)*	Forward	Reverse	Source
BCL2L11(h)	TAAgTTCTgAgTgTgACCgAgA	gCTCTgTCTgTAgggAggTAgg	PrimerBank† (323362951c1)
CLU (h)	gCAAgACACTgCTCAgCAAC	CCATCATggTCTCATTgCAC	Primer3
CLU (m)	TTCCCggAAgTgTgTAACgA	AgAAACTCCTCTAgCTgCTgg	Primer3
DLC1 (h)	CgTTgTTTTAAgAACCTggAggA	ATTAACCCTCACACCCACCA	Primer3
DLC1 (m)	gTTCCCTATTgATATTgCgCTgg	CTTCCggTgAggACTgATCTC	Primer3
GAPDH (h)	ACAgTCAgCCgCATCTTCTT	ACgACCAAATCCgTTgACTC	Primer3
HPRT1 (h)	CCTggCgTCgTgATTAgTgAT	AgACgTTCAgTCCTgTCCATAA	PrimerBank (164518913c1)
HPRT1 (m)	AgTgTTggATACAggCCAgAC	CgTgATTCAAATCCCTgAAgT	Reference ‡
SDHA (h)	gCAACAgAAgAAgCCCTTTg	gTTTTgTCgATCACgggTCT	Primer3
TNFAIP3 (h)	CAAggAAACAgACACACgCA	CCATTCATCATTCCAgTTCCgA	Primer3
TNFAIP3 (m)	AAACCAATggTgATggAAACTg	gTTgTCCCATTCgTCATTCC	Reference ‡

*h=human; m=mouse.

†Designed using primer3.

‡ Maelfait, J. *et al.* A20 (Tnfaip3) deficiency in myeloid cells protects against influenza A virus infection. *PLoS Pathog* **8**, e1002570, doi:10.1371/journal.ppat.1002570 (2012)

Supplementary Table 2. Antibodies used for Western Blotting and Immunofluorescence*

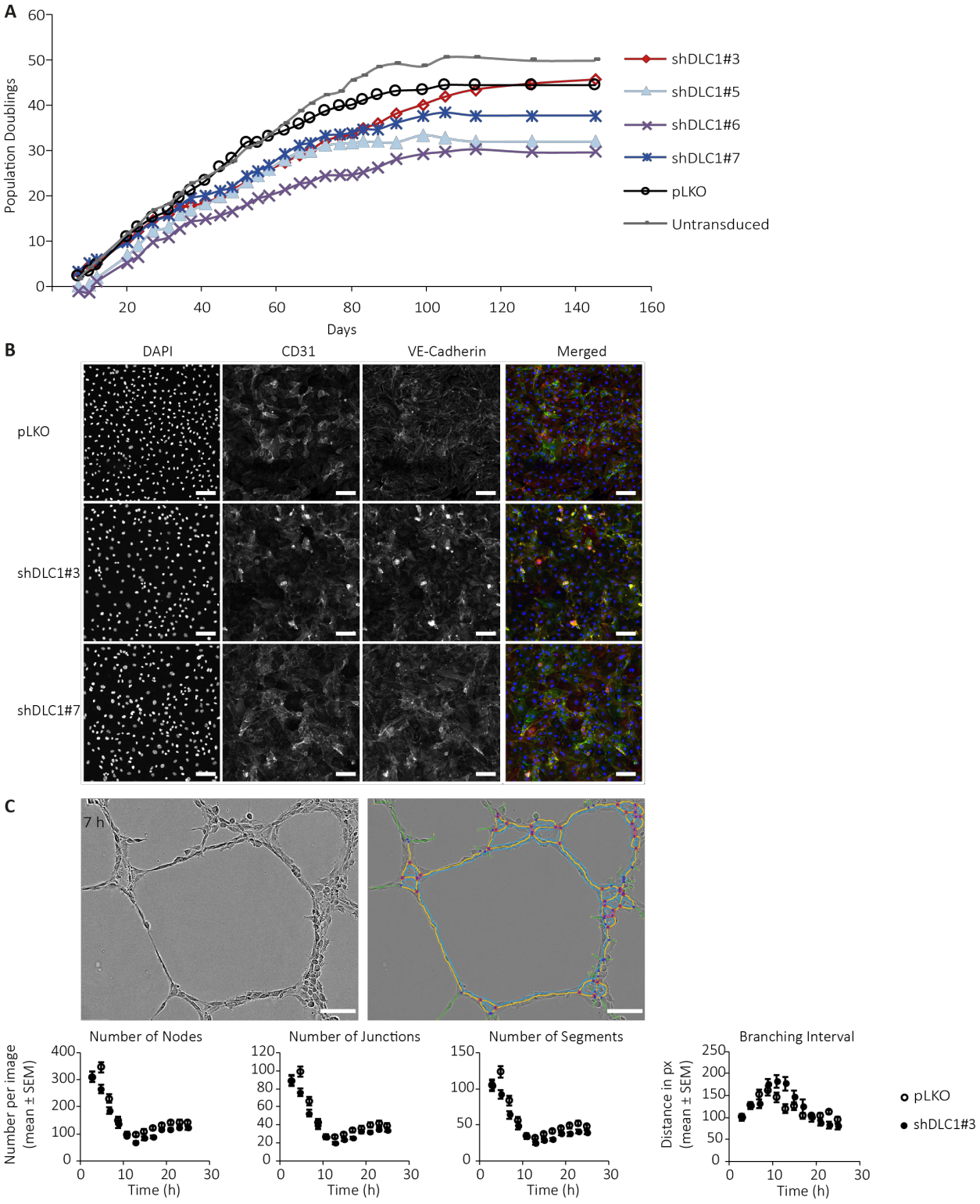
Target	Host	Source	Catalog number	Dilution WB	Dilution IF
Actin	Go	Santa Cruz	sc-1615	1:1000	—
BCL2L11/BIM	Rb	AbCam	ab32158	1:1000	—
CD31	Mo	DAKO	M0823	—	1:50
Clusterin	Go	Santa Cruz	sc-6419	1:200	—
Clusterin	Rb	AbCam	ab69644	1:1000	1:100
DLC1	Go	AbCam	ab21200	1:1000	—
DLC1	Mo	BD	612020	1:200	1:50
DLC1	Rb	Sigma	HPA017753	1:1000	1:100
DLC1	Rb	Santa Cruz	sc32931	1:200	—
GAPDH	Mo	American Research Products	31-M001	1:1000	—
IκBα	Mo	Cell Signaling	4814	1:1000	—
Ki67	Rb	AbCam	ab16667	—	1:100
p65	Rb	Cell Signaling	4764	1:1000	—
pIκBα (Ser32/36)	Mo	Cell Signaling	9246	1:1000	—
pMLC	Mo	Cell Signaling	3675	1:1000	1:100
p-p65 (Ser536)	Rb	Cell Signaling	3033	1:1000	—
Rho	Mo	Millipore	05-778	3 ug/ml	—
TNFAIP3/A20	Rb	AbCam	ab92324	1:1000	1:50
TNFAIP3/A20	Rb	Cell Signaling	5630	1:1000	—
V5	Mo	Invitrogen	46-0705	1:5000	—
VE-Cadherin	Rb	Cell Signaling	2500	—	1:100

*Go=goat; Rb=rabbit; Mo=mouse; WB=western blot; IF=immunofluorescence

Supplementary Table 3. Source of angiosarcoma tissue

Patient	Sex	Age, y	Location	Primary	Chemotherapy*	Origin
1	Male	101	Skin	primary	pre	trauma
2	Male	91	Skin	primary	post	idiopathic
3	Male	77	Skin	primary	pre	idiopathic
4	Male	75	Skin	primary	post	idiopathic
5	Male	77	Skin	primary	post	trauma
6	Male	64	Skin	primary	pre	trauma

*pre=prior chemotherapy; post=after chemotherapy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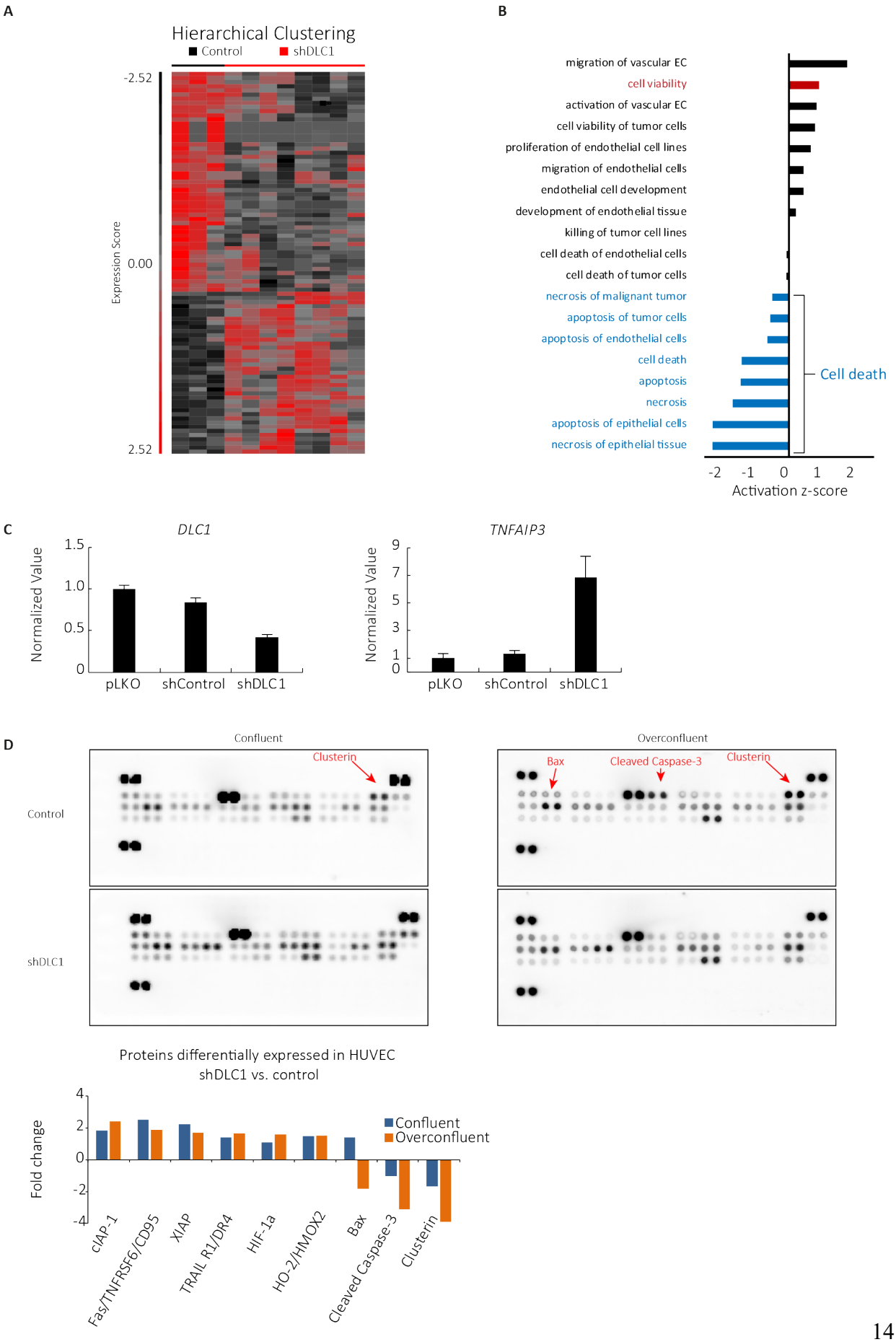
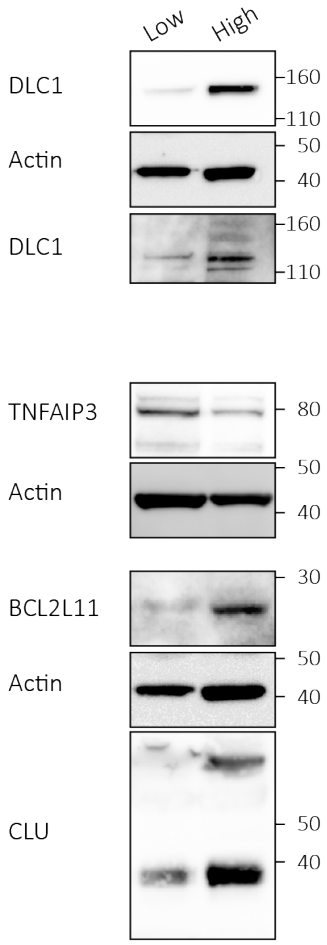
Population Doublings
Days
shDLC1#3
shDLC1#5
shDLC1#6
shDLC1#7
pLKO
Untransduced
B
DAPI
CD31
VE-Cadherin
Merged
pLKO
shDLC1#3
shDLC1#7
C
7 h
Number of Nodes
Number of Junctions
Number of Segments
Branching Interval
Number per image (mean ± SEM)
Distance in px (mean ± SEM)
Time (h)
pLKO
shDLC1#3

Supplementary Figure 1. Characterization of DLC1-silenced HUVEC. Effects of DLC1 silencing on HUVEC propagated under optimal culture conditions **(A)**. Surface CD31 and VE-Cadherin in HUVEC after DLC1 silencing **(B)**. HUVEC tube formation on extracellular matrix after DLC1 silencing (**C**); upper row: representative brightfield image with enhanced contrast (left) and image analyzed by the "Angiogenesis Analyzer" plugin for ImageJ (right); lower panels: angiogenic measurements. Scale bars: B, 100 μm; C, 200 μm.


A
Hierarchical Clustering
Control
shDLC1
-2.52
0.00
2.52
Expression Score
B
migration of vascular EC
cell viability
activation of vascular EC
cell viability of tumor cells
proliferation of endothelial cell lines
migration of endothelial cells
endothelial cell development
development of endothelial tissue
killing of tumor cell lines
cell death of endothelial cells
cell death of tumor cells
necrosis of malignant tumor
apoptosis of tumor cells
apoptosis of endothelial cells
cell death
apoptosis
necrosis
apoptosis of epithelial cells
necrosis of epithelial tissue
Cell death
-2 -1 0 1 2
Activation z-score
C
DLC1
Normalized Value
1.5
1.0
0.5
0
pLKO
shControl
shDLC1
TNFAIP3
Normalized Value
9
7
5
3
1
0
pLKO
shControl
shDLC1
D
Confluent
Overconfluent
Control
shDLC1
Clusterin
Bax
Cleaved Caspase-3
Clusterin
Proteins differentially expressed in HUVEC
shDLC1 vs. control
Fold change
4
2
0
-2
-4
Confluent
Overconfluent
cIAP-1
Fas/TNFRSF6/CD95
XIAP
TRAIL R1/DR4
HIF-1a
HO-2/HMOX2
Bax
Cleaved Caspase-3
Clusterin

Supplementary Figure 2. Comparative analysis of DLC1-silenced and control HUVEC. (**A**) Hyerarchical clustering of differentially expressed genes (p-value < 0.05 and fold change >1.5; shown as z-score (data standardized to mean of zero and variance of one)). (**B**) Cell death-related or endothelial-related functions distinguishing DLC1-silenced HUVEC from control identified using Ingenuity Pathway Analysis. (**C**) Effects of DLC1 silencing in HUVEC on TNFAIP3 mRNA levels (relative mRNA levels by qPCR normalized to HPRT1), shControl: shRNA non-mammalian control. (**D**) Time-dependent accumulation of clusterin, Bax and cleaved-caspase-3 in control and DLC1-silenced HUVEC grown as confluent or superconfluent cultures.

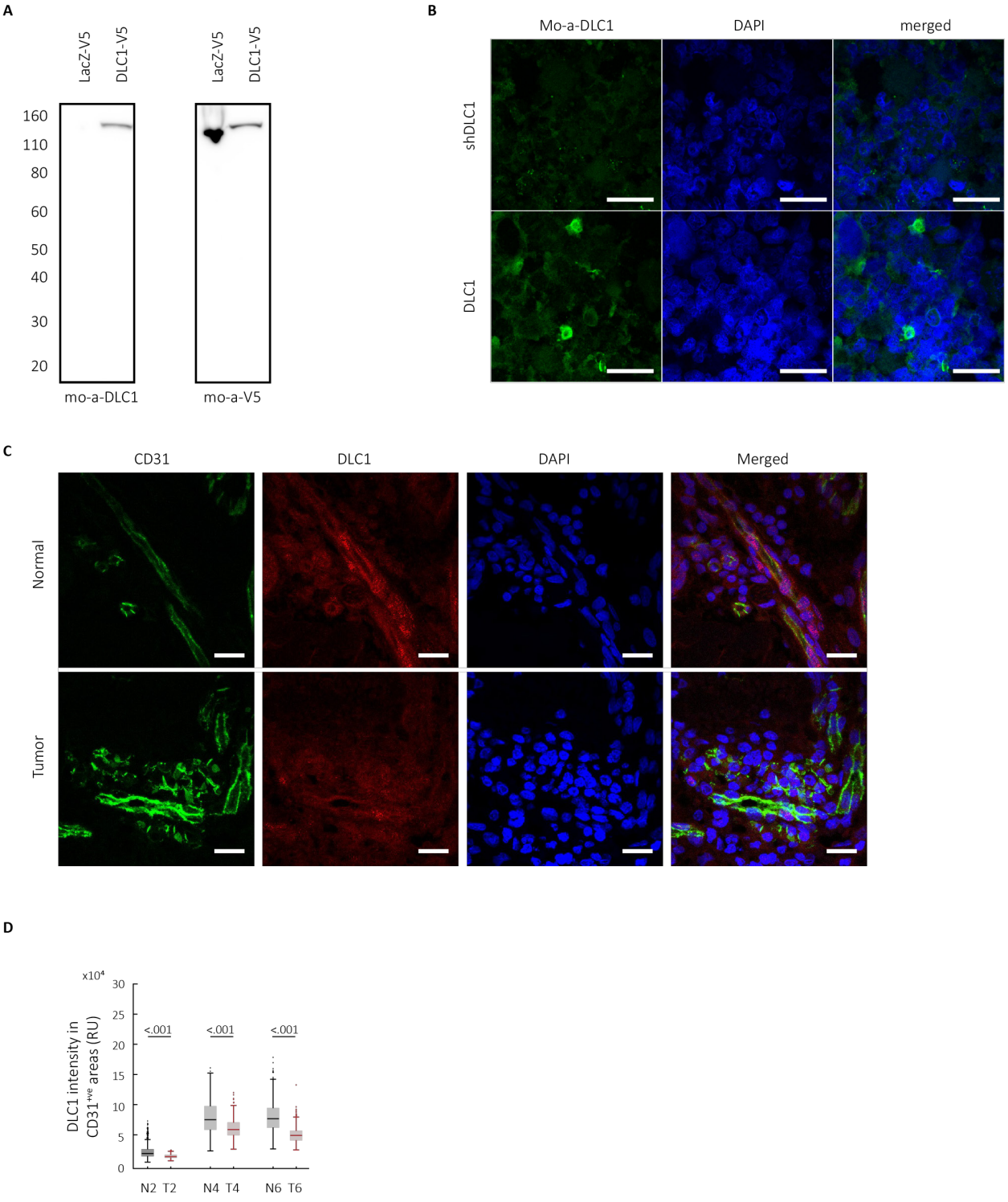
A



B

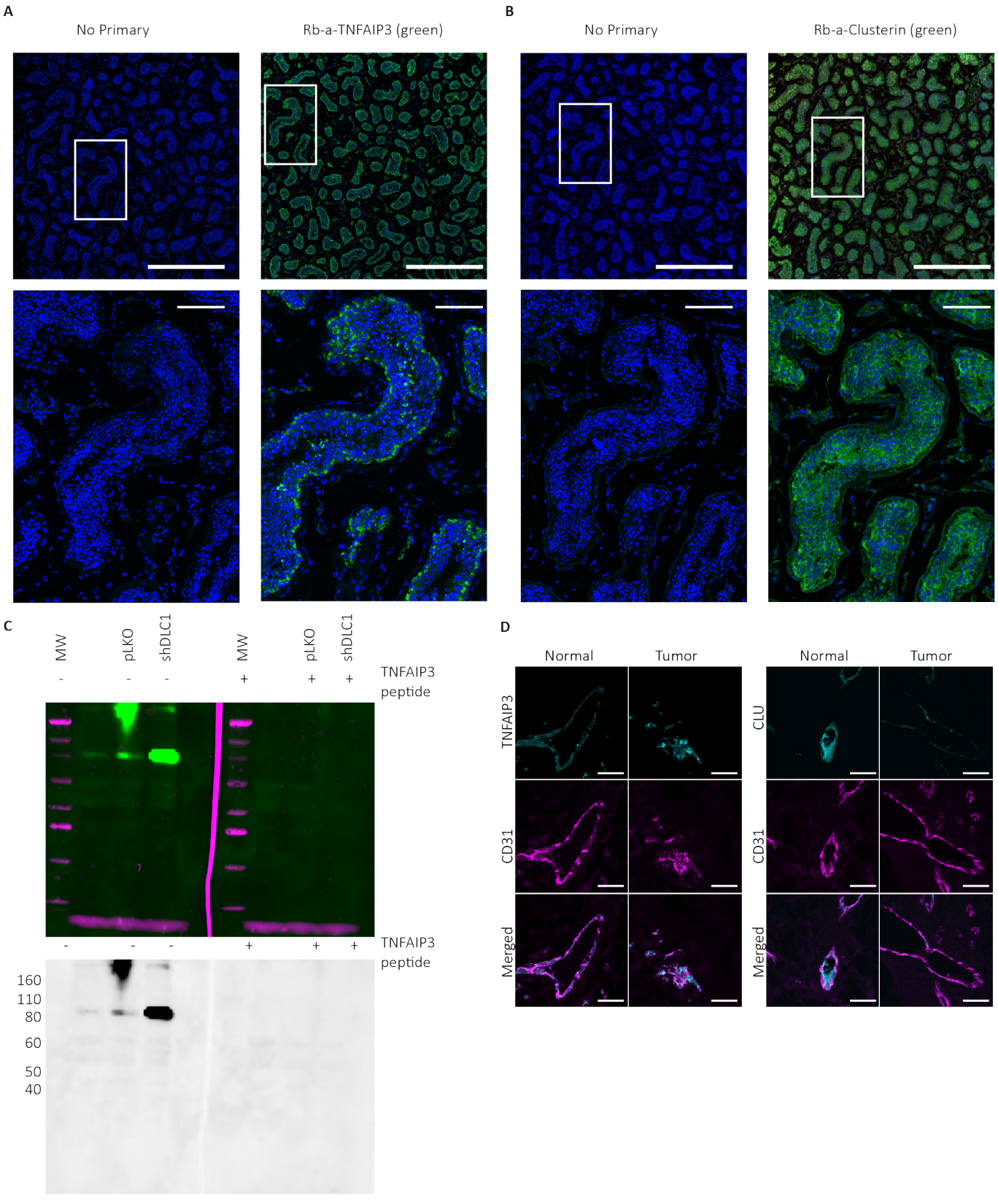
	Low Density	High Density
DLC1	Lower	Higher
TNFAIP3	Higher	Lower
BCL2L11	Lower	Higher
CLU	Lower	Higher

Supplementary Figure 3. Detection of endogenous DLC1, BCL2L11/BIM and clusterin in HUVEC grown at low or high cell density. Changes in DLC1 (detected with either BD (top) or Sigma (bottom) antibodies), TNFAIP3/A20, BCL2L11/BIM and clusterin protein levels observed in HUVEC grown at low and high cell densities (**A**). Summary correlation of relative protein levels in HUVEC grown at low or high density (**B**).



Supplementary Figure 4. Validation of antibodies directed against DLC1. **(A)** HEK293 cells were transfected with pCDNA3.1 lacZ-V5 or pCDNA3.1 DLC1-V5. Immunoblot detection with mouse DLC1 antibody (BD, 612020) and the anti-V5 tag in duplicate protein samples. (**B**)

293T/17 cells were transfected with DLC1 or shDLC1, and a cell pellet was embedded in paraffin prior to immunostaining with mouse monoclonal DLC1 antibody (mo-a-DLC1; BD). Each slide contains a section of each pellet. Staining of Angiosarcoma samples (**C)**: representative staining; (**D):** quantitation of staining in patients 2, 4 & 6 with the rabbit DLC1 antibody (Sigma, HPA017753); Mann-Whitney U test. Scale bars B and C: 20 μm.


A
No Primary
Rb-a-TNFAIP3 (green)
B
No Primary
Rb-a-Clusterin (green)
C
MW
pLKO
shDLC1
MW
pLKO
shDLC1
- - - + + +
TNFAIP3 peptide
- - - + + +
TNFAIP3 peptide
160
110
80
60
50
40
D
Normal
Tumor
Normal
Tumor
TNFAIP3
CD31
Merged
CLU
CD31
Merged

Supplementary Figure 5. Validation of TNFAIP3/A20 immunostaining. (**A**) Upper: 10X tiling of Normal Testis (slides from abcam ab4373) with or without primary antibody against TNFAIP3. Bottom: magnification of boxed area with (right) or without (left) primary antibody. Validation of clusterin immunostaining (**B**) Upper: 10X tiling of Normal Testis (slides from abcam ab4373) with or without primary antibody against clusterin. Lower: magnification of boxed area with (right) or without (left) primary antibody. Validation of TNFAIP3/A20 immunoblotting (**C**) Western Blot detection of TNFAIP3 in control and DLC1-silenced HUVEC lysates in the presence (+) or absence (-) of the immunizing peptide (ab175807); left side overlay of the ECL detection (green) and the brightfiled image to show the molecular weight markers (magenta), right, ECL. (**D**) TNFAIP3/A20, clusterin and CD31 immunofluorescence detected in angiosarcoma biopsies, including normal and tumor areas; representative images. Scale bars: A and B: upper 1 mm, lower 100 μm; D: 30 μm.