ONLINE SUPPLEMENTARY MATERIALS AND METHODS

Histological analysis

After fixed in 4% formalin and embedded in paraffin, tissues were cut into 4-μm sections. H&E staining were performed following the conventional protocol [1]. Immunohistochemistry (IHC) staining was performed using rabbit antibodies for DLC3 (Proteintech, Wuhan, China), MACC1 (Abnova, Taipei, China) and Ki-67 (Cell signaling technology, Danvers, MA) and stained by Dako Envision System (Dako, Glostrup, Denmark). The protein expressions were analyzed by a semi-quantitative method [1]. The intensities of the protein were scored as 0 (negative), 1 (weak), 2 (medium) or 3 (intense), while the staining extent were scored as 0 (0%), 1 (1%-25%), 2 (26%-50%), 3 (51%-75%) or 4 (76%-100%) according to the stained cells in percentage. Multiplying the staining intensity and extent scores together gets the final protein expression score.

Western blotting and Immunofluorescence

For western blotting, Goat Anti-Rabbit antibodies of DLC1 (Abcam, Cambridge, MA, US), DLC3 (Proteintech), MACC1 (Abnova), JNK (Abcam), phosphorylated-JNK1/2/3 at sites Y185/Y185/Y223 (Abcam), c-JUN (Cell Signaling Tech., Danvers, MA, US), phosphorylated-c-JUN at site S63 (Cell Signaling Tech.), E-cadherin (Cell Signaling Tech.), vimentin (Cell Signaling Tech.) and GAPDH (Cell Signaling Tech.) were used. For immunofluorescence, antibodies of DLC3 (Proteintech) and MACC1 (Abcam), E-cadherin (Cell Signaling) and vimentin (Cell Signaling) were used.

RhoA activity assay

RhoA activity was measured using a RhoA activation assay kit (cytoskeleton, Denver, OH). Briefly, freshly prepared cell lysates of 800 μg total protein were incubated with 50 μg rhotekin-RBD beads at 4°C on a rotator for 1h. After centrifugation, supernatant removal and carefully washing, the bead pellet was suspended in 2× Laemli buffer and boiled for 2 min. Then, the prepared samples were subjected for Western blotting using the anti-RhoA antibody included in the kit. Total RhoA expressions were also blotted as reference.

Protein array assay

To find out DLC3 downstream signaling, protein array assay was performed using Human Phospho-kinase array kit (R&D Systems, Minneapolis, MN) and following the manufacturer's protocol.

qRT-PCR

Trizol kit (Life science, Carlsbad, CA) for cells and tissues total RNA extraction and a reverse transcriptase (Roche, Penzberg, Germany) were used according to the protocols as recommended. qRT-PCR was performed using SYBR Green I Master kit (Roche) on a LightCycler 480 system. Primer sequences involved in present study are listed in online supplementary Table 1.

Dual-luciferase reporter assay

According to the documented functional binding site for AP-1 in the MACC1 promoter [2], mutated MACC1 reporter fragments was generated (see online supplementary Table 2). To detect the influence of DLC3 on the MACC1 transcription, MACC1 and its corresponding mutated reporter fragments were cloned into the luciferase pGL3 vector. Luciferase activities of each groups was detected using Dual-Luciferase Reporter Assay System (Promega, Madison, WI, US) according to the manufacturer's instructions.

ROS detection and cell apoptosis assays

ROS was measured by fluorescent DCFH-DA dye (Jiancheng, Nanjing, China) and observed under fluorescent microscope after 6 hours glucose deprivation. Cell apoptosis was analyzed by staining Annexin V-FITC and Propidium Iodide after 12 hours glucose deprivation, and detected by flow cytometry analysis using the FACSCanto II system (BD Biosciences, San José, CA).

Cell viability, proliferation and colony formation assays

Cells viability was measured by methyl-thiazolyl-tetrazolium (MTT) assay as previously described [3]. Cell proliferation ability was reflected by EdU incorporation assay using the EdU Assay Kits (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instruction and observed under fluorescent microscope. For colony formation, trypsinized cells were suspended in 2 mL of complete medium (Sigma, St.

Louis, MO, US) in complete medium. After 14 days culturing, cells were observed by crystal violet.

Migration, invasion and glucose chemotaxis assays

For scratch healing assay, trypsinized cells were seeded on 24-well culture plates (5×10^4/well). Upon confluence, cells were scratched with a sterile 200 μL pipette tip. Wound closure was observed at indicated time point. For Boyden chamber transwell assay, cells were planted in serum free medium in the upper chamber, which consisted of 8 μm membrane filter inserts (5×10^4/ml, 200 μL/well) with Matrigel. The lower chamber was complete medium with serum as chemoattractant. After 48 hours culturing, cells at upper chamber were fixed by fixed in 4% paraformaldehyde and stained by crystal violet. Cells were photographed under microscope. For glucose chemotaxis assay, cells were seeded on the upper chamber coated with Matrigel (BD Biosciences) and incubated with RPMI 1640 glucose free medium, while the lower chamber was supplemented with RPMI 1640 high glucose medium (2 g/L) as attractant. Cells were cultured for 48 hours before crystal violets staining.

Glucose uptake and pH evaluation

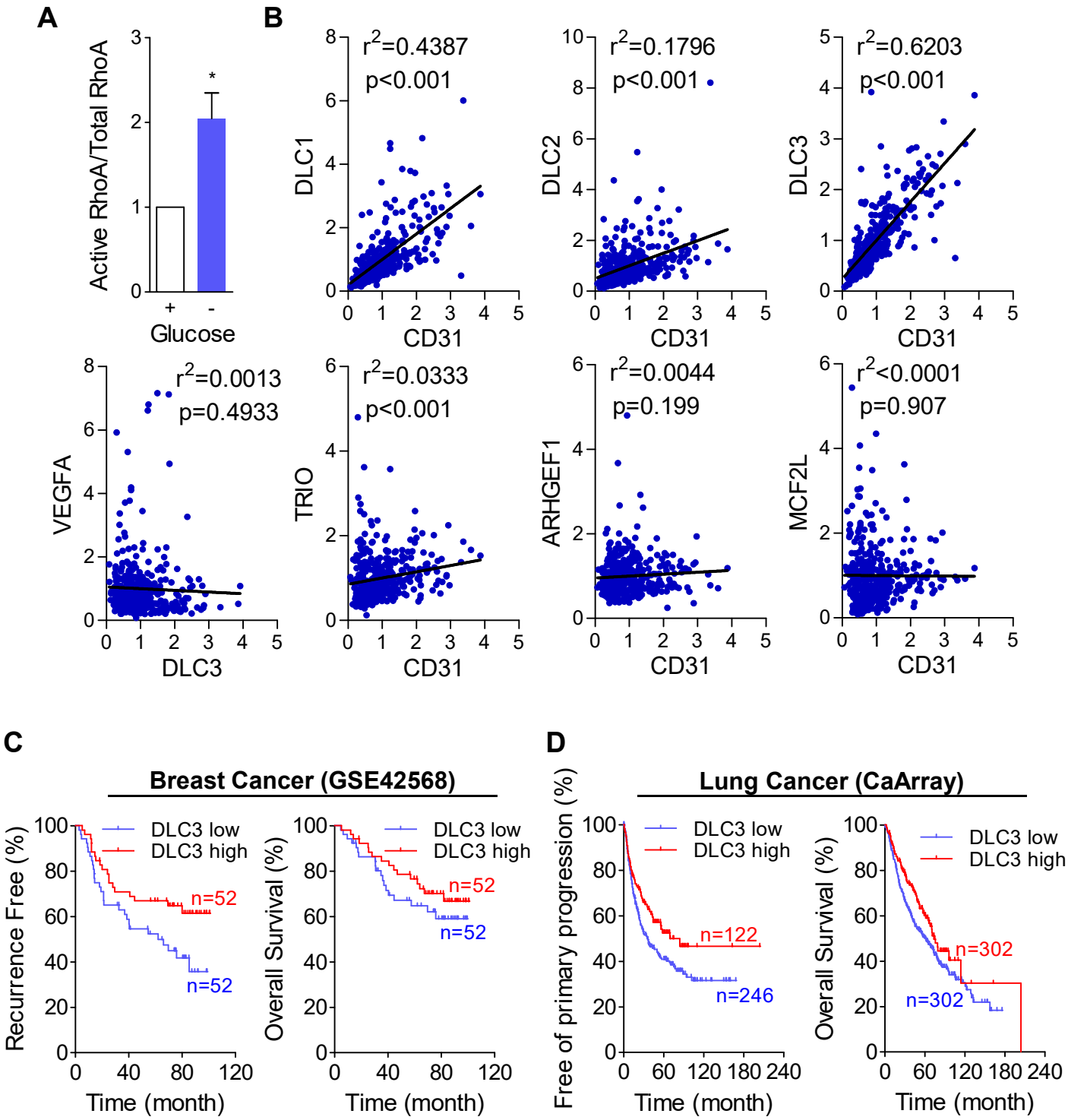
Cells were seeded on 6-well plates (5×10^5/well) and cultured in 2 ml complete medium. The 24-hour conditioned medium and the original medium (baseline control) were collected. Glucose uptake was determined using a glucose assay kit by conventional enzymatic methods (Randox, Antrim, UK). Total protein of cells was detected by

conventional BCA method (Beyotime, Haimen, China). The pH value was detected by a pH meter.

Reference

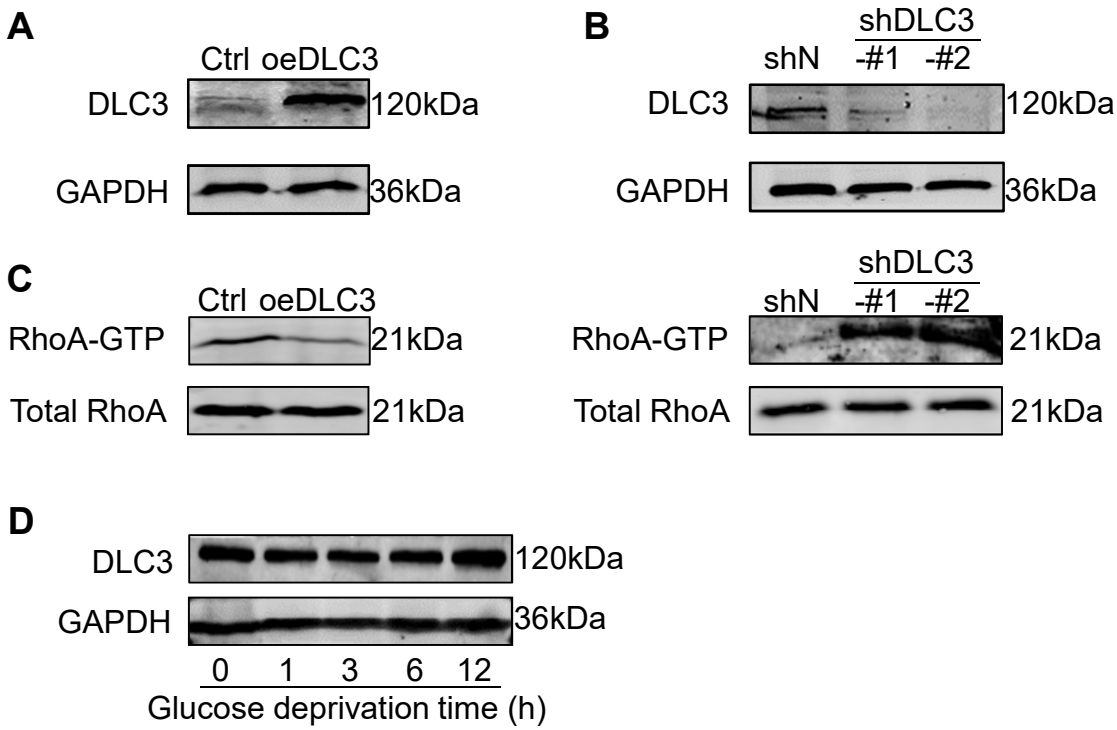
1. Wang L, Wu Y, Lin L, Liu P, Huang H, Liao W, et al. Metastasis-associated in colon cancer-1 upregulation predicts a poor prognosis of gastric cancer, and promotes tumor cell proliferation and invasion. International Journal of Cancer. 2013; 133: 1419-30.
2. Juneja M, Ilm K, Schlag PM, Stein U. Promoter identification and transcriptional regulation of the metastasis gene MACC1 in colorectal cancer. Mol Oncol. 2013; 7: 929-43.
3. Wang L, Lin L, Chen X, Sun L, Liao Y, Huang N, et al. Metastasis-associated in colon cancer-1 promotes vasculogenic mimicry in gastric cancer by upregulating TWIST1/2. Oncotarget. 2015; 6: 11492-506.

Online Supplementary Figures and Tables



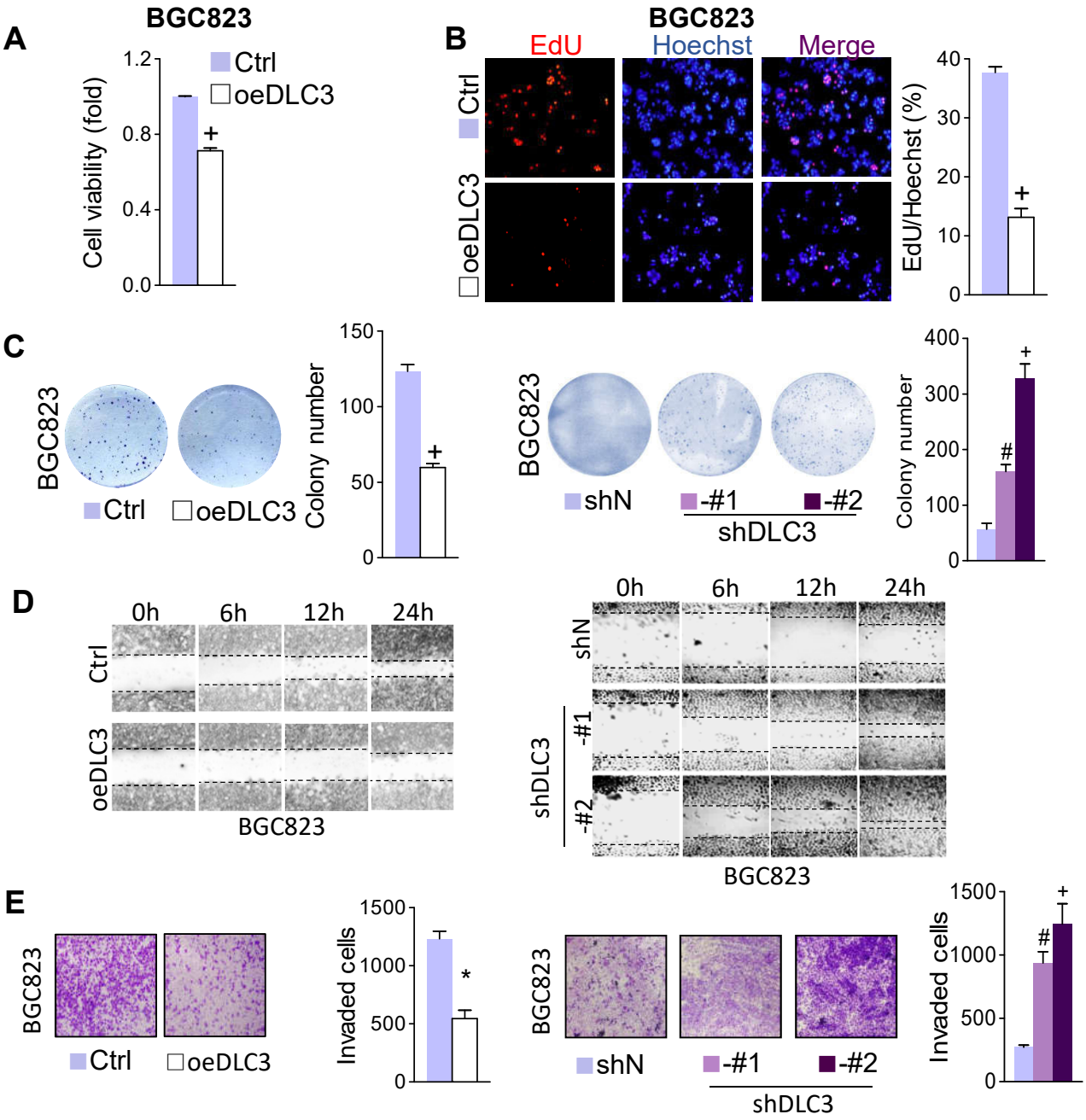
Supplementary Figure S1 DLC3 is associated with perfusion status in tumor microenvironment, and DLC3 low expression indicates poor cancer prognosis.

(A) Quantified of the RhoA activity changes upon glucose deprivation by three independent repeats. (B) Using TCGA GC tissue data, the mRNA expressions of DLC family members in correlation with CD31, and three representative GEF members (TRIO, ARHGEF1 and MCF2L) were analyzed. Angiogenic factor VEGFA in correlation with DLC3 was also analyzed. (C and D) Using online Kaplan-Meier plotter for bioinformatic analysis, DLC3 downregulation was associated with poor survival in (C) breast cancer (GSE42568) and (D) lung cancer (CaArray) respectively.



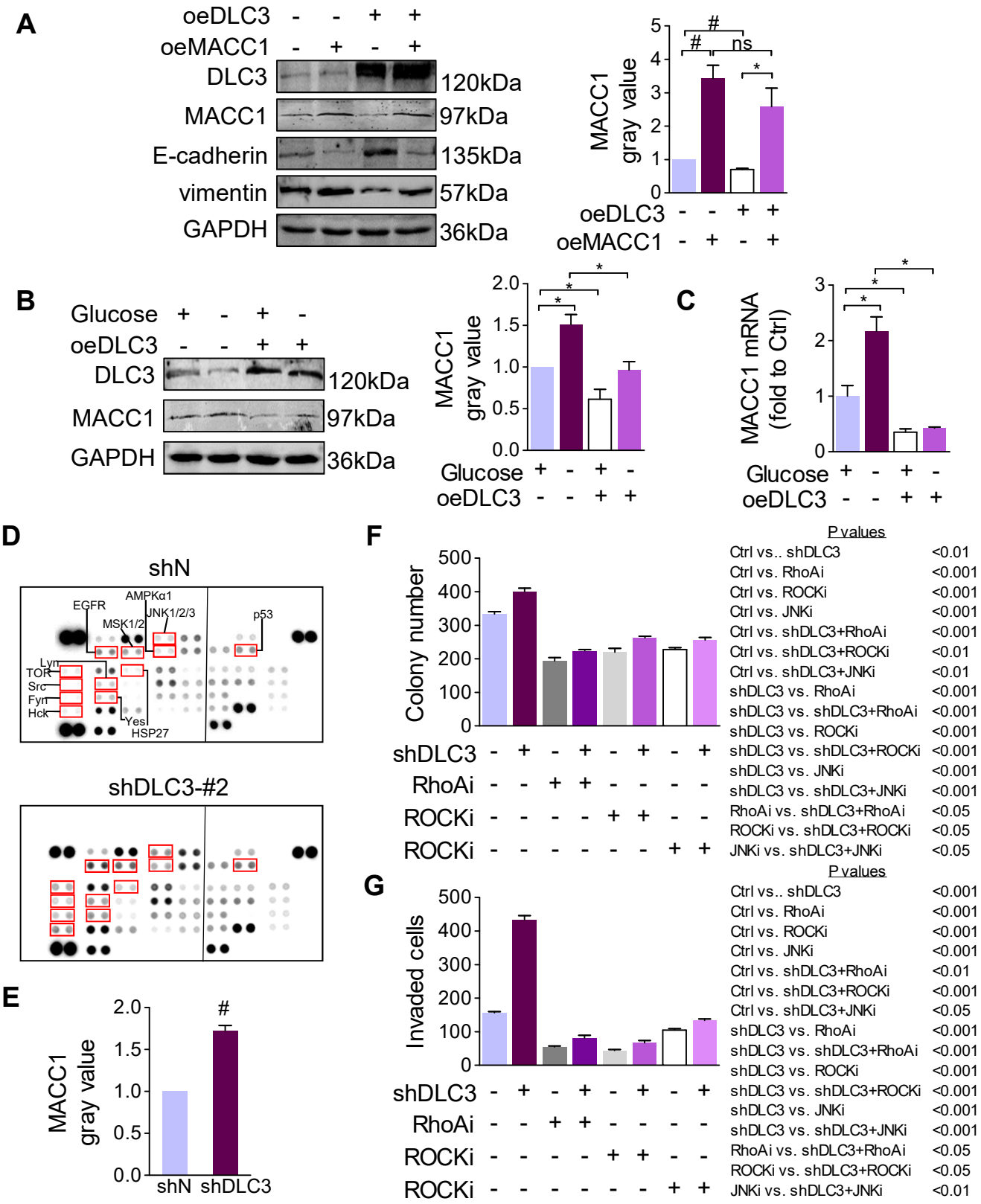
Supplementary Figure S2 Constructing DLC3-overexpression and -silenced GC cell models.

DLC3 was successfully (A) overexpressed and (B) silenced in MKN45 cells. (C) Correspondingly, RhoA activities were altered by DLC3. (D) Glucose deprivation did not influence DLC3 expression in oeDLC3 cells.



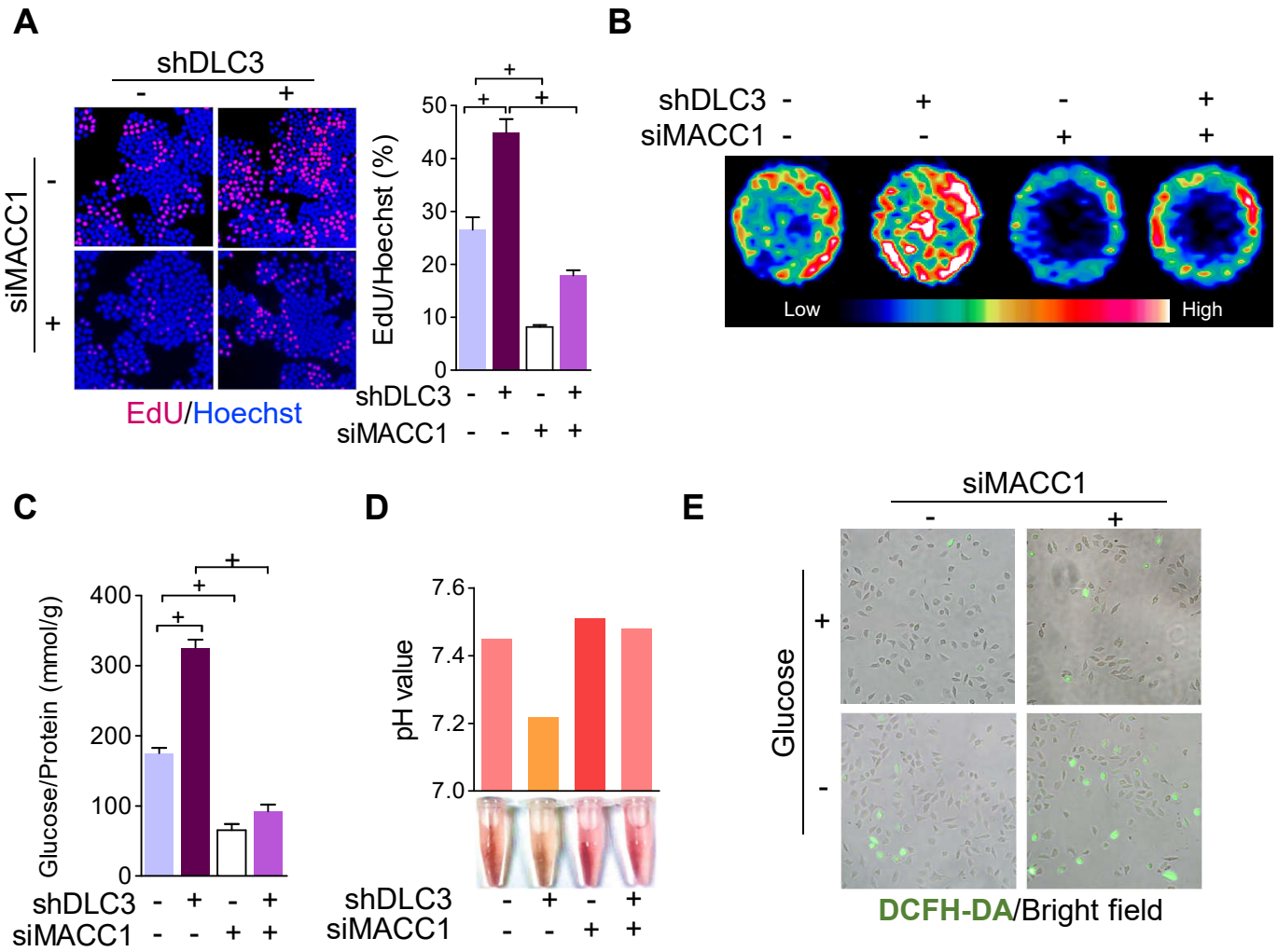
Supplementary Figure S3 DLC3 suppressed proliferation and migration in BGC823 cells.

(A) The viability and (B) DNA replication of BGC823 were suppressed by DLC3 overexpression. (C) DLC3 influenced colony formation of BGC823. (D) The migration and (E) invasiveness was inhibited by DLC3 overexpression and enhanced by its silencing. *P<0.05, #P<0.01, +P<0.001.

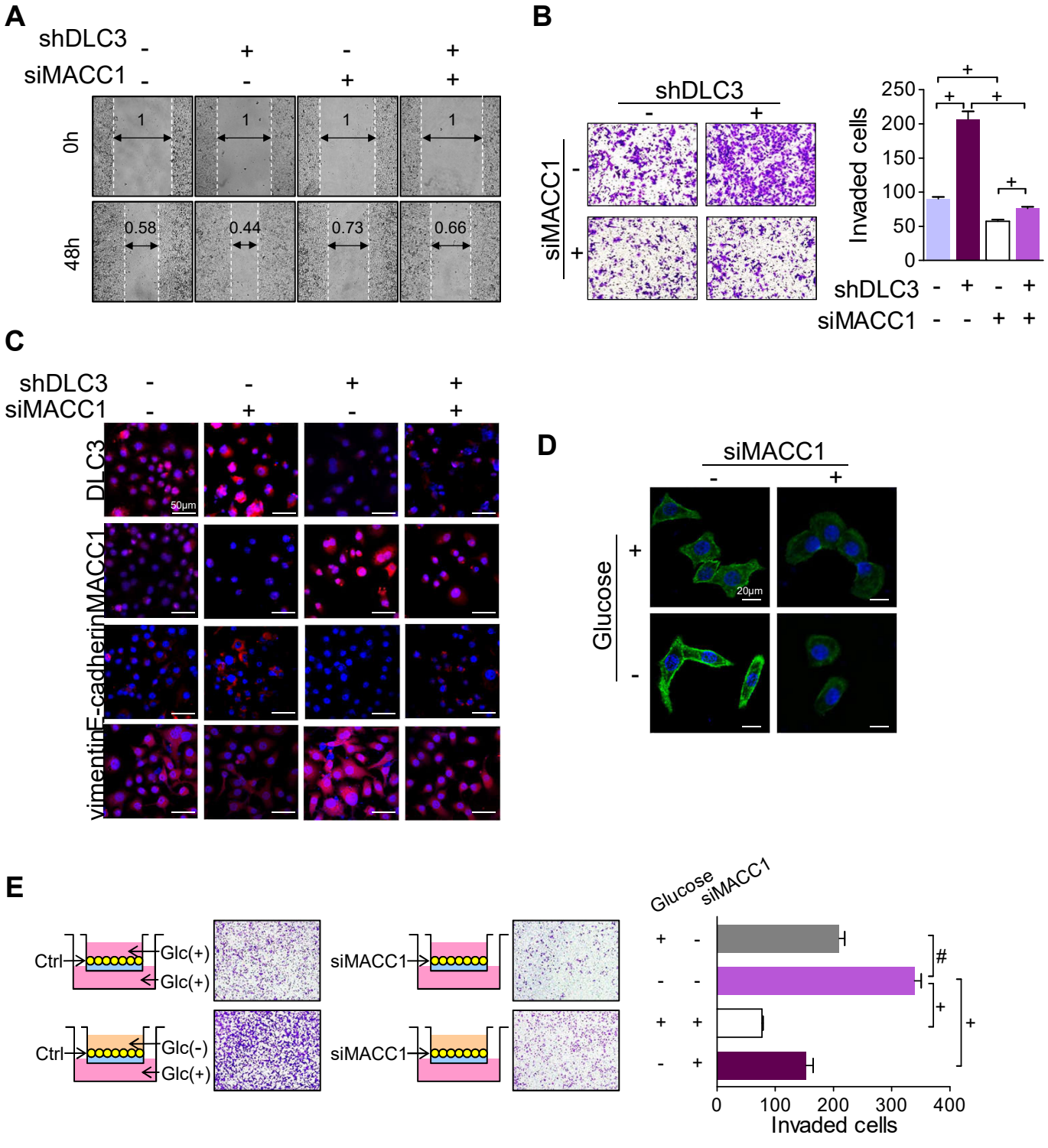


Supplementary Figure S4 DLC3 inhibited MACC1 expression via RhoA/JNK/AP-1 signaling in MKN45 cells.

(A) OeMACC1 did not influence DLC3 expression, but oeDLC3 significantly downregulated MACC1 expression. OeMACC1 reversed the epithelial mesenchymal transition marker alterations induced by oeDLC3 in MKN45 cells. Right panel: semi-quantified MACC1 relative gray values (n=3). (B and C) Glucose deprivation upregulated (B) MACC1 protein and (C) mRNA expressions via DLC3 in MKN45 cells. (B) Right panel: semi-quantified MACC1 relative gray values (n=3). (D) In protein array assay, shDLC3 enhanced the phosphorylation of JNK and several other protein kinases. (E) Semi-quantified MACC1 relative gray values of shDLC3 and corresponding control (n=3). (F and G) RhoA, ROCK or JNK inhibitors suppressed shDLC3-promoted MKN45 (F) colony formation and (G) invasion. Quantified results related to Figure 6G and H.



Supplementary Figure S5 The DLC3/MACC1 axis modulated glycolysis remodeling.
(A) Indicated by EdU staining, siMACC1 significantly reversed shDLC3 accelerated DNA-replication in MKN45 cells. (B) The DLC3/MACC1 axis regulated cellular ^{18}F-FDG uptake in culturing plates. (C) Silencing MACC1 reduced glucose uptake in shDLC3 MKN45 cells. (D) The DLC3/MACC1 axis influenced acidosis in the conditioned medium. (E) SiMACC1 further increased glucose deprivation-promoted ROS generation. #P<0.01, +P<0.001.



Supplementary Figure S6 The DLC3/MACC1 axis modulated GC cell invasion and glucose chemotaxis.

(A and B) The DLC3/MACC1 axis modulation on MKN45 cell motility was confirmed by (A) scratch healing migration assay and (B) transwell invasion assays. (C) DLC3/MACC1 axis modulation on epithelial and mesenchymal marker expressions were confirmed by immunofluorescence. (D and E) Silencing MACC1 (D) reversed the glucose-deprivation-induced cytoskeleton changes and (E) inhibited glucose chemotaxis in MKN45 cells. *P<0.05, #P<0.01, +P<0.001.

Supplementary Table S1 The qRT-PCR primer sequences used in this study

Target		Sequence
MACC1	F	GAGTTAGTCGCACGTCTCATCC
	R	AGTGAGCACTCCAGGTATACAG
DLC3	F	GACGGAGCAGTCCCTCCT
	R	CTTTTGGCCTCGGCTTCT
β-actin	F	TGGCACCCAGCACAATGAA
	R	CTAAGTCATAGTCCGCCTAGAAGCA

Supplementary Table S2 The functional binding site for AP-1 in MACC1 promoter