Supporting information

Supplementary Materials and Methods

Cell lines and transfection

To generate stable OLFM4 knockdown and control cell lines, HT-29 cells were transfected with human OLFM4 small hairpin RNA (shRNA) plasmid for three pooled targets or nonspecific scramble control shRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) using Lipofectamine 2000 (Thermofisher Scientific, Waltham, MA, USA) followed by selection with 4 μg/ml puromycin (Santa Cruz Biotechnology) for 4 weeks. After establishment of puromycin-resistant cell lines, OLFM4 expression was confirmed by western blot analysis and qRT-PCR and stably OLFM4-silenced cell lines (shOLFM4) and control cell lines (CTL) were selected. To verify knockdown experiments by shRNA, small interfering RNA (siRNA) was used for transient transfection.

Transient knockdown of OLFM4 was achieved by 12 h transfection of small interfering RNA (siRNA) or non-targeting control (AccuTarget, Bioneer, Daejeon, South Korea) into HT-29 cells using Lipofectamine 2000 (Life Technologies, Rockville, MD, USA).

Cell proliferation assay

After subculture and treatment, media were collected every 0, 24, and 48 h and frozen at -70°C; 100 μl of the media was dispensed into 96-well plates, and 10 μl of CCK-8 agent (Dojindo Molecular Technologies, MD, USA) was added. The plate was incubated at 37°C for 2 h, and absorbance was measured by a microplate reader at 450 nm. Each assay was performed in triplicate.

Wound healing assay and invasion assay

Cells were seeded in 24-well plates at 5×10^5 cells per well. After 24 h, a scratch was made in the cell monolayer using a 200-μl pipette tip. Images were taken at 0, 24, and 48 h using a BX41 light microscope (Olympus Optical, Tokyo, Japan). Wound size was measured by Image J software (NIH, Bethesda, MD, USA).

Invasion assay was performed using Boyden chambers equipped with an 8-μm cell culture insert (BD Biosciences, San Jose, CA, USA) that was coated with reconstituted Growth Factor Reduced BD Matrigel (10 μg/cm^2; 50 μg/ml; BD Bioscience). The cells were seeded in 12-well plates at a concentration of 2.5×10^5/well and cultured for 48 h following treatment with anti-OLFM4 antibody. Pretreated cells were seeded in the top chamber with serum-free medium; normal culture medium was added in the bottom chamber. The Matrigel invasion chamber was incubated for 24 h in a humidified tissue culture incubator. After 24 h, the non-invasive cells were removed from the upper surface of the separating membrane by gentle scrubbing with a cotton swab, and the invading cells were stained with 1 μg/ml 4', 6-diamidino-2-phenylindole (DAPI). Cells were then counted under a fluorescence microscope at a magnification of ×40.

Quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted using TRIzol Reagent (Thermofisher Scientific) and reverse-transcribed using a SuperScript First-Strand Synthesis Kit (Thermofisher Scientific) according to the manufacturer's protocol. Reactions were performed in triplicate using SYBR Green master mix (Applied Biosystems, Foster City, CA, USA). PCR was performed using primers

listed in Supplementary Table 2. Samples were amplified in a StepOne Plus real-time PCR system (Applied Biosystems) for 40–45 cycles using the following conditions: 95°C for 30 sec, 60–62°C for 1 min, and 72°C for 30 sec. Quantitative analysis was performed using the relative standard curve method and the results were determined as the relative expression or fold change compared with the control, *GAPDH or bACTIN*.

Western blot analysis

HT-29 cells were lysed in RIPA lysis buffer (Thermofisher Scientific) with protease inhibitor (Thermofisher). Protein samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Thermofisher). Membranes were incubated with anti-OLFM4 (Santa Cruz Biotechnology) and anti-β-actin (Sigma-Aldrich, St. Louis, MO, USA) antibodies. Immunoreactive bands were visualized with Enhanced Chemiluminescence Western Blot Detection Reagent (Amersham Biosciences, Freiburg, Germany). Anti-OLFM4 antibody was purchased from Abcam (Cambridge, UK).

Histological and image analysis

Mouse colons were extracted postmortem and opened by making a longitudinal incision. A portion of the rectum was excised to make a tissue section. The section was fixed in 10% buffered formalin and embedded in paraffin. Paraffin-embedded tissue was cut into 10 μm–thick sections. Formalin-fixed, paraffin-embedded colon sections were deparaffinized in xylene and ethanol and rehydrated in water. Sections were placed on a slide glass and stained with hematoxylin-eosin (H&E).

Images were evaluated by Image J software (v1.4.6h, National Institutes of Health, Washington DC, USA) (1).

Immunofluorescence stain

Cells were fixed with 10% neutral buffered formalin for 30 min at room temperature and washed with phosphate-buffered saline (PBS). Deparaffinized colon sections or cells were blocked with 5% BSA for 1 h at room temperature and incubated with anti-olfactomedin-4 antibody (1:100, clone 13G2.1, Merck Millipore (Burlington, MA, USA), PerCP-cyanine5.5 anti-Gr-1 antibody (1:100, RB6-8C5, BDbiosicence, CA, USA), PerCP-cyanine5.5 anti-CD66b antibody (1:500, G10F5, BD Biosciences), or PE-cyanine7 F4/80 antibody (1:100, BM8, eBioscience) diluted in blocking buffer overnight at 4°C. Cells were then washed with PBS, incubated with the secondary antibody, Alexa Fluor 488 goat anti-rabbit IgG H&L (1:200, A-11008, Invitrogen, Cambridge, UK) for 1 h at room temperature, and nuclear stained with diamidino-2-phenylindole (DAPI, 300 nM, D1306, Thermo Fisher Scientific) for 3 min. Confocal images were obtained using a Zeiss LSM700 confocal microscope (Carl Zeiss AG, Oberkochen, Germany).

Analysis of gene expression databases

Public database for gene expression analysis was obtained from Gene expression omnibus (GEO). Information for OLFM4 mutation and survival of colon cancer patients was downloaded from NCBI and cBioPortal website, an open-access database that is publicly available at http://www.cbioportal.org (2).

Supplementary Tables

Supplementary Table 1. Clinical characteristics of healthy controls and patients

Characteristic	Healthy control (n=7)	Dysplasia (n=18)	Adenocarcinoma (n=24)
Age (years, mean ± SD)	36.8±4.2	47.1±19.2	60.9±17.0
Male gender	5 (62.5%)	14 (77.8%)	20 (76.9%)
T classification[1]			
T1			4 (15.4%)
T2/3/4			22 (84.6%)
N classification[2]			
N0			17 (65.3%)
N1/2/3			9 (34.7%)
Stage			
Stage I/II			9 (34.7%)
Stage III/IV			17 (65.3%)

[1]T1, tumor invades lamina propria or submucosa; T2, tumor invades muscularis propria or subserosa; T3, tumor penetrates serosa; T4, tumor invades adjacent structures

[2]N0, no regional lymph node metastasis; N1, metastasis in 1–6 regional lymph nodes; N2, metastasis in 7 to 15 regional lymph nodes; N3, metastasis in more than 15 regional lymph nodes.

Supplementary Table 2. List of sequences of primers used for qRT-PCR

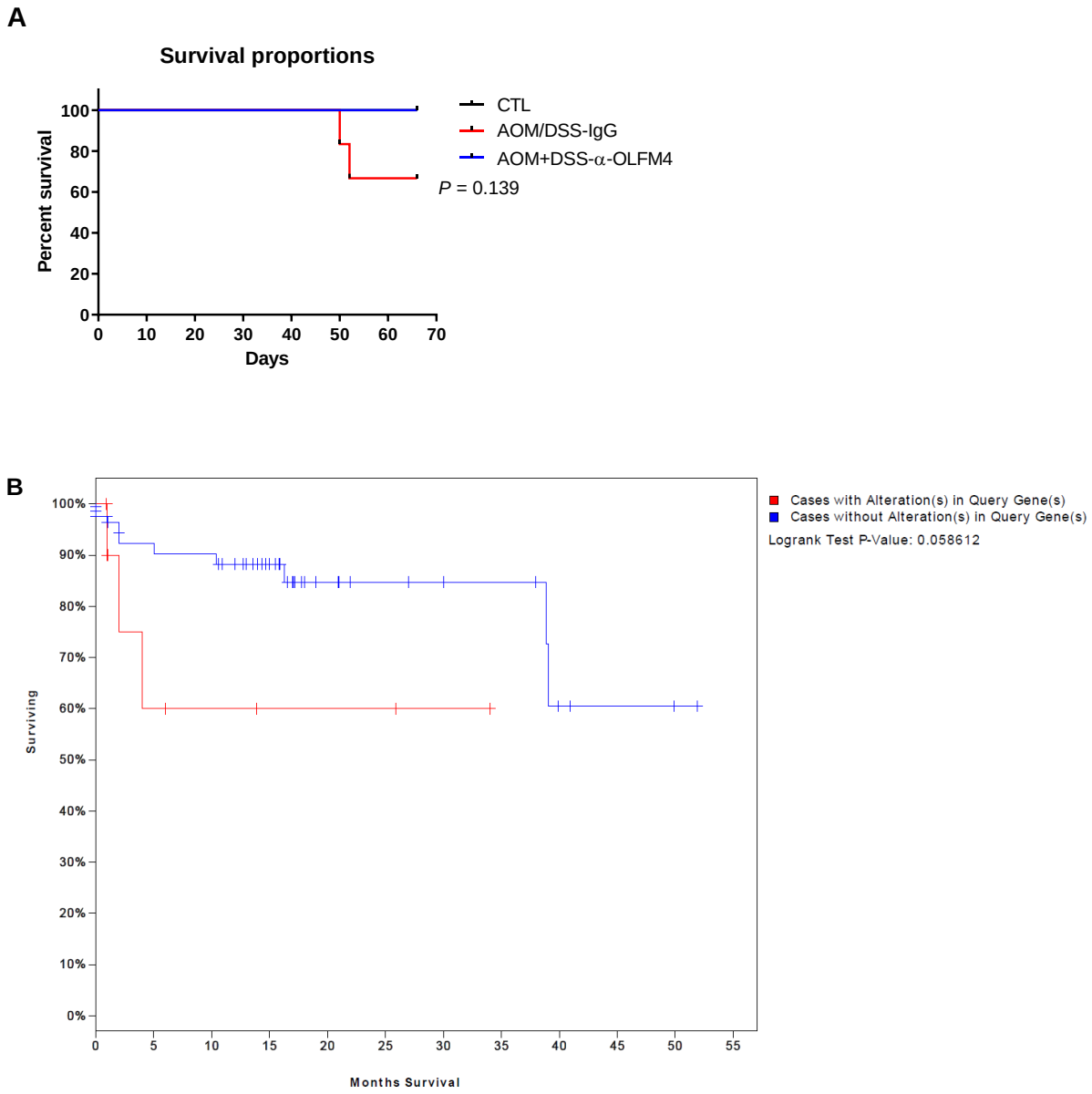
Gene	Sequence (5′–3′)
Human	
OLFM4	F: AGCTCTTTCCCAGGTGTTGA
	R: AAGCGTTCCACTCTGTCCAC
NANOG	F: AGTCCCAAAGGCAAACAACCCACTTC
	R: TGCTGGAGGCTGAGGTATTTCTGTCTC
SOX2	F: GGGAAATGGGGAGGGGTGCAAAAGAGG
	R: TTGCGTGAGTGTGGATGGGATTGGTG
OCT4	F: GACAGGGGGAGGGGAGGAGCTAGG
	R: CTCCCTCCAACCAGTTGCCCCAAAC
ZFP42	F: ACTGGTACCTCGGATTTCAAATGGAGAGGTCCTGC
	R: AATCTGGCTAGCAGTGGAAACGTGGACTGCCCGCG
GAPDH	F: GAAGGTGAAGGTCGGAGTC
	R: GAAGATGGTGATGGGATTTC
bACTIN	F: CTCTTCCAGCCTTCCTTCCTG
	R: CAGCACTGTGTTGGCGTACAG
ECAD	F: CCTAGATGAACCTTATGAGGCCA
	R: GCTGTAGAGGAGACGAGCATTAT
VIM	F: TTGTACCATTCTTCTGCCTCCTG
	R: TGTCCAAATCGATGTGGATGTTTC
NCAD	F: TGTTTGATATGAAGGCAGTGG
	R: TCAGTCATCACCTCCACCAT
TWIST	F: TGAGCAAGATTCAGACCCTCA
	R: ATCCTCCAGACCGAGAAGG
SNAI1	F: GGCAATTTAACAATGTCTGAAAAGG
	R: GAATAGTTCTGGGAGACACATCG

F: forward primer, **R**: reverse primer

Gene	Sequence (5′–3′)
Mouse	
Olfm4	F: CACCGAACTCACCCAAGTGTT
	R: CACTGTGCAGATACACCTGCC
Tnfa	F: CAAAGGGAGAGTGGTCAGGT
	R: ATTGCACCTCAGGGAAGAGT
Il1b	F: GCAACTGTTCCTGAACTCAACT
	R: ATCTTTTGGGGTCCGTCAACT
Tgfb	F: ACCATGCCAACTTCTGTC
	R: CGGGTTGTGTTGGTTGTAGA
Ahr	F: GGCTTTCAGCAGTCTGATGTC
	R: CATGAAAGAAGCGTTCTCTGG
β-Actin	F: AGTGTGACGTTGACATCCGT
	R: TGCTAGGAGCCAGAGCAGTA

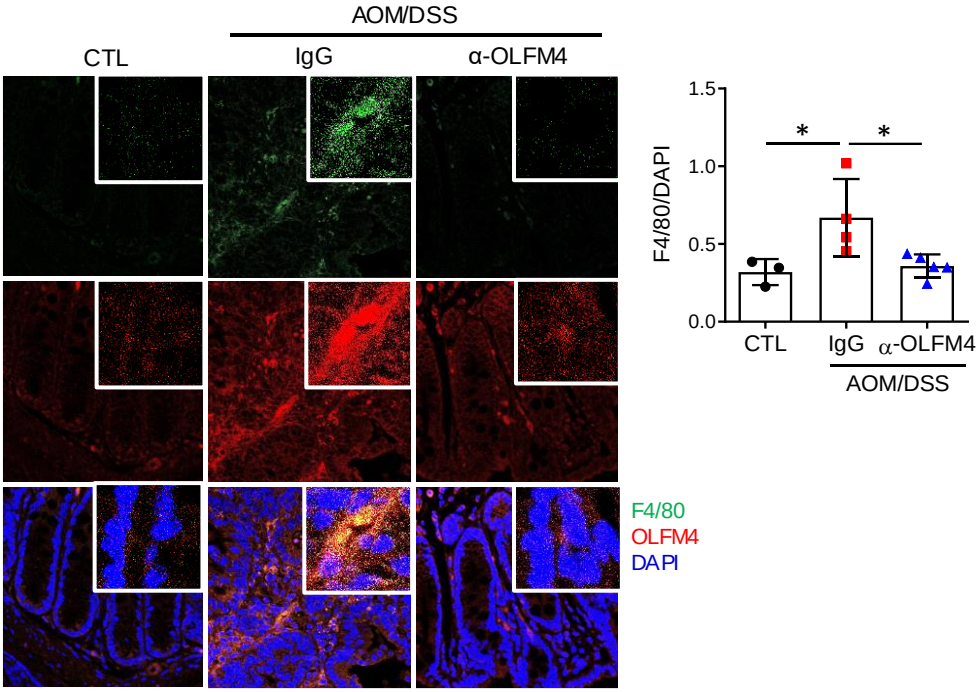
F: forward primer, **R**: reverse primer

Supplementary figures



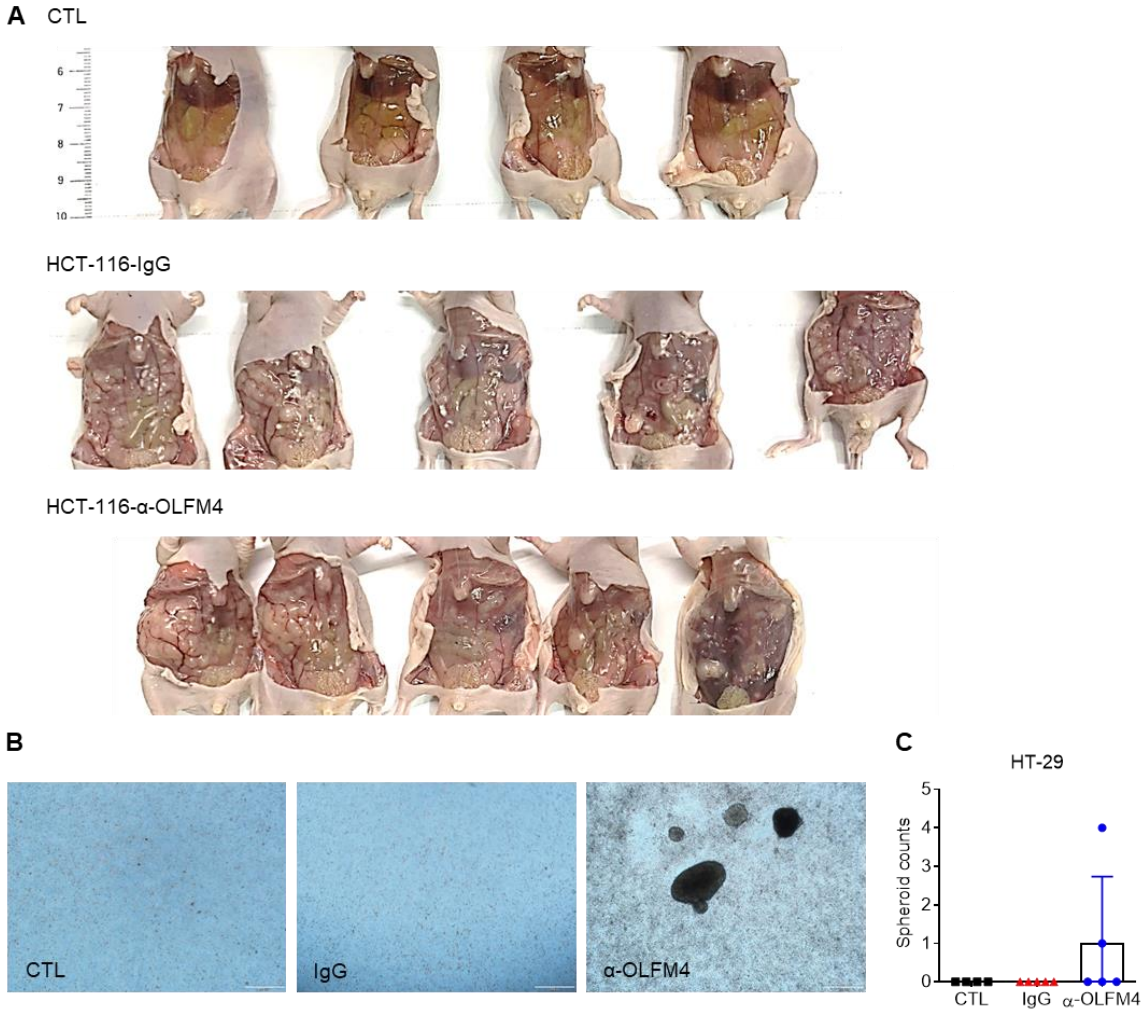
Supplementary Fig. 1. Survival analyses of animal model and human public data. Survival analyses were performed using Kaplan–Meier plots for overall survival. Differences in survival curves were compared using a log-rank test. (A) Survival curves of mice in AOM/DSS CAC model. (B) Overall survival in OLFM4 wild-type and mutated patients subdivided according to OLFM4 mutation site. CRC patients with missense mutation of OLFM4 in the cBioPortal database were analysed.

CTL, untreated control; AOM, treated with azoxymethane; DSS, treated with dextran sodium sulfate; IgG, treated with isotype control antibody; α-OLFM4, treated with anti-OLFM4 antibody.

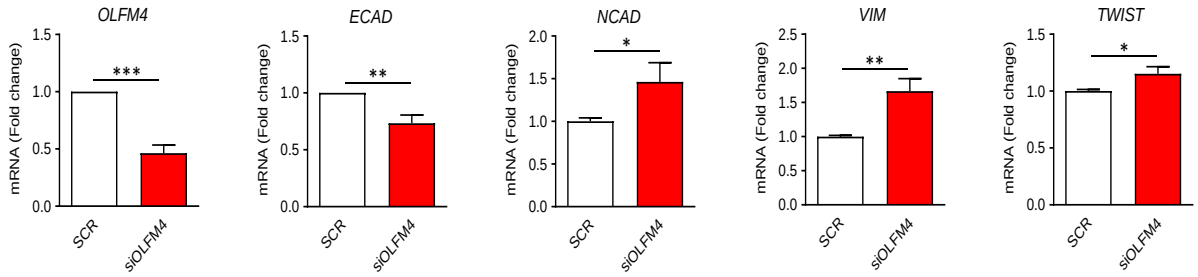


Supplementary Fig. 2. OLFM4 positive macrophages are reduced by anti-OLFM4. Mice treated with AOM and DSS received either neutralizing anti-OLFM4 (α-OLFM4) or control antibody (IgG) nine times of water until the 9th week. Immunofluorescence analysis of OLFM4-high macrophages. Representative images (left) and quantification of $F4/80^{+}$ (green color) cells (right). Scale bar, 20 μm.

Data are expressed as mean ±S.E.M. Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparisons post-test. *$P < 0.05$.



Supplementary Fig. 3. OLFM4 neutralizing antibody inhibits metastatic nodule formation. (A) Macroscopic view of the intraperitoneal tumor spread in HCT-116 i.p. injected mice. (B) Representative images of the peritoneal spheroid of HT-29 colon cancer cells in peritoneal ascites. Scale bar, 500 μm. (C) Peritoneal spheroid formation of HT-29 colon cancer cells in peritoneal ascites.



Supplementary Fig. 4. OLFM4 knockdown promotes epithelial-mesenchymal transition. HT-29 cells were transfected with scramble siRNA (SCR) or siRNA against OLFM4 (siOLFM4). mRNA levels of representative EMT markers were determined. Gene expressions of OLFM4, E-cadherin, N-cadherin, vimentin, and Twist were assessed using qRT-PCR.

Data are expressed as mean ±S.E.M. Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparisons post-test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$.

References

1. Ruifrok AC, Johnston DA. Quantification of histochemical staining by color deconvolution. Anal Quant Cytol Histol 2001;23:291-9.

2. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov 2012;2:401-4.