Supplementary materials and methods

RNA extraction and qRT-PCR

Total RNA was extracted using TRIzol reagent (Takara) according to the manufacturer's instructions. cDNAs were synthesized using a Reverse Transcription Kit (Takara). Quantitative real-time PCR was carried out using SYBR Green I (Takara) in triplicate. The results were normalized to the expression of GAPDH. The primer sequences used for qRT-PCR are listed in Table S1. Relative quantification of mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blot assay (WB)

Protein lysates were prepared and quantified by a bicinchoninic acid (BCA) protein assay (KeyGen Biotech). Equivalent amounts of protein lysates were separated using 10% SDS-PAGE and transferred onto a PVDF membrane. Then, the membrane was incubated with the primary antibody at 4°C overnight, followed by the appropriate second antibody. The bands were visualized by Pierce ECL Western Blotting Substrate (Thermo Scientific).

Immunohistochemistry (IHC)

The 3 μm-thick tissue sections were deparaffinized and rehydrated, and incubated with primary antibody at 4°C overnight. Prior to incubation with anti-SSH3 (1:100 dilution; Proteintech Group, Chicago, MA, USA), and anti-Ki67 antibodies (1:100 dilution; Bioworld Technology Inc., St. Louis Park, MN, USA), the sections were heated in 0.01 M sodium citrate buffer, pH 6.0 for antigen retrieval, and incubated in 3% H_2O_2 to inhibit endogenous peroxidase activity. On the next day, the sections were washed and incubated with the secondary antibody for 30 minutes at room temperature. Finally, the slides were developed using a DAB chromogen kit and counterstained with Mayer's hematoxylin.

The total SSH3 immunostaining score was calculated as the sum of the percentage positivity of stained tumor cells and the staining intensity. The percentage positivity was scored from 0 to 3, with 0 for < 10%, 1 for 10-30%, 2 for 31-50%, and 3 for > 50%. The staining intensity was scored from 0 to 3, with 0 for no staining, 1 for weakly stained, 2 for moderately stained, and 3 for strongly stained. Both the percentage positivity of cells and staining intensity were determined in a double-blinded manner. Subsequently, SSH3 expression was calculated as the value of percentage positivity score × staining intensity score, which ranged from 0 to 9. The final expression level of SSH3 was defined as 'low' (0-4) and 'high' (5-9).

Transwell assay

A Boyden chamber with 8-μm-pore filter membrane was used for the in vitro migration assay. Briefly, cells (5×10^4) in culture medium containing 1% fetal bovine serum were seeded in the upper chamber, and culture medium with 10% fetal bovine serum was added in the lower chamber as a chemoattractant. Cells that migrated to the lower surface of the filter were fixed in 4% paraformaldehyde and stained with Giemsa. The migratory cells were counted (10 random 200 × fields per well). Three independent experiments were performed and the data were presented as the mean ± s.e.m.

Wound healing assay

Cells were allowed to grow to confluence, and the monolayer was scratched with a 100 μl pipette tip. The remigration of cells to close the wound was assessed by live cell imaging. The percentage of open area was measured with the software at defined time points (0 and 48 h).

Table S1. Primer sequences used for qRT-PCR (5' to 3')

Gene	Forward primer	Reverse primer
SSH3	TCCAGGTATTGCACCAAGC	GCCATAGCCGTCCACTCAT
GAPDH	GGUGACUAUUCAACCGCAUTT	AUGCGGUUGAAUAGUCACCTT

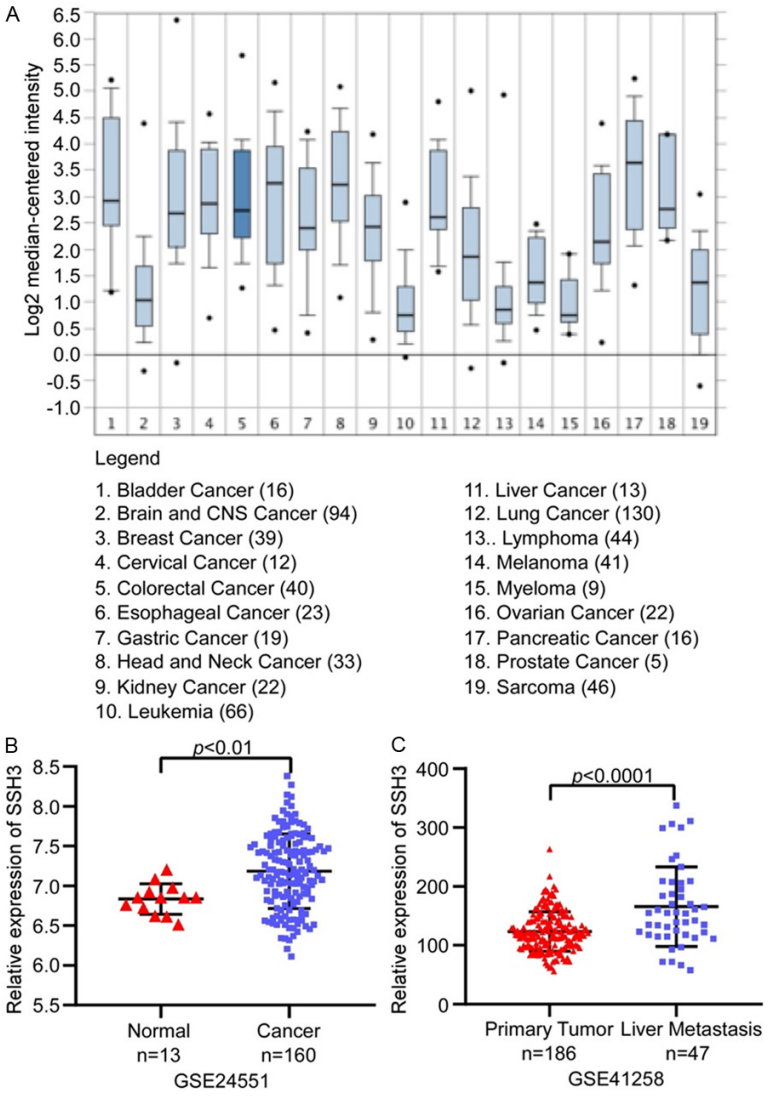


Figure S1. Expression of SSH3 in tumors. A. The analyses of SSH3 expression in various types of tumors by using the public database Oncomine (https://www.oncomine.org/resource/login.html). B. The analyses of SSH3 expression in CRC by using GEO dataset (GSE24551). C. The analyses of SSH3 expression in CRC by using GEO dataset (GSE41258).

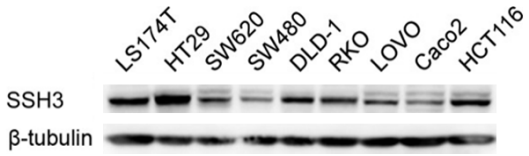


Figure S2. Expression of SSH3 in CRC cells. The expression of SSH3 in different types of CRC cells using Western blot. β-tubulin was used as a loading control.

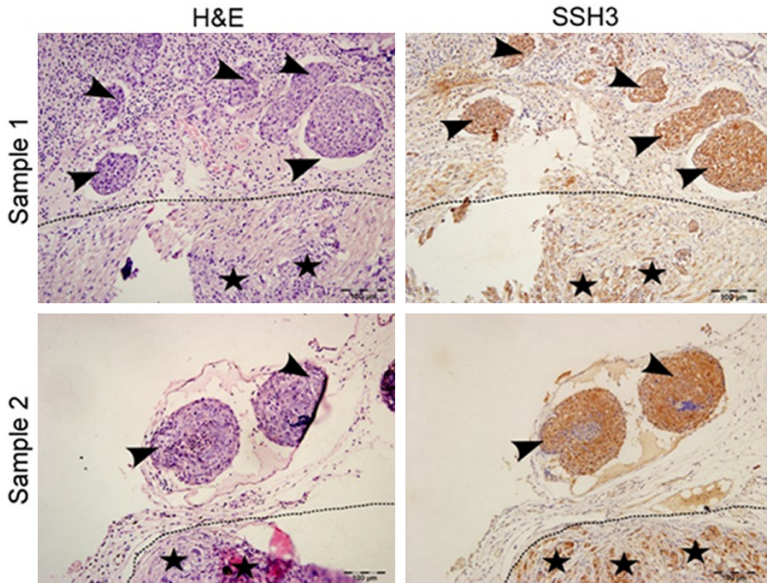


Figure S3. Expression of SSH3 in the primary tumor and the tumor thrombus of the orthotopic mice. SSH3 expression was tested in the primary tumor and the tumor thrombosis of the orthotopic mice using IHC.

A

Query protein	Q8TE77
Gene names	SSH3 SSH3L
Protein name	Protein phosphatase Slingshot homolog 3
Function	Protein phosphatase which may play a role in the regulation of actin filament dynamics. Can dephosphorylate and activate the actin binding/depolymerizing factor cofilin, which subsequently ... view more

Statistics

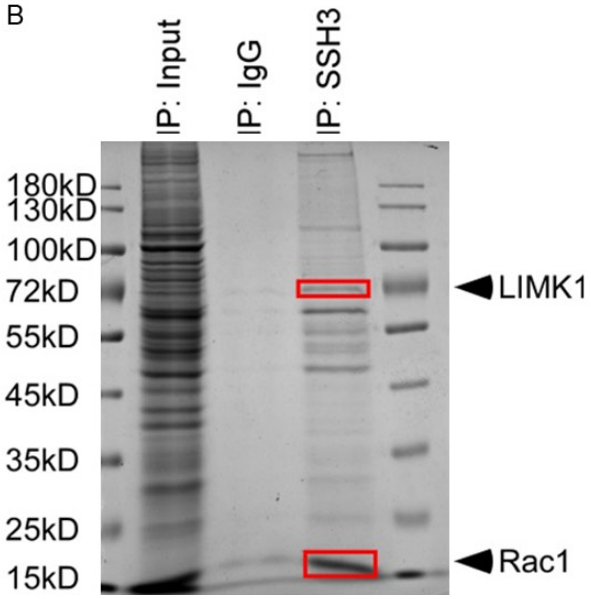
High confidence predictions (Score>0.5): 61

All predictions (Score>0.1): 139

Interactions in database: 3

Interactor	Gene names	Organism	Prediction code	Prediction LR	Database LR	Final prob.
P53667	LIMK1 LIMK	human	S T G E M C P	17.9	1802.0	0.98

B



C

Accession number in the database	Score	Mass	Number of matching secondary spectrum	Number of matching peptides	Protein abundances
LIMK1_HUMAN	219	71815	10 (5)	8 (4)	0.21
RAC1_HUMAN	349	21835	16 (15)	10 (10)	6.37

Figure S4. Candidate proteins interacted with SSH3. A. Candidate proteins interacted with SSH3 using the public database PrePPI. B and C. Candidate proteins interacted with SSH3 using the mass spectrometry (MS).