The RAS-interacting chaperone UNC119 drives the RASSF6–MDM2–p53 axis and antagonizes RAS-mediated malignant transformation

Takanobu Shimizu[1], Takeshi Nakamura[1], Hironori Inaba[2], Hiroaki Iwasa[1,*], Junichi Maruyama[1], Kyoko Arimoto-Matsuzaki[1], Takao Nakata[2,3], Hiroshi Nishina[4], and Yutaka Hata[1,3,*]

From the [1]Department of Medical Biochemistry, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo 113-8519, Japan

[2]Department of Cell Biology, Graduate School of Medical and Dental Science, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan

[3]Center for Brain Integration Research, Tokyo Medical and Dental University, Tokyo, 113-8519, Japan.

[4]Department of Developmental and Regenerative Biology, Medical Research Institute, Tokyo Medical and Dental University, Tokyo 113-8510, Japan.

Running title: UNC119 regulates RAS-mediated apoptosis

*To whom correspondence should be addressed: Hiroaki Iwasa and Yutaka Hata: Department of Medical Biochemistry, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo 113-8519, Japan; hiwammch@tmd.ac.jp and yuhammch@tmd.ac.jp ;Tel 81-3-5803-5164; Fax 81-3-5803-0121

Keywords: apoptosis, KRAS, TP53, tumor suppressor, Ras association domain family member 6 (RASSF6), human retina gene 4 (HRG4), cancer, cell signaling, cell proliferation, GTPase

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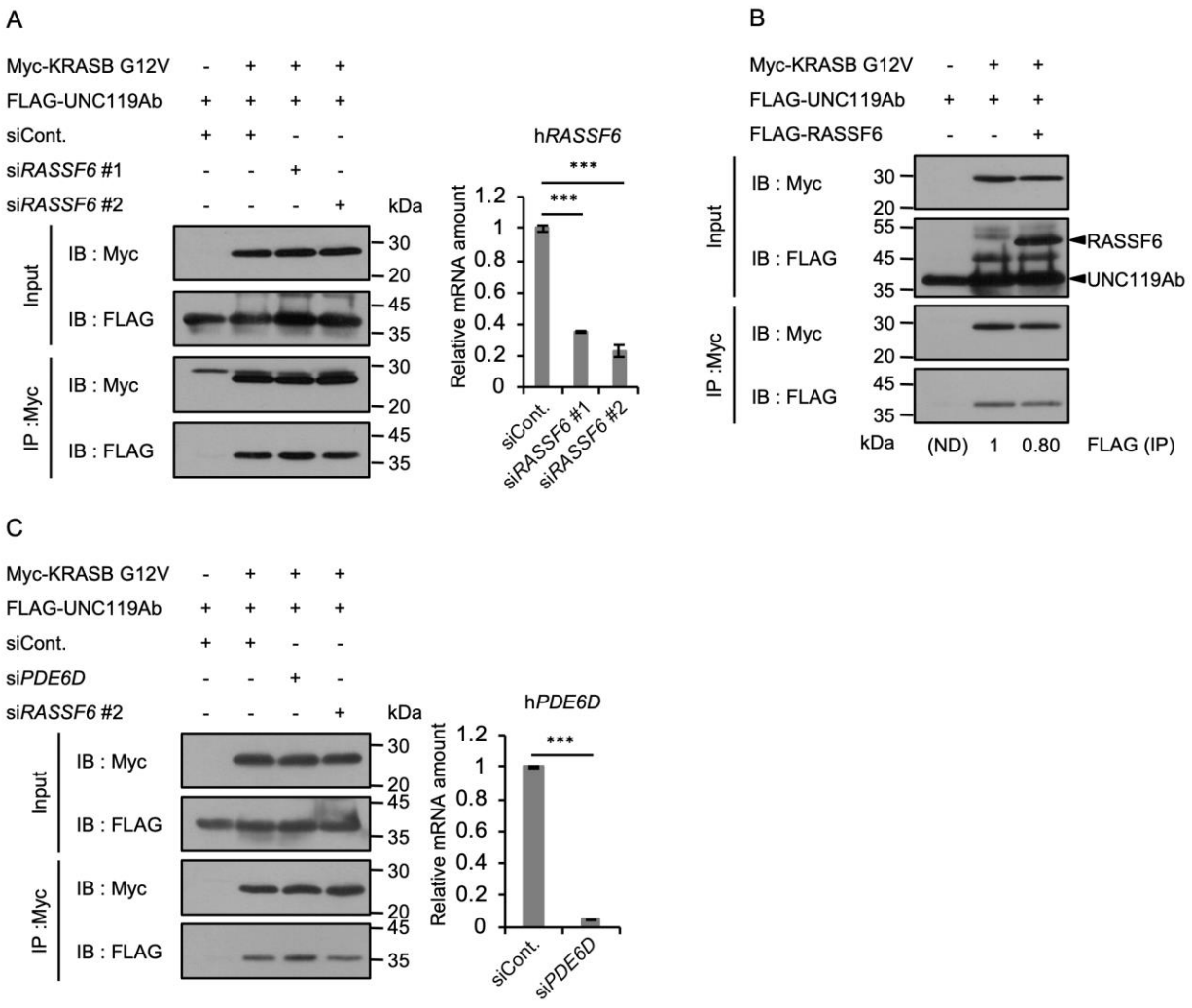

Fig. S1. RASSF6 or PDEδ is not involved in the interaction between KRASB G12V and UNC119Ab. Myc-tagged proteins and FLAG-tagged proteins were expressed in HEK293FT cells and Myc-proteins were immunoprecipitated. **(A)** and **(C)** Cells were transfected with control siRNA (siCont), *RASSF6* siRNA (si*RASSF6*#1, #2) or *PDE6D* siRNA in advance. The efficiency of knockdown was confirmed by qRT-PCR (bar graphs). ***, $p < 0.001$. 24 h later, cells were transfected with pCIneoMyc-KRASB G12V (Myc-KRASB G12V) and pCIneoFHF-UNC119Ab (FLAG-UNC119Ab). **(B)** HEK293FT cells were transfected with pCIneoMyc-KRASB G12V, pCIneoFHF-UNC119Ab, and pCIneoFHF-RASSF6 (FLAG-RASSF6) as indicated. 24 h after transfection, Myc-KRASB G12V was immunoprecipitated. The signals were measured by use of ImageJ (the numbers under the image). Statistical analyses were performed with One-way ANOVA with Tukey's test for Fig. S1A and two tailed Student's t test for Fig. S1C. Three experiments for (A), (B), and (C) were performed by two members.

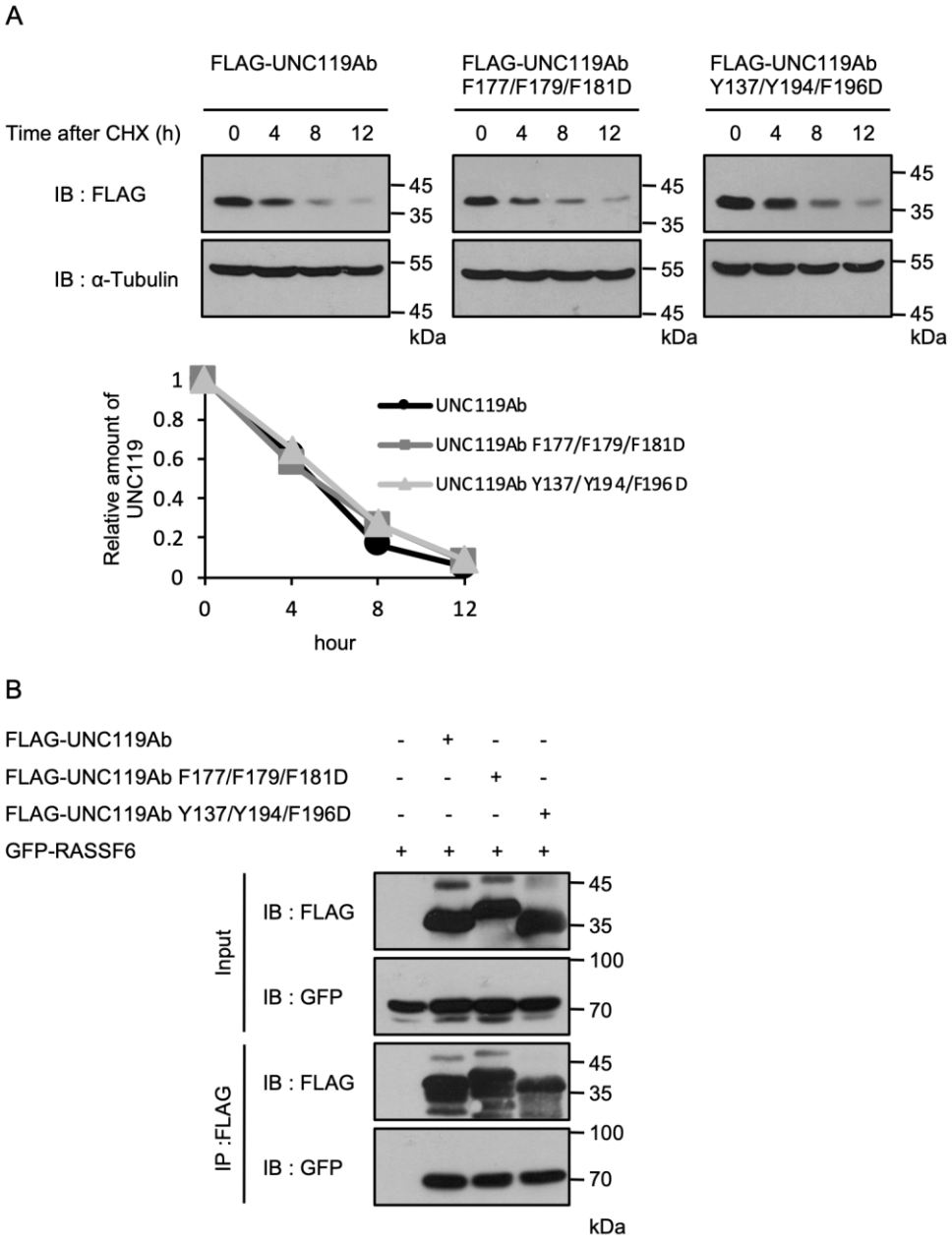

Fig. S2. Characterization of UNC119Ab F177/F179/F181D and UNC119Ab Y137/Y194/F198D mutants. **(A)** FLAG-tagged proteins were expressed in HEK293FT cells. The cells were treated with 50 mg/l cycloheximide and harvested at the indicated time points. Whole cell lysates were immunoblotted. The signals detected with anti-FLAG and α-tubulin antibodies were measured by ImageJ and the ratio between two signals were calculated for each point. The value at time 0 was set to 1. **(B)** FLAG- and GFP-tagged proteins were expressed in HEK293FT cells. Immunoprecipitates with anti-DYKDDDDK tag beads were immunoblotted with anti-GFP antibody. Three experiments were performed.

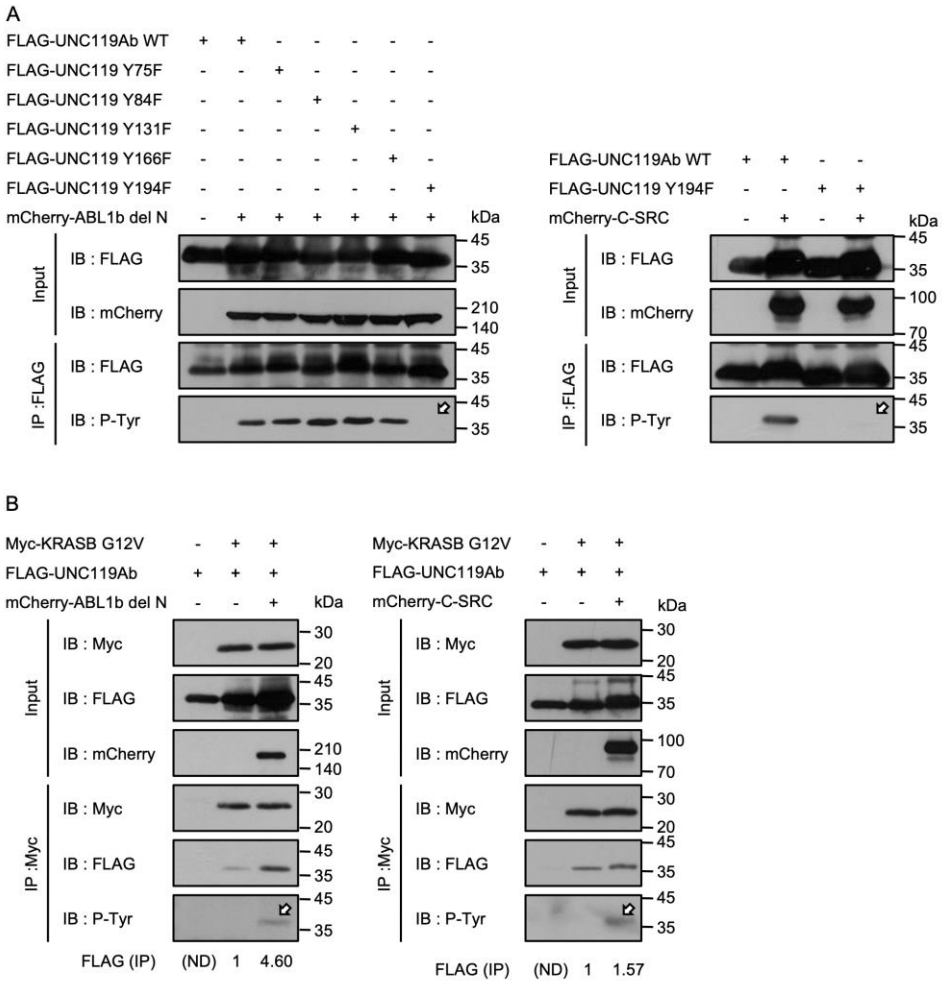

Fig. S3. UNC119Ab is tyrosine-phosphorylated at Tyr194 and tyrosine-phosphorylated UNC119Ab interacts with KRASB G12V. (A) HEK293FT cells were transfected with pCIneoFHF-UNC119Ab, pCIneoFHF-UNC119Ab Y75F, pCIneoFHF-UNC119Ab Y84F, pCIneoFHF-UNC119Ab Y131F, pCIneoFHF-UNC119Ab Y166F, pCIneoFHF-UNC119Ab Y194F, and pCIneomCherry-ABL1b del N (the left). The immunoprecipitates with anti-DYKDDDDK tag beads were immunoblotted with anti-FLAG and anti-phosphotyrosine antibodies. UNC119Ab Y194F was not tyrosine-phosphorylated (an arrow). The similar experiment was performed by using pCIneoFHF-UNC119Ab, pCIneoFHF-UNC119Ab Y194F, and pCIneomCherry-C-SRC (the right). **(B)** HEK293FT cells were transfected with pCIneoMyc-KRASB G12V, pCIneoFHF-UNC119Ab, pCIneomCherry-ABL1b delN, and pCIneomCherry-C-SRC. Myc-KRASB G12V was immunoprecipitated. Immunoprecipitates were immunoblotted with the indicated antibodies. In the presence of ABL1b delN and C-SRC, the amount of co-immunoprecipitated UNC119Ab was increased. The signals were measured by use of ImageJ (the numbers under the image). The immunoblotting with anti-phosphotyrosine antibody demonstrates that the co-immunoprecipitated UNC119Ab was tyrosine-phosphorylated (arrows). Three experiments for (A), (B), and (C) were performed by two members.

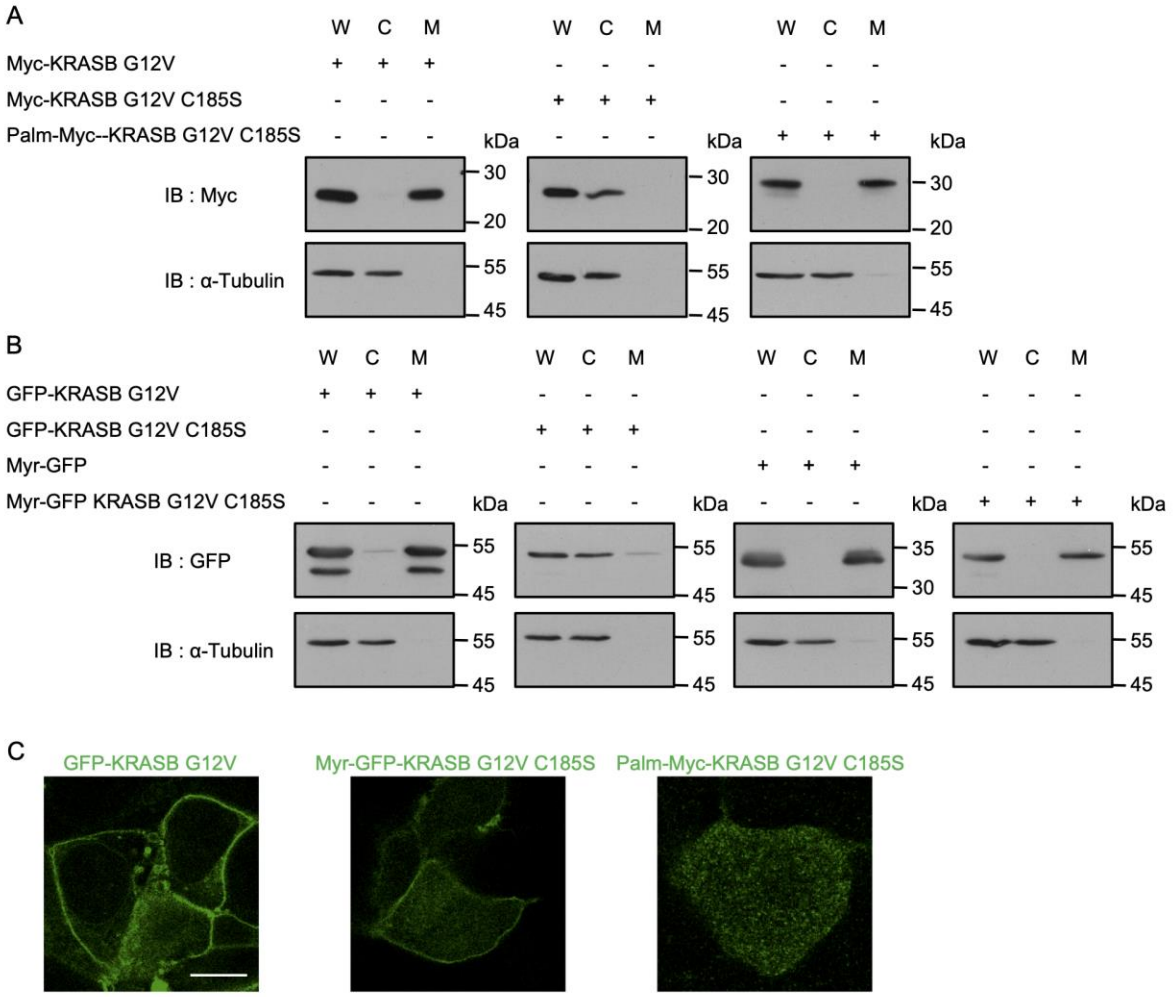

Fig. S4. The subcellular distribution of membrane-anchoring KRASB mutants. HEK293FT cells were transfected with pCIneoMyc-KRASB G12V, pCIneoMyc-KRASB G12V C185S, or pCIneoFHF-Palm-Myc-KRASB G12V C185S in **(A)**, and pCIneoGFP-KRASB G12V, pCIneoGFP-KRASB G12V C185S, pCIneoMyr-GFP, or pCIneoMyr-GFP-KRASB G12V C185S in **(B)**. 48 h later, cells were harvested and the subcellular fractionation was performed as described in Experimental procedures. W, C, and M stand for the whole cell lysates, the cytosolic fraction, and the membrane fraction, respectively. The comparative amount of each sample was charged onto each lane. α-Tubulin was used as a cytosolic marker. Myc-KRASB G12V C185S was recovered exclusively in the cytosolic fraction, whereas Myc-KRASB G12V and Palm-Myc-KRASB G12V C185S were detected only in the membrane fraction. Similarly, GFP-KRASB G12V, Myr-GFP, and Myr-GFP-KRASB G12V C185S were recovered in the membrane fraction. **(C)** Immunofluorescence of KRASB constructs. HEK293FT cells were transfected with pCIneoGFP-KRASB G12V, pCIneoMyr-GFP-KRASB G12V C185S, and pCIneoPalm-Myc-KRASB G12V C185S. Myc-tagged proteins were detected by anti-Myc antibody. Bar, 10 μm. Two experiments for (A) and three experiments for (B) and (C) were performed.

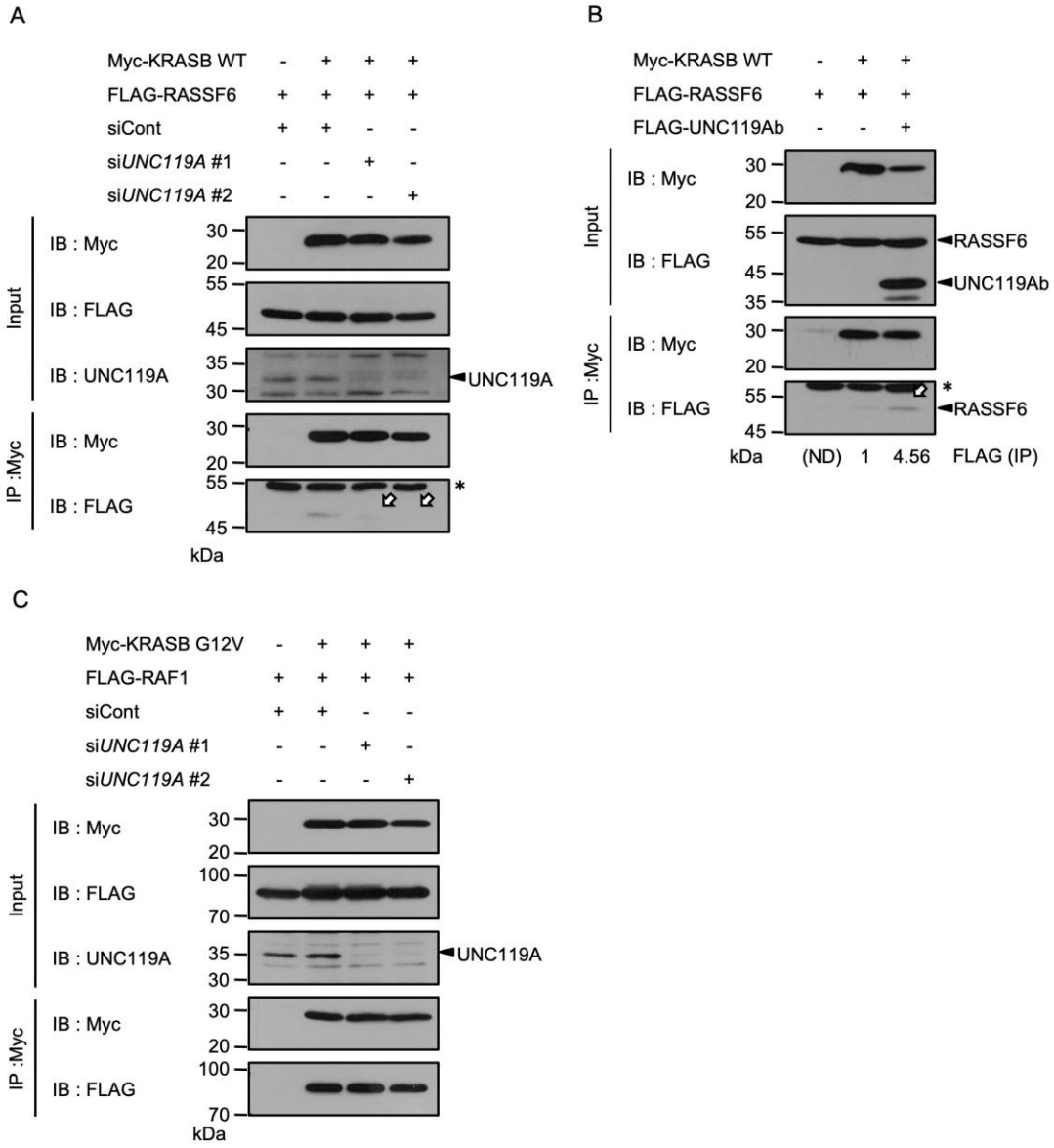

Fig. S5. ***UNC119Ab*** **silencing attenuates the interaction between RASSF6 and KRASB WT, whereas UNC119Ab co-expression enhances it. (A)** and **(B)** The experiments described as for Fig. 5A and Fig. 5B were performed by using KRASB WT instead of KRASB G12V. Asterisks indicate IgG light chain. pCIneoMyc2-KRASB WT, pCIneoFHF-RASSF6 (FLAG-RASSF6), and pCIneoFHF-UNC119Ab (FLAG-UNC119Ab), were used. *UNC119A* silencing reduced the amount of co-immunoprecipitated FLAG-RASSF6 with KRASB WT, while UNC119A co-expression increased it (white arrows). The signals were measured by use of ImageJ (the numbers under the image). **(C)** *UNC119A* was silenced by two siRNAs (si*UNC119A*#1, #2). Myc-KRASB G12V and FLAG-RAF1 were expressed in HEK293FT cells and 48 h after transfection, Myc-KRASB G12V was immunoprecipitated. Four experiments for (A) and three experiments for (B) and (C) were performed.

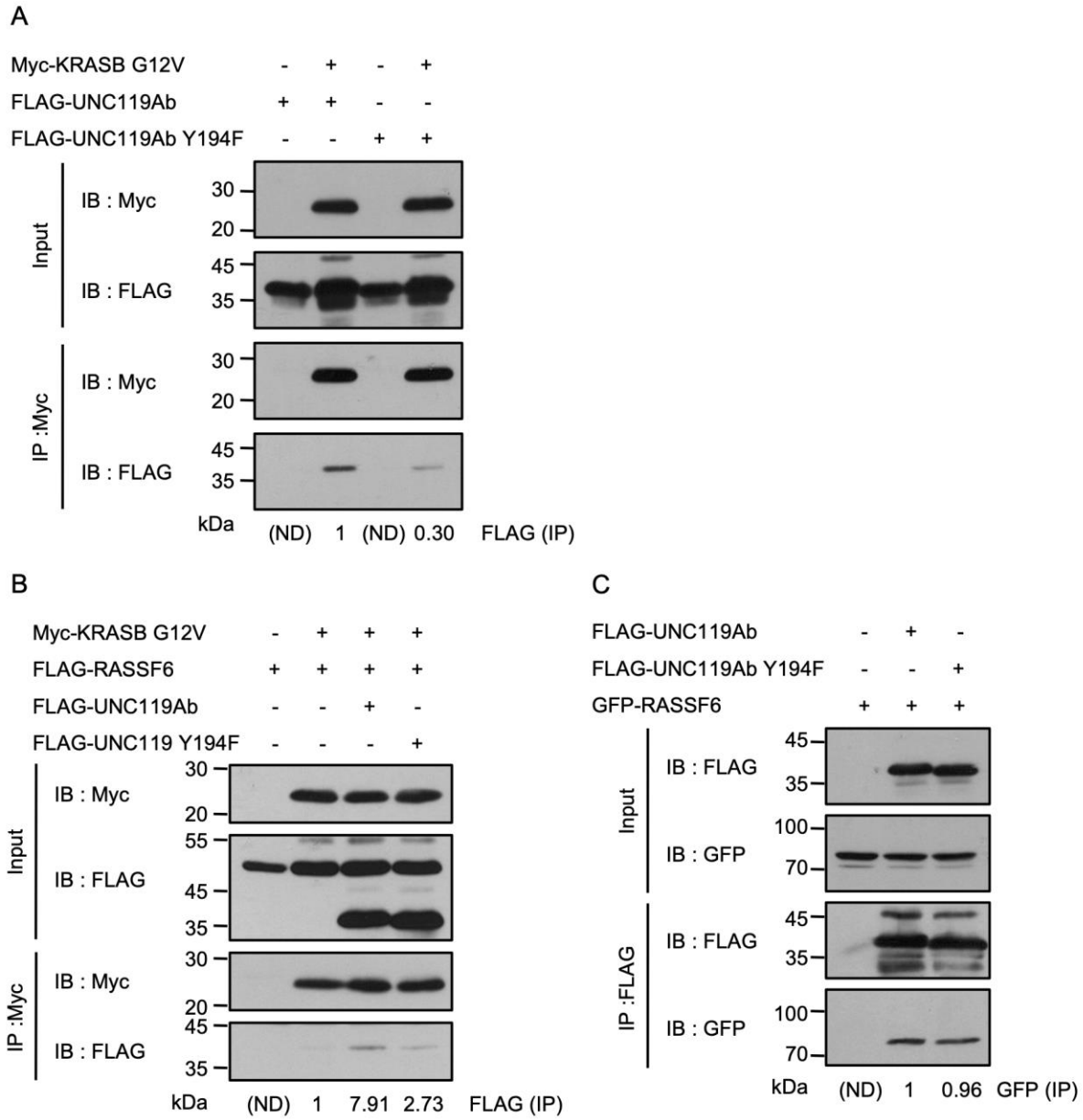

Fig. S6. UNC119Ab Y194F does not enhance the interaction between KRASB G12V and RASSF6 as efficiently as UNC119Ab. (A), **(B)**, and **(C)** HEK293FT cells were transfected with pCIneoMyc-KRASB G12V, pCIneoFHF-UNC119Ab, pCIneoFHF-UNC119Ab Y194F, pCIneoFHF-RASSF6, and pCIneoGFP-RASSF6 as indicated. The immunoprecipitation was performed by using anti-Myc antibody or anti-DYKDDDDK tag beads. UNC119Ab Y194F binds RASSF6 but does not bind KRASB G12V as efficiently as UNC119Ab. Accordingly, UNC119Ab Y194F does not enhance the interaction between KRASB G12V and RASSF6 as much as UNC119Ab. The signals were measured by use of ImageJ (the numbers under the image). Three experiments were performed for (A), (B), and (C).

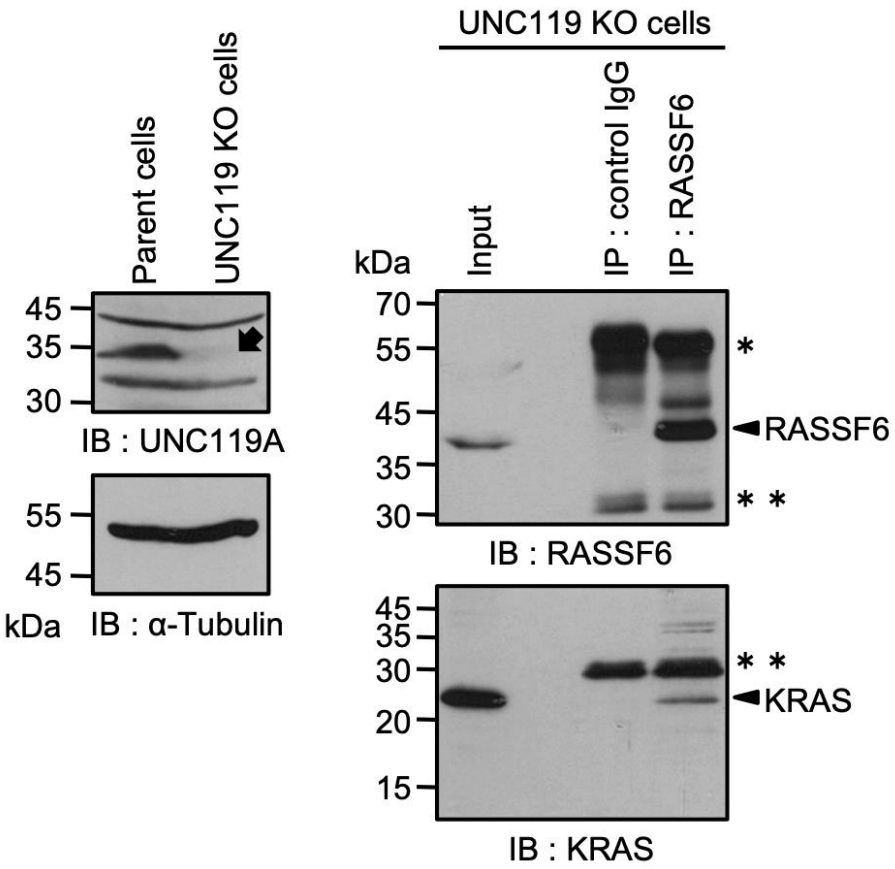

Fig. S7. RASSF6 and KRASB G12V interact with each other in UNC119A-negative background. UNC119A-knockout SW480 cells were prepared by CRISPR/Cas9. UNC119A deletion was confirmed by the immunoblotting (the left, an arrow). RASSF6 was immunoprecipitated from UNC119A-knockout SW480 cells. The immunoprecipitates were immunoblotted with anti-RASSF6 and anti-KRAS antibodies (the right). Arrowheads indicate the immunoprecipitated RASSF6 and KRAS. * and ** indicate IgG heavy and light chains, respectively. Three experiments were performed.

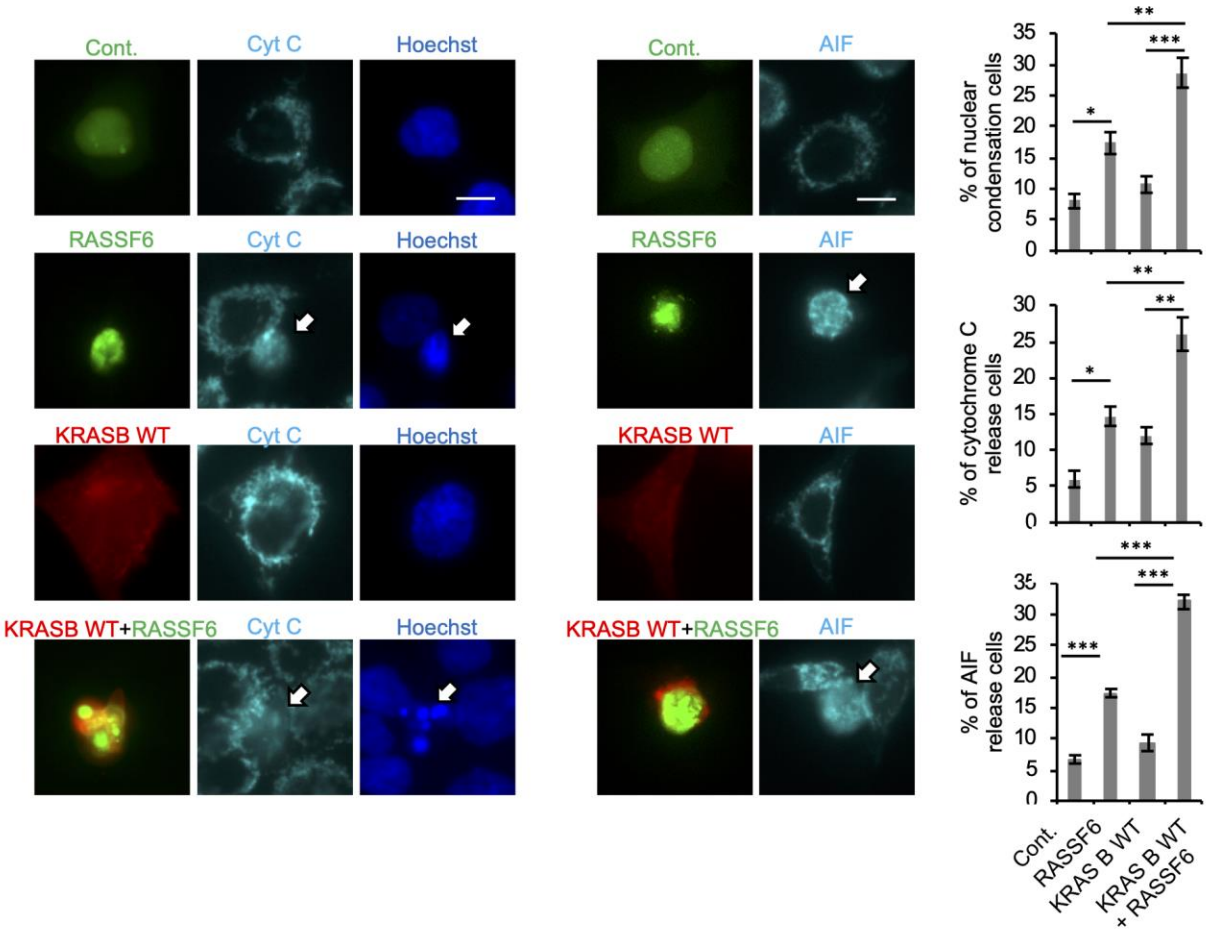

Fig. S8. RASSF6 and KRASB WT synergistically induce apoptosis. HCT116 cells were transfected with pBudCGFP-SUMO (Cont), pCIneoGFP-RASSF6 (RASSF6), and pCIneoMyc2-KRASB WT (KRASB WT). 24 h later, cells were immunostained with anti-cytochrome C or AIF antibody. Nuclei were visualized with Hoechst 33342. 50 cells were observed, and the ratios of cells with nuclear condensation, cytochrome-C release, and AIF release were calculated. The data are means with SEM. *, $P < 0.05$; **, $P < 0.01$; ***, and $P < 0.001$. Bar, 10 μm. Statistical analyses were performed with One-way ANOVA with Tukey's test. Three experiments were performed.

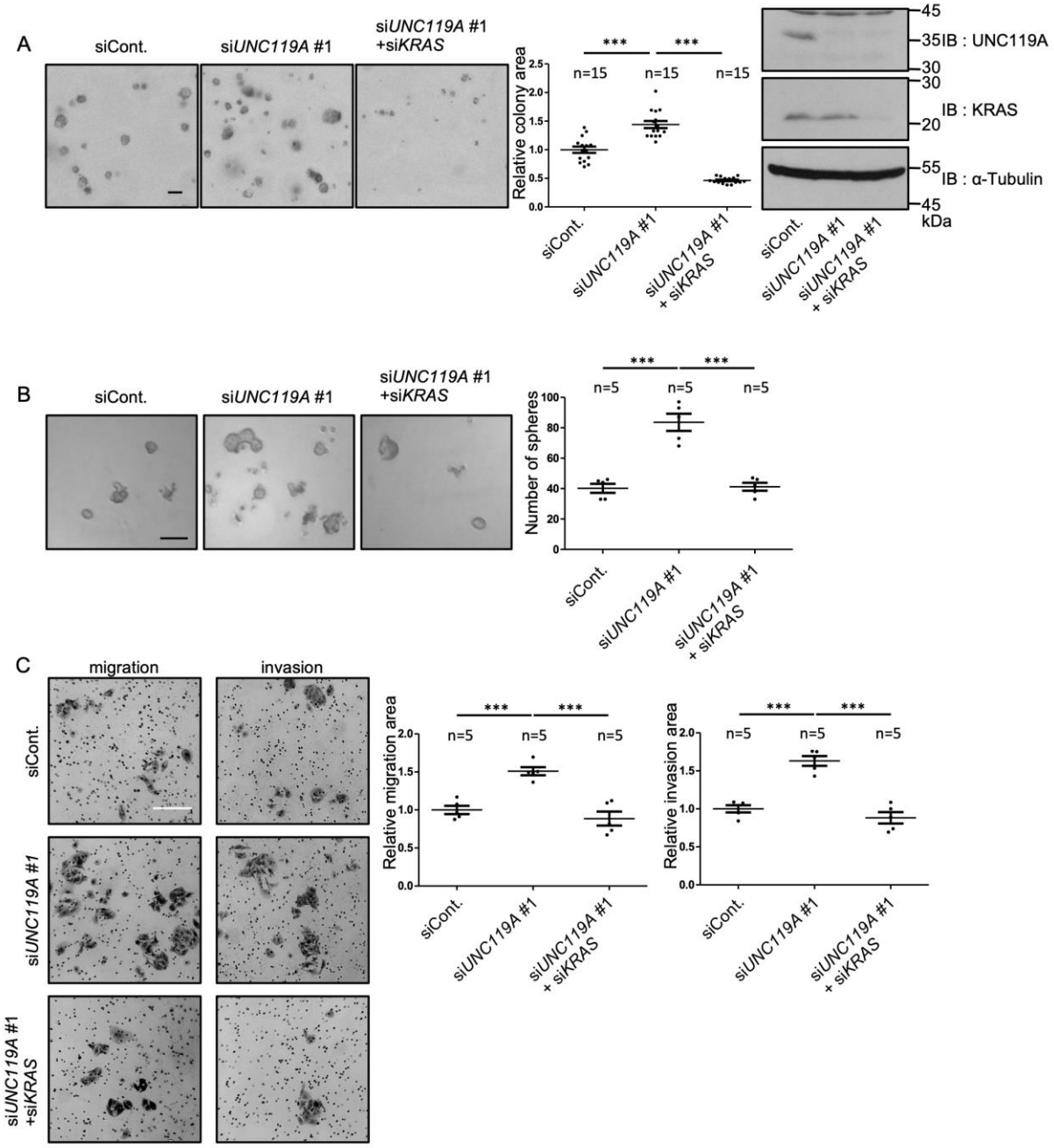

Fig. S9. *UNC119A* silencing enhances malignant transformation in human colon cancer SW480 cells.

(A), **(B)**, and **(C)** SW480 cells were transfected with control siRNA (siCont), *UNC119* siRNA (si*UNC119*#1), and *KRAS* siRNA (si*KRAS*). Soft agar colony formation, sphere formation, and transwell (migration and invasion) assays were performed as described for Fig. 9B, 9C, and 9D. The immunoblottings demonstrate the suppression of UNC119A and KRAS. ***, $p < 0.001$. Scale bars, 100 μm for (A) and (B); and 200 μm for (C). Three experiments were performed. Data are means with SEM. Numbers of samples are shown in Figures. Statistical analyses were performed with One-way ANOVA with Tukey's test.

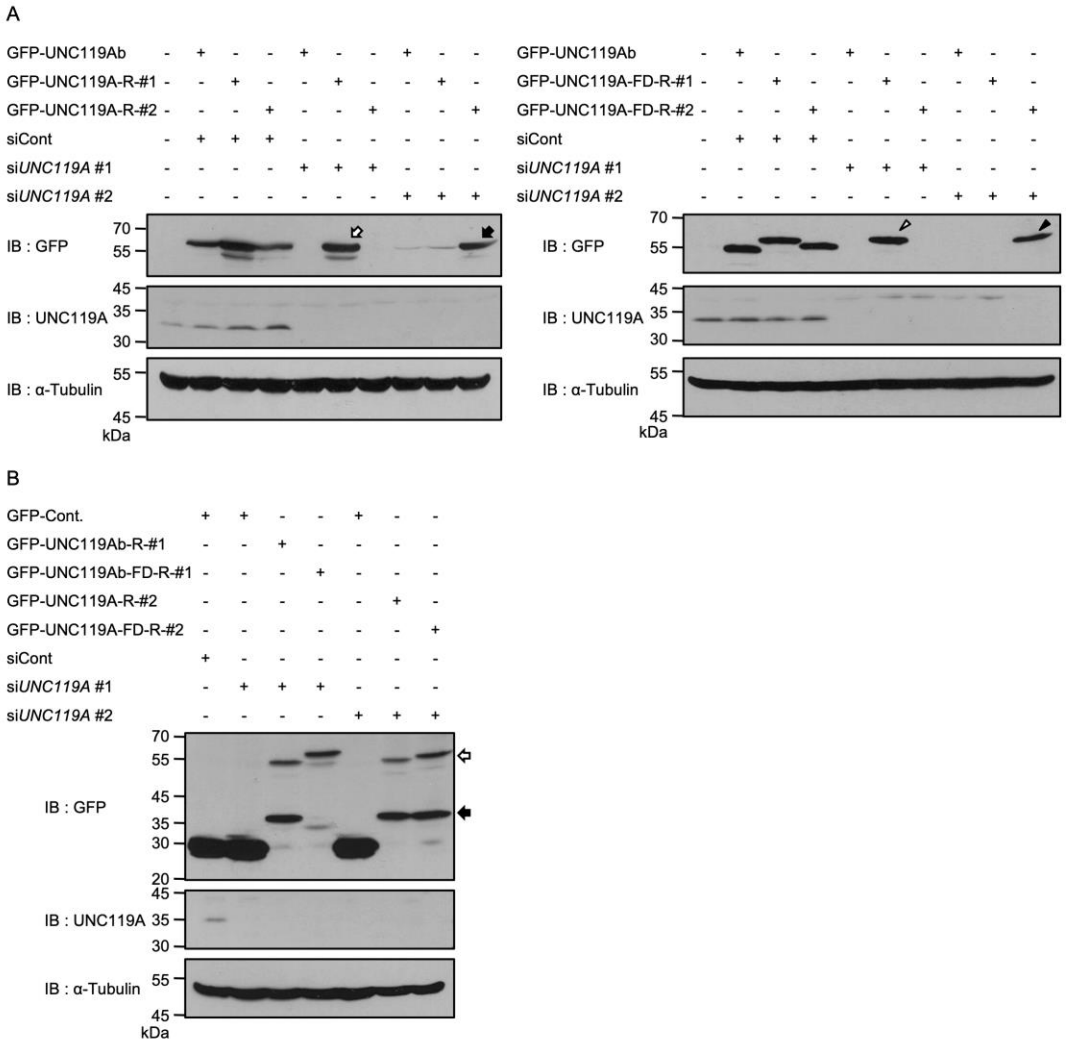

Fig. S10. Generation of A549 cells expressing RNA interference-resistant UNC119Ab and UNC119Ab F177/F179/F181D. To corroborate the results shown in Fig. 9, we generated RNA interference-resistant constructs against si*UNC119* #1 and #2 (R-#1 and R-#2) based on GFP-UNC119 and -UNC119A F177/F179/F181D (FD-R-#1 and FD-R-#2). **(A)** HEK293FT cells were transfected with these constructs and immunoblotted with the indicated antibodies. GFP-UNC119A-R-#1 (a white arrow) and -#2 (a black arrow) was expressed after transfection with si*UNC119A* #1 and si*UNC119A* #2, respectively. Similarly, GFP-UNC119A-FD-R-#1 (a white arrowhead) and -#2 (a black arrowhead) were resistant against si*UNC119A* #1 and #2, respectively. **(B)** A549 cells expressing RNA interference-resistant UNC119A proteins were generated as described in Experimental procedures. The immunoblotting demonstrates that RNA interference-resistant UNC119A proteins were expressed (a white arrow) under RNA interference, while endogenous UNC119A was suppressed. GFP antibody detected signals with about 35 kDa (a black arrow), which may be degraded products. Three experiments were performed.

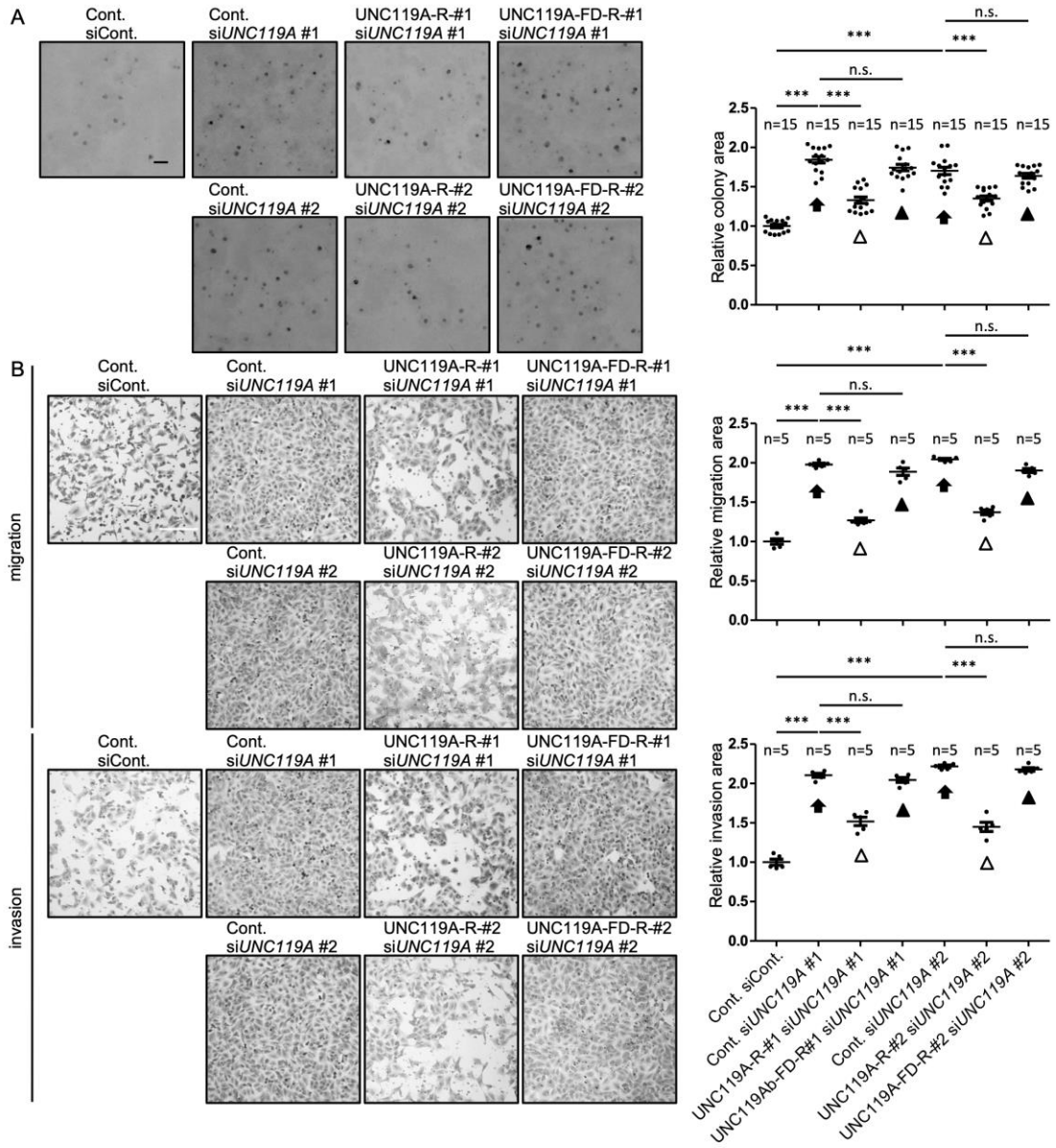

Fig. S11. *UNC119A* silencing enhances malignant transformation in human lung cancer A549 cells. By using A549 cells described in Fig. S8, soft agar colony formation in **(A)** and Transwell (migration and invasion) assays in **(B)** were performed as described for Figure 9. RNA interference with si*UNC119A* #1 and #2 promotes colony formation, migration, and invasion (black arrows). UNC119A-R-#1 and -#2 attenuated the effect of si*UNC119A* #1 and #2, respectively (white arrowheads), while UNC119A-FD-R-#1 and –#2 did not (black arrowheads). Fifteen independent fields in (A) and five wells in (B) were evaluated. Statistical analyses were performed with One-way ANOVA with Tukey's test. ***, $p < 0.001$; n.s., not significant. Three experiments were performed.

Table 1 *KRAS* mutations in cancers with *UNC119*-at various expression levels

(A) Pancreatic adenocarcinoma

UNC119	High	Middle	Low	SUM
KRAS				
Mutated	28	38	44	110
Wild	29	19	14	62
SUM	57	57	58	172

(B) Colon adenocarcinoma

UNC119	High	Middle	Low	SUM
KRAS				
Mutated	62	77	79	218
Wild	115	100	99	314
SUM	177	177	178	532

(C) Lung adenocarcinoma

UNC119	High	Middle	Low	SUM
KRAS				
Mutated	35	62	58	155
Wild	135	108	112	355
SUM	170	170	170	510

(D) Pancreatic adenocarcinoma

UNC119	High	Middle	Low	SUM
TP53				
Mutated	30	36	36	102
Wild	27	21	22	70
SUM	57	57	58	172

(E) Colon adenocarcinoma

UNC119	High	Middle	Low	SUM
APC				
Mutated	130	132	125	387
Wild	47	45	53	145
SUM	177	177	178	532

(F) Lung adenocarcinoma

UNC119	High	Middle	Low	SUM
LRP1B				
Mutated	78	50	48	176
Wild	92	120	122	334
SUM	170	170	170	510

(A) Chi-square, 9.19, p=0.0101 (B) Chi-square, 3.91, p=0.142 (C) Chi-square, 11.8, p=0.00273

(D) Chi-square, 1.59, p=0.452 (E) Chi-square, 0.914, p=0.633 (F) Chi-square, 14.6, p=0.000661

Table S2. List of oligonucleotides

Name	Sequence
H4222 linker for pCIneoMyc2	5’-aatttggcggcggcggcggcggccaattga-3’
H4223 linker for pCIneoMyc2	5’-cgcgtcaattggccgccgccgccgccgcca-3’
H4065 KRAS	5’-acgcgtatgactgaatataaacttgtggtagttgg-3’
H3250 KRASA	5’-gtcgactacattataatgcatttttttaatttt-3’
H4066 KRASB	5’-agtcgacttacataattacacactttgtctttgac-3’
H4244 HRAS	5’-acgcgtatgacggaatataagctggtggtggtg -3’
H4245 HRAS	5’-agtcgactcaggagagcacacacttgcagctcatg -3’
H4246 NRAS	5’-acgcgtatgactgagtacaaactggtggtgg-3’
H4247 NRAS	5’-agtcgacttacatcaccacacatggcaatcccata-3’
H4250 RAP1B	5’-acgcgtatgcgtgagtataagctagtcgttcttg-3’
H4259 RAF1	5’-acgcgtatggagcacatacagggagcttggaagac-3’
H4260 RAF1	5’-agtcgactagaagacaggcagcctcggggacgtgg-3’
H4019 SRC	5’- acgcgtatgggtagcaacaagagcaagcccaagg-3’
H4020 SRC	5’- agtcgacctagaggttctccccgggctggtactgg-3’
H4072 KRASA C180S	5’-aagactcctggctctgtgaaaattaaaaaatgca-3’
H4073 KRASA C180S	5’-tttaattttcacagagccaggagtcttttcttctt-3’
H4074 KRASA C186S	5’- agtcgacttacattataatggatttttttaattttc -3’
H4067 KRASB C185S	5’-agtcgacttacataattacagactttgtctttgac-3’
H4384 KRAS G60/RAP1B T61	5’-ctcgacacagcaggtacggagcaatttacagcaat-3’
H4385 KRAS G60/RAP1B T61	5’-tgtaaattgctccgtacctgctgtgtcgagaatat-3’
H4450 KRAS Q63/RAP1B F64	5’-tcaagaggagtttacagcaatgagggattt-3’
H4451 KRAS Q63/RAP1B F64	5’-ttgctgtaaactcctcttgacctgctgtgt-3’
H4452 KRAS E76/RAP1B G77	5’-gactggggagggatttgcattagtttattc-3’
H4453 KRAS E76/RAP1B G77	5’-atgcaaatccctccccagtcctcatgtact-3’
H4454 KRAS N86/RAP1B Q87	5’-cataaataatcagtccacatttaacgattt-3’

H4455 KRAS N86/RAP1B Q87	5’-atgtggactgattatttatggcaaatacac-3’
H3975 UNC119Ab Y75F	5’-caccggtgacttcctctgctcccctgaggagaata-3’
H3976 UNC119Ab Y75F	5’-aggggagcagaggaagtcaccggtgatccgctgca-3’
H3977 UNC119Ab Y84F	5’-ggagaatatcttcaagatcgactttgtcaggtttg-3’
H3978 UNC119Ab Y84F	5’-aaagtcgatcttgaagatattctcctcaggggagc-3’
H3979 UNC119Ab Y131F	5’-ctttgtccgcttccagttcacgcctgccttcctcc-3’
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H3984 UNC119Ab Y194F	5’-agggggggaagtcgaaaatgtgctcgcaggtgttct-3’
H4486 UNC119Ab K92/R94A	5’-tcaggtttgcgattgcggacatggactcaggcact-3’
H4487 UNC119Ab K92/R94A	5’-tccatgtccgcaatcgcaaacctgacaaagtcgat-3’
H4497 UNC119Ab F177/F179/F181D	5’-acgaccacgatggcgactgcatccccagcagcaag-3’
H4498 UNC119Ab F177/F179/F181D	5’-cagtcgccatcgtggtcgtcgaagcttttgagtag-3’
H4456 UNC119Ab Y194/F196D	5’-gagcacattgacgacgacccccctctctccgagga-3’
H4457 UNC119Ab Y194/F196D	5’-agagggggggtcgtcgtcaatgtgctcgcaggtgtt-3’
H4458 UNC119Ab Y137D	5’-gttcacgcctgccgacctccgcctgaggcaggtgg-3’
H4459 UNC119Ab Y137D	5’-cctcaggcggaggtcggcaggcgtgaactggtagc-3’
H3818 ABL1b delN N83	5’-acgcgtaacctttcgttgcactgtatgattttgttg-3’
H3819 ABL1b	5’-agtcgactacctctgcactatgtcactgatttccttcac-3’
H4540 rat PSD-95	5’-agctagcatggactgtctctgtatagtg-3’
H4544 rat PSD-95/Myc2	5’-ctcctcgctgataagcttctgctccatttcatcttggtagcggtattt-3’
H4520 pCIneo reverse	5’-agaacctgaaacataaaatgaat-3’
H2962 mouse Lck-GFP	5’ gctagccatggggtgcgtctgctcatcaaatatggtgagcaagggcgagg-3’
H543 pCIneo reverse	5’-caattgctcgaagcattaaccct-3’
H4610 UNC119A CR1 for CRISPR	5’-caccgcagaacggttgcccatcaac-3’

H4611 UNC119A CR1 for CRISPR	5’-aaacgttgatgggcaaccgttctgc-3’
H4686 si*UNC119A* #1 resistant	5’-gaacacttgtgaacatatttacgacttccccctct-3’
H4687 si*UNC119A* #1 resistant	5’-aaatatgttcacaagtgttcttgctgctggggatgc-3’
H4688 si*UNC119A* #2 resistant	5’-aggcactattttagaaaccagctactcaaaagctt-3’
H4689 si*UNC119A* #2 resistant	5’-ctggtttctaaaatagtgcctctcgatcatgcgga-3’

Table S3. The primary antibodies.

Antigen	Company	Catalog number	Application and Dilution
anti-α-Tubulin	Medical and Biological Laboratories	PM054	WB 1:1000
anti-GFP	Medical and Biological Laboratories	598	WB 1:1000
anti-MYC	Medical and Biological Laboratories	562	IF 1:200
anti-GFP	Santa Cruz Biotechnology	sc-9996	WB 1:1000
anti-AIF	Santa Cruz Biotechnology	sc-13116	IF 1:200
anti-p53	Santa Cruz Biotechnology	sc-126	IF 1:200 WB 1:1000
anti-HA	Sigma-Aldrich	clone 12CA5	WB 1:2
anti-DYKDDDDK-tag	FUJIFILM Wako Pure Chemical Co.	014-22383	WB: 1:1000
anti-Myc clone	American Type Culture Collection	9E10	WB 1:2
anti-mCherry	GeneTex	GTX59788	WB 1:1000
anti-Cytochrome C	BD Biomedicals	556432	IF 1:200
anti-BAX	BD Biomedicals	610982	WB 1:250
anti-p21	Abcam	ab109199	WB 1:1000
anti-KRAS	Proteintech Group	12063-1-AP	WB 1:500
anti-KRAS	Abgent	AT2650a	WB 1:500
anti-RASSF6	Proteintech Group	11921-1-AP	WB 1:1000, IP
anti-UNC119	Abnova	H00009094-M01	WB 1:1000 IP

WB=Western blotting; IF=Immunofluorescence; IP=immunoprecipitation