The Elderly's Organs are Ready for Functional Rejuvenation

Running title: Young cells' origin, SLIT2, is a new rejuvenation factor

Supplementary Movie. 1 to 2

Supplementary Table. 1 to 4

Supplementary Fig. 1 to 22

Supplementary Movie. 1 Representative Erk1/2-KTR-mClover translocation patterns for each cell type. Cells were starved for 24 hrs with serum free media before experiments. Fluorescent images were acquired every 15 sec for 30 mins with 100 msec exposure time after serum (20%) stimulation. Green: Erk1/2-KTR-mClover. Blue: nuclear region dyed by Hoechst.

Supplementary Movie. 2 Old-aged mice activity is improved significantly by administration of rmSLIT2. PBS or rmSLIT2 protein was injected intraperitoneally six times for three weeks. At the end, the recoded movement of each mouse is shown (left). Representative video of each group was displayed (right).

Supplementary Tables

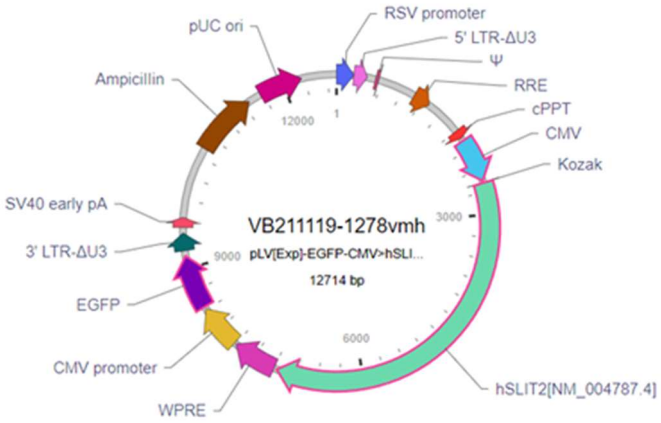
Table. 1 The real-time PCR primer sequence

Gene name	Forward Primer	Reverse Primer
Human		
p53	GCC CAA CAA CAC CAG CTC CT	CCT GGG CAT CCT TGA GTT CC
$p21^{Waf1}$	ATT AGC AGC GGA ACA AGG AGT CAG ACA T	CTG TGA AAG ACA CAG AAC AGT ACA GGG T
$p16^{INK4A}$	CTC GTG CTG ATG CTA CTG AGG A	GGT CGG CGC AGT TGG GCT CC
IL6	AAT TCG GTA CAT CCT CGA CGG	GGT TGT TTT CTG CCA GTG CC
IL1β	CTG TCC TGC GTG TTG AAA GA	TTG GGT AAT TTT TGG GAT CTA CA
IL8 (CXCL8)	CTG GCC GTG GCT CTC TTG	CCT TGG CAA AAC TGC ACC TT
MIP1α (CCL3)	TGTTGCCAAACAGCCACAC	CAGAGCAAACAATCACAAACACAC
MIP3a (CCL20)	ATGTGCTGTACCAAGAGTTT	TTACATGTTCTTGACTTTTT
MCP2 (CCL8)	TATCCAGAGGCTGGAGAGCTAC	TGGAATCCCTGACCCATCTCTC
CCL2	AGC AGC AAG TGT CCC AAA GA	TTG GGT TTG CTT GTC CAG GT
CCL5	TCC CAC AGG TAC CAT GAA GGT C	GCA ATG TAG GCA AAG CAG CAG
CXCL1	CTT GCC TCA ATC CTG CAT C	CCT TCT GGT CAG TTG GAT TTG
SAA1	TTT CTG CTC CTT GGT CCT GG	CTC TGG CAT CGG TGA TCA CT
SLIT2	GTC AAT GAC CTA CTG GCC TCA TTC	TGA TTG CTA CAC ACC ATT TCG TTT C
CXCL12	TGC CAG AGC CAA CGT CAA G	CAG CCG GGC TAC AAT CTG AA
IL1rα	AGA GGA AAA CAA ACC CAC AAG G	CTG GCC GGT GAC ATT ACA GAT
IL11	GGA CCA CAA CCT GGA TTC CCT G	AGT AGG TCC GCT CGC AGC CTT
IL18BP	GTG TCC AGC ATT GGA AGT GAC C	GGA GGT GCT CAA TGA AGG AAC C
Caveolin1	CCA AGG AGA TCG ACC TGG TCA A	GCC GTC AAA ACT GTG TGT CCC T
Caveolin2	TTC TCT TTG CCA CCC TCA GCT G	GAA GCA TCG TCC TAC GCT CGT A
KPNA2	CTG TTG GCT CTC CTT GCA GTT C	GCA GGA TTC TTG TTG CGG CAA AG
KPNB1	CTG CTT CCT GAA GCT GCC ATC A	CTT CAG CCA GAC TGG AGA AAG C
MMP1	AAG CGT GTG ACA GTA AGC TA	AAC CGG ACT TCA TCT CTG
MMP3	GGA CAA AGG ATA CAA CAG GGA CCA	GAA CCG AGT CAG GTC TGT GAG TG
MMP9	CAC GAC GTC TTC CAG TAC CGA GA	CAT AGG TCA CGT AGC CCA CTT GGT
Fbox30	ACA AAT GGA GAC TGT GTG GCA TC	GCC ATT AGG CAA AGC ACT GGA TG
Fbox32	CAC TGG TCC AAA GAG TCG GCA A	GCA CAA AGG CAG GTC AGT GAA G
Trim63	AAG CCA GTG GTC ATC TTG CCG T	CTC CAG ACA TGG ACA CTG AGC T
Col4A1	TGT TGA CGG CTT ACC TGG AGA C	GGT AGA CCA ACT CCA GGC TCT C
Col4A2	GGA TAA CAG GCG TGA CTG GAG T	CTT TGC CAC CAG GCA GTC CAA T
Col4A3	GGA CAA AGG AGA ACC AGG TCT C	AGT GCT GCC CAA ATC TCC TCT G
Col3A1	TGG TCT GCA AGG AAT GCC TGG A	TCT TTC CCT GGG ACA CCA TCA G
Col1A1	GAT TCC CTG GAC CTA AAG GTG C	AGC CTC TCC ATC TTT GCC AGC A
AQP3	CCG TGA CCT TTG CCA TGT GCT T	TTG TCG GCG AAG TGC CAG ATT G
NHE2	AAG GAA GGT CAC GTC CAG TG	TGT CTC TCA CTT GTG TCG GC
NHE3	TCT CGG CCA TCG AGG ACA TA	ACA TTC AGG ATC CGG TCT CG
ATP1A1	GGC AGT GTT TCA GGC TAA CCA G	TCT CCT TCA CGG AAC CAC AGC A
SCNN1A	GTG CCT ACA TCT TCT ATC CGC G	GTC TGA GGA GAA GTC AAC CTG G
SFTPD	AAG TGG GCT TCC AGA TGT TG	CTG TGC CTC CGT AAA TGG TT
SOX2	AGG GCC GGA CAG CGA ACT G	TTT GCA CCC CTC CCA TTT C
OCT4	CCT GAA GCA GAA GAG GAT CAC C	AAA GCG GCA GAT GGT CGT TTG G
TNF	CTC TTC TGC CTG CTG CAC TTT G	ATG GGC TAC AGG CTT GTC ACT C
SBLC	CACACATCTACTCTCTTACAGTTCTAT	TTATCTGGGAGGACCAATGTAATC
SAL RNA1	TGCATGTGTGTGTGTGTGTG	CTCTGGGAATCTGGAACCAA
ROR	GCC TGA GAG TTG GCA TGA AT	AAA ACC TCA CTC CCA TGT GC
MYH2	GAC AGC CAA GAA GAG GAA ACT GG	ACC TGC CAT CTC TTC TGT GAG G
MYBPC1	CCT ATG AGG TCC GCA TCT TTG C	GTC GTG TCA GTG ACA GAG TCC A
ROBO1	GGC CCC ACT CCC CCT GTT CG	TCC TCT TCT GGC GCA TCC GTA TCC
ROBO2	GTT TGT GTT GCG AGG AAC TAT CT	GTT TTG TCG GAA GTC ATC TCG TA
ROBO3	CAG TGT CCG ATG GAA GAA GG	GTC CAT CTC CTG CAC ATT GG
ROBO4	GAC ACT TGG CGT TCC ACC TC	AGA GCA AGG AGC GAC GAC AG
Actin	CCC TGG CAC CCA GCA C	GCC GAT CCA CAC GGA GTA C
Mouse		
NHE3	TCT GTT TGT CAG CAC CAC TCT C	TCA CGA TGC TCG CTC CTC TTC
SCNN1A	TGA TGG TGG CTT CAA CGT GAG G	AGT GCA GTC TCC GTA GTT GCC T
Actin	TTG AAC ATG GCA TTG TTA CCA ACT	TCA AAC ATG ATC TGG GTC ATC TTT

Table. 2 SLIT2 lentivirus plasmid sequence

Vector ID	VB211119-1278vmh
Vector Name	pLV[Exp]-EGFP-CMV>hSLIT2[NM_004787.4]

Vector Map



hSLIT2 sequence

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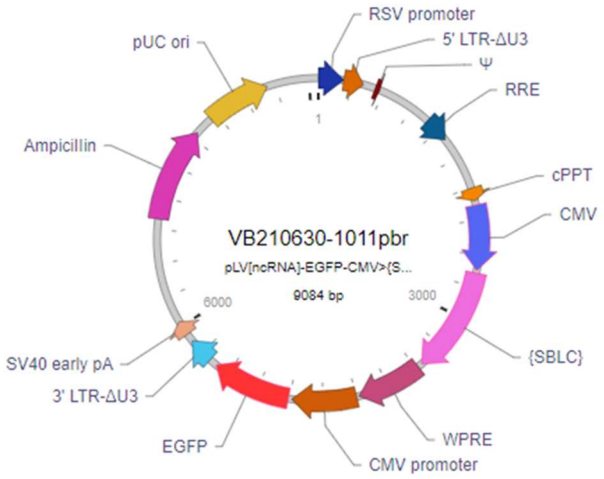
Table. 3 SBLC lentivirus plasmid sequences

Vector ID	VB210630-1011pbr
Vector Name	pLV[ncRNA]-EGFP-CMV>{SBLC}

Vector Map



SBLC sequence

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106 **Table S4. The shRNA sequences**

Gene	Target Sequence
shSLIT2	#1; ACTAGAGAGACTGCGTTTAAA
shp21	#1; CGCTCTACATCTTCTGCCTTA
shSAA1	#1; CTGACATGAGAGAAGCCAATT
	#2; CATCGGCTCAGACAAATACTT

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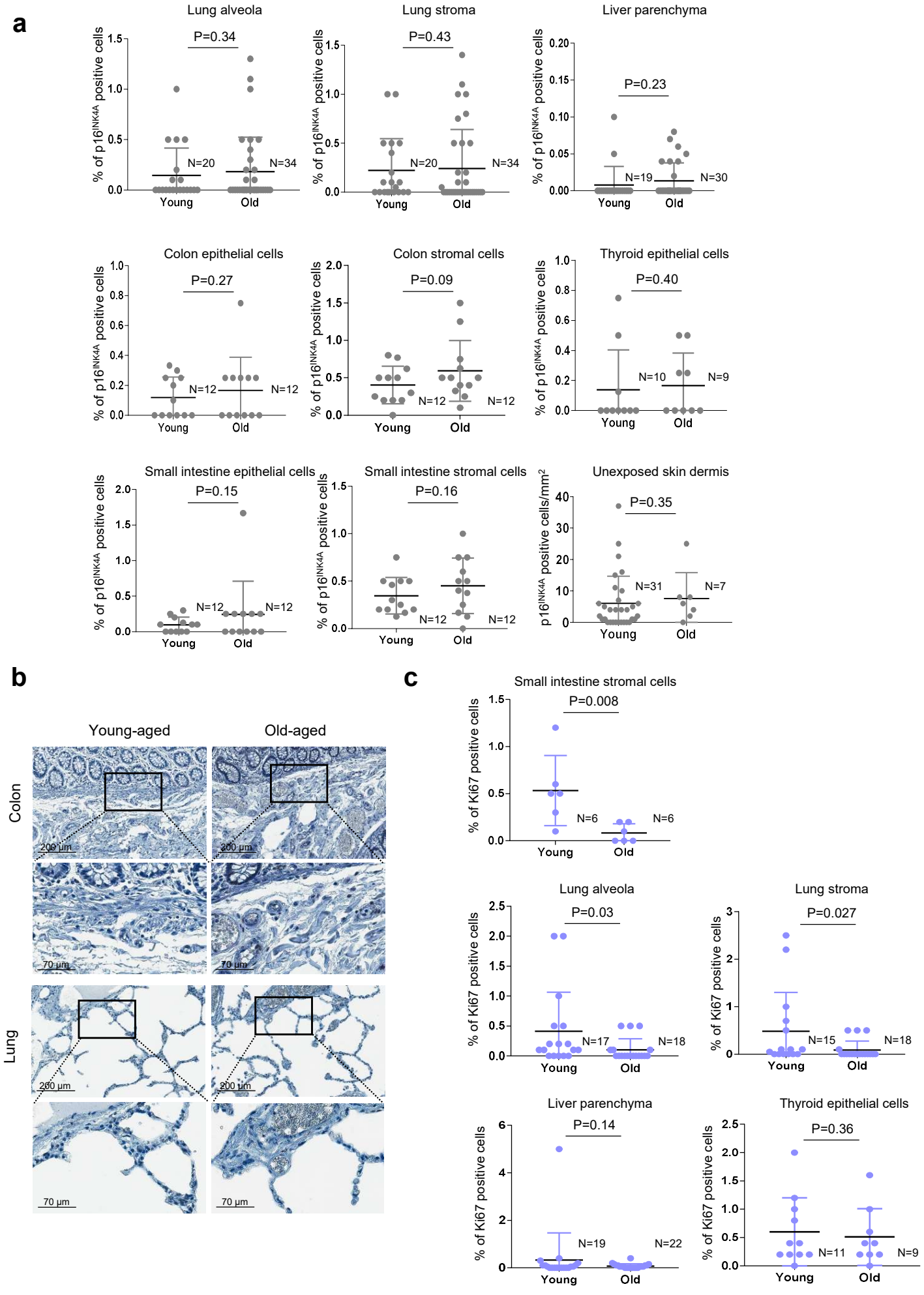
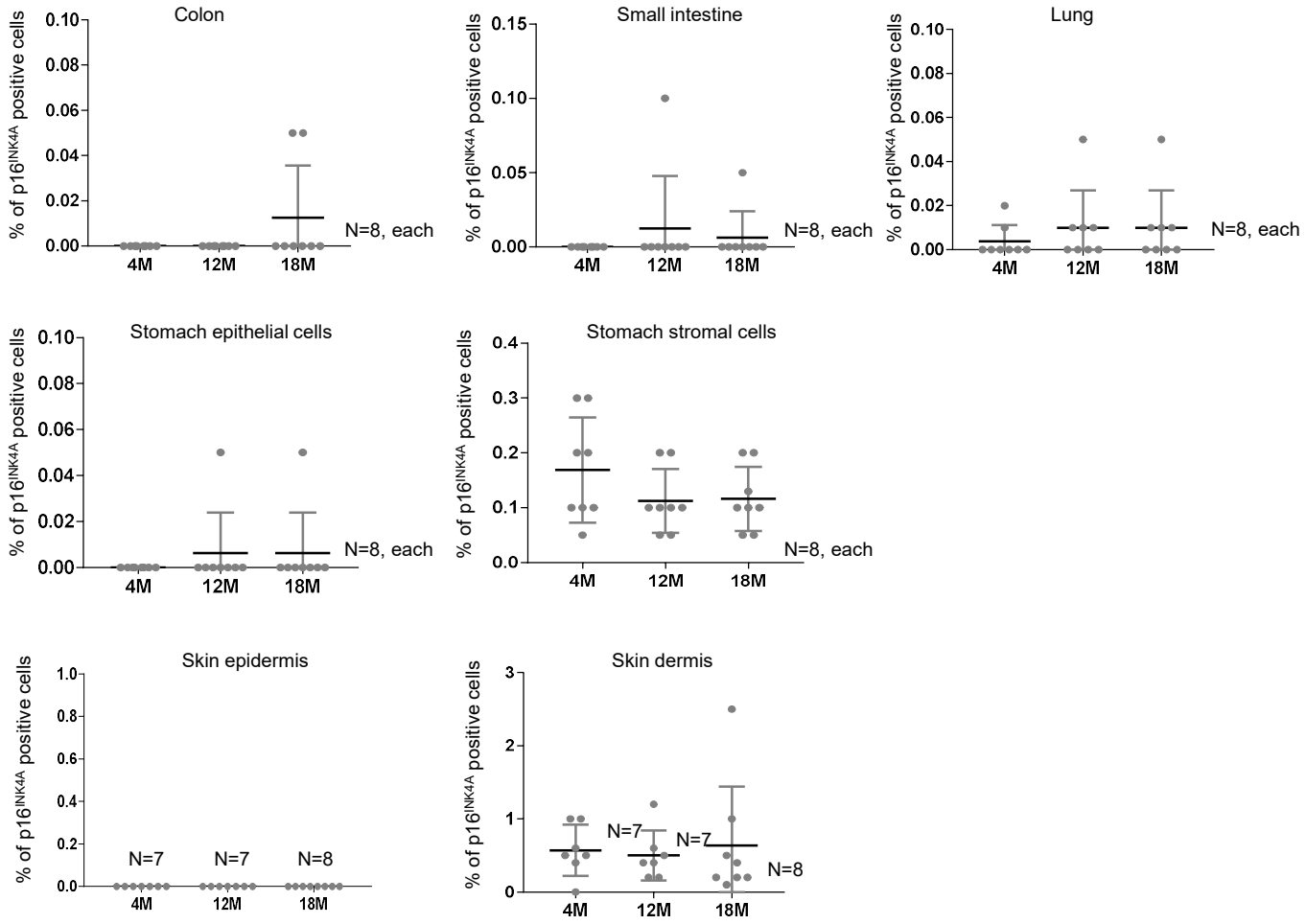
Supplementary Fig. 1



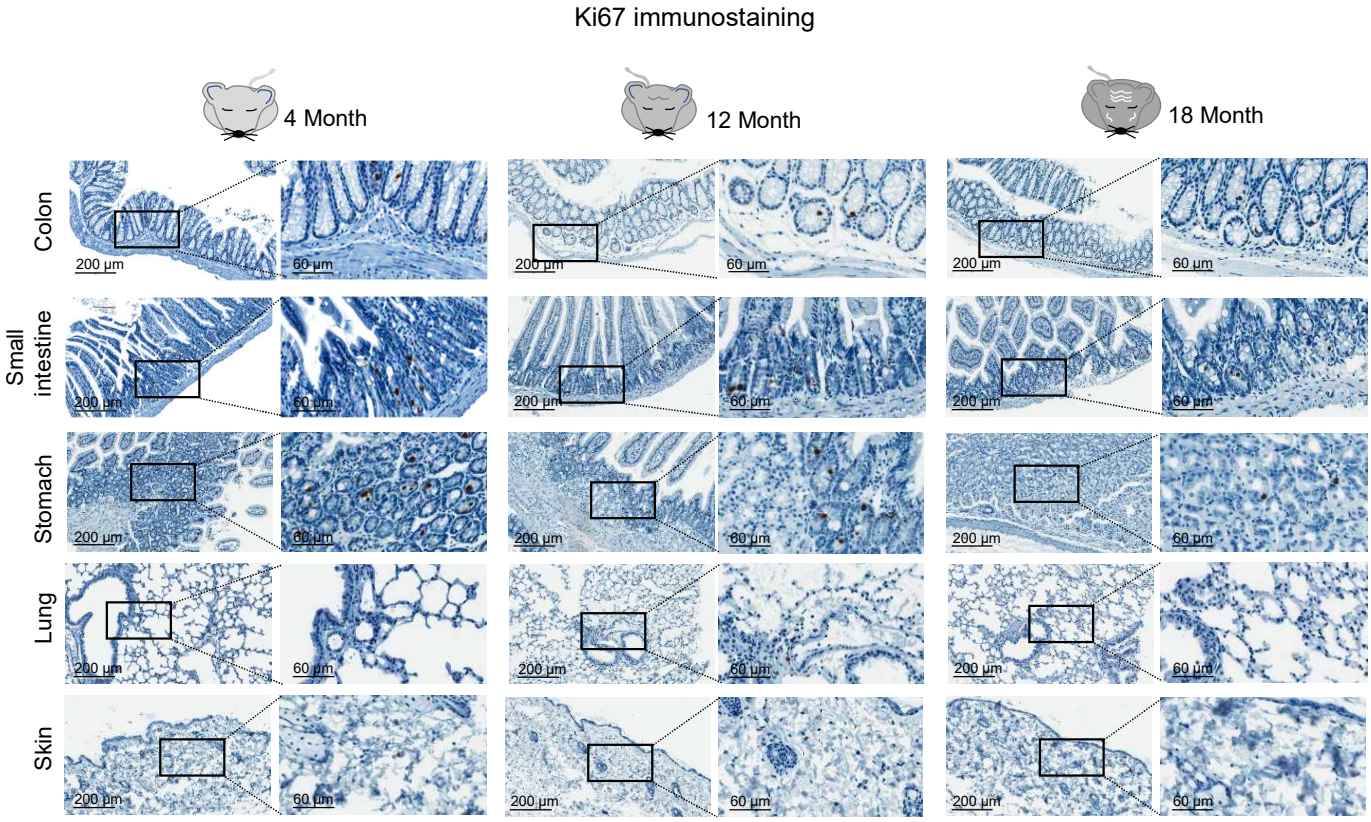
Supplementary Fig. 1 p16^{INK4A}, trimethyl-H3K9 and Ki67 expression in tissue from elderly and young subjects. Immunostained p16^{INK4A} (**a**) and Ki67(**c**) positive cells were counted in the epithelial or stromal region of the specified human tissues and presented in a dot graph. The *p* value was calculated using the Mann-Whitney U test. **b** Trimethyl-H3K9 immunostaining in human colon and lung tissues.

Supplementary Fig. 2



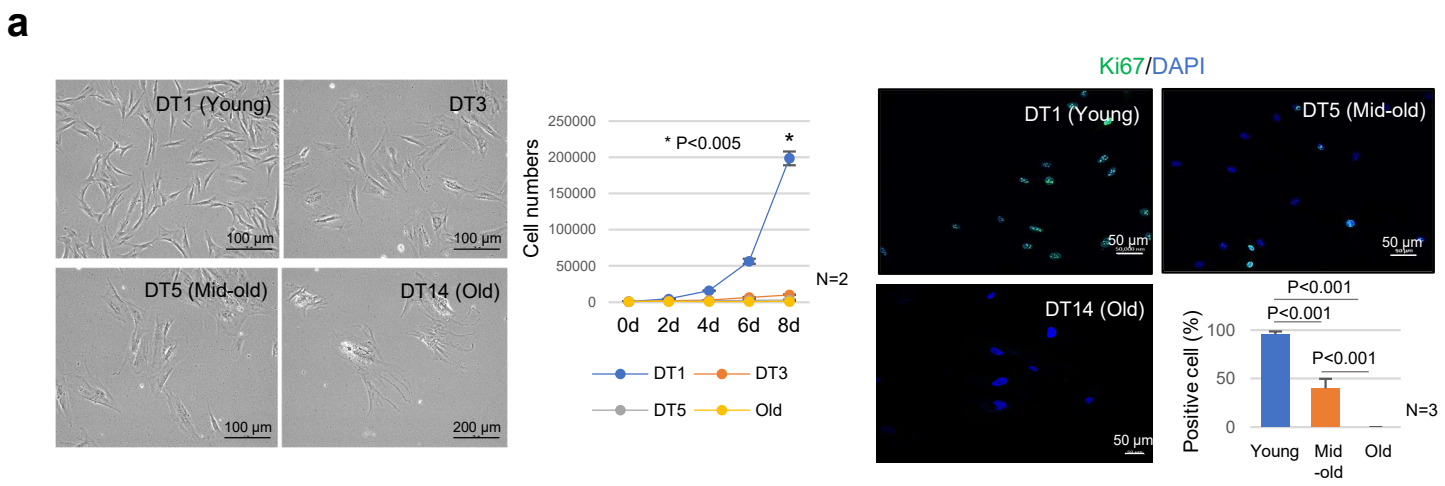
Supplementary Fig. 2 p16^{INK4A} positive cells were sparsely identified in tissue from aged mice. Immunostained p16^{INK4A} positive cells were counted in the specified male mouse tissues and presented in a dot graph. The *p* value was calculated using the Mann-Whitney U test.

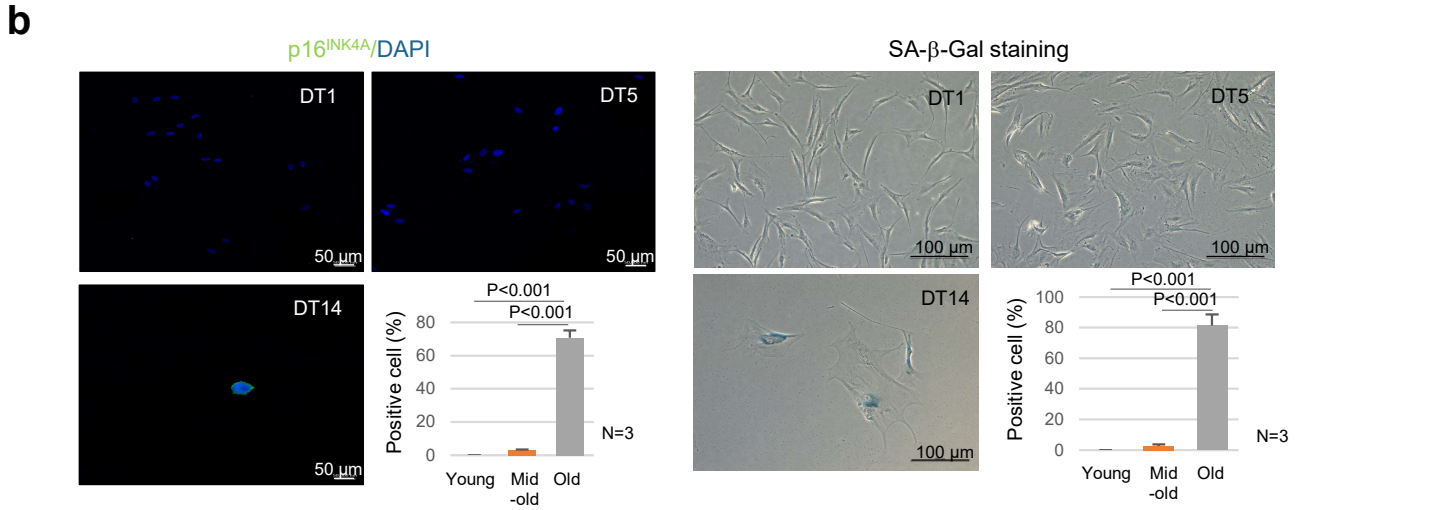
Supplementary Fig. 3

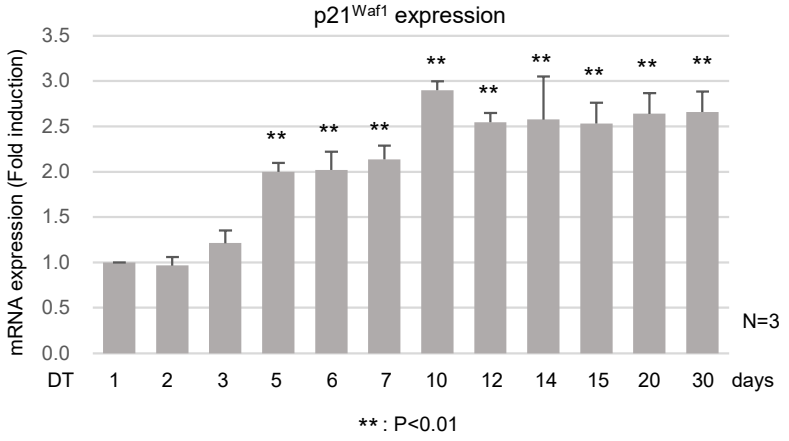


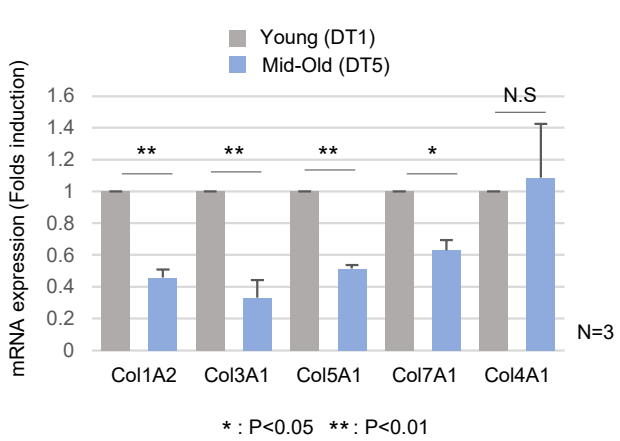
Supplementary Fig. 3 Ki67 expression in old-aged mouse tissues. Ki67 immunostaining in the specified tissues from young (4-month-old) and old (12- and 18-month-old) male mice.

Supplementary Fig. 4



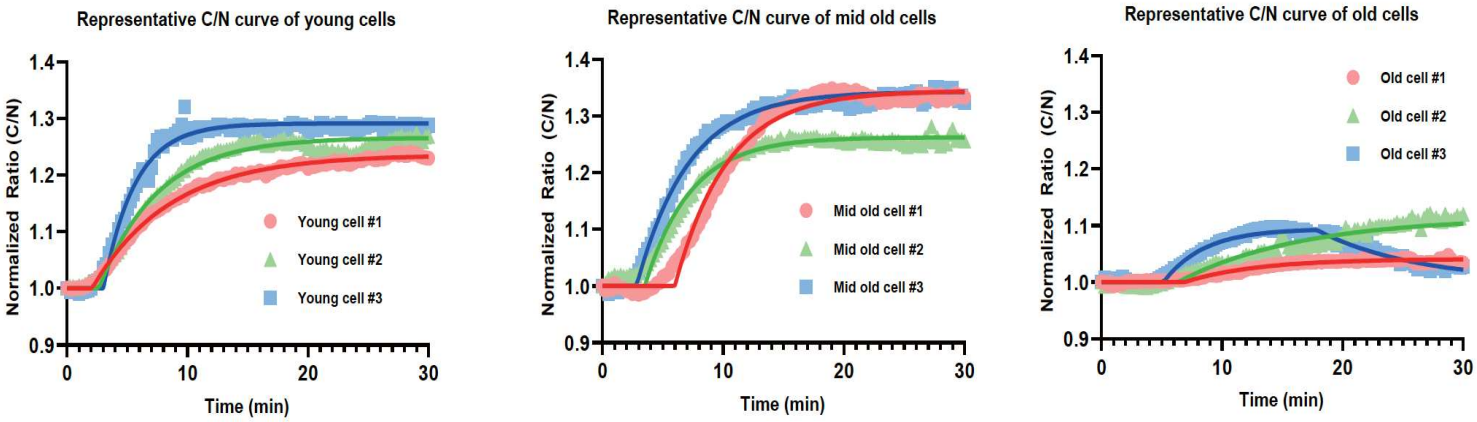






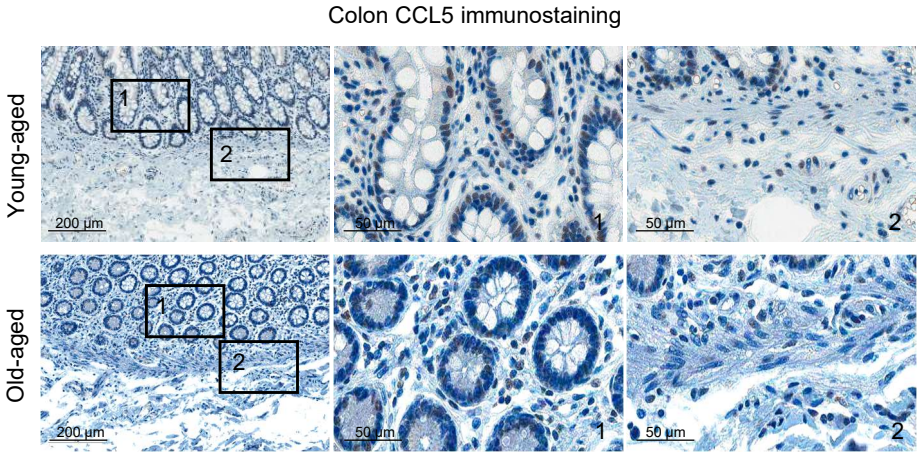
Supplementary Fig. 4 Characterization of young, mid-old, and old fibroblasts. a Representative morphology images of young (DT1), mid-old (DT5), and fully senescent (DT14) fibroblasts (left). Cell proliferation was determined by cell growth rate (middle) and Ki67 immunostaining (right). Cell growth rates were measured over a period of 8 days. At the beginning of the experiment, 1×10^3 cells were plated, and two independent experiments were conducted. The percentage of Ki67-positive cells was calculated in 134 cells per group. A representative Ki67 fluorescence image is shown and representative of three independent experiments. **b** $p16^{INK4A}$ expression in mid-old and fully senescent cells. Fibroblasts were immunostained with p16 ^{INK4A} antibody (left, *n* = 164) and SA-β-gal staining (right, *n* = 255). The percentage of positive cells was calculated and representative of three independent experiments. **c** $p21^{Waf1}$ mRNA level in a cDNA microarray performed during replicative senescence is presented as a bar graph. **d** Collagen (Col1A2, Col3A1, Col5A1, Col7A1, and Col4A1) mRNA levels in young (DT1) and mid-old (DT5) fibroblasts. The *p* value was calculated using the Mann-Whitney U test.

Supplementary Fig. 5



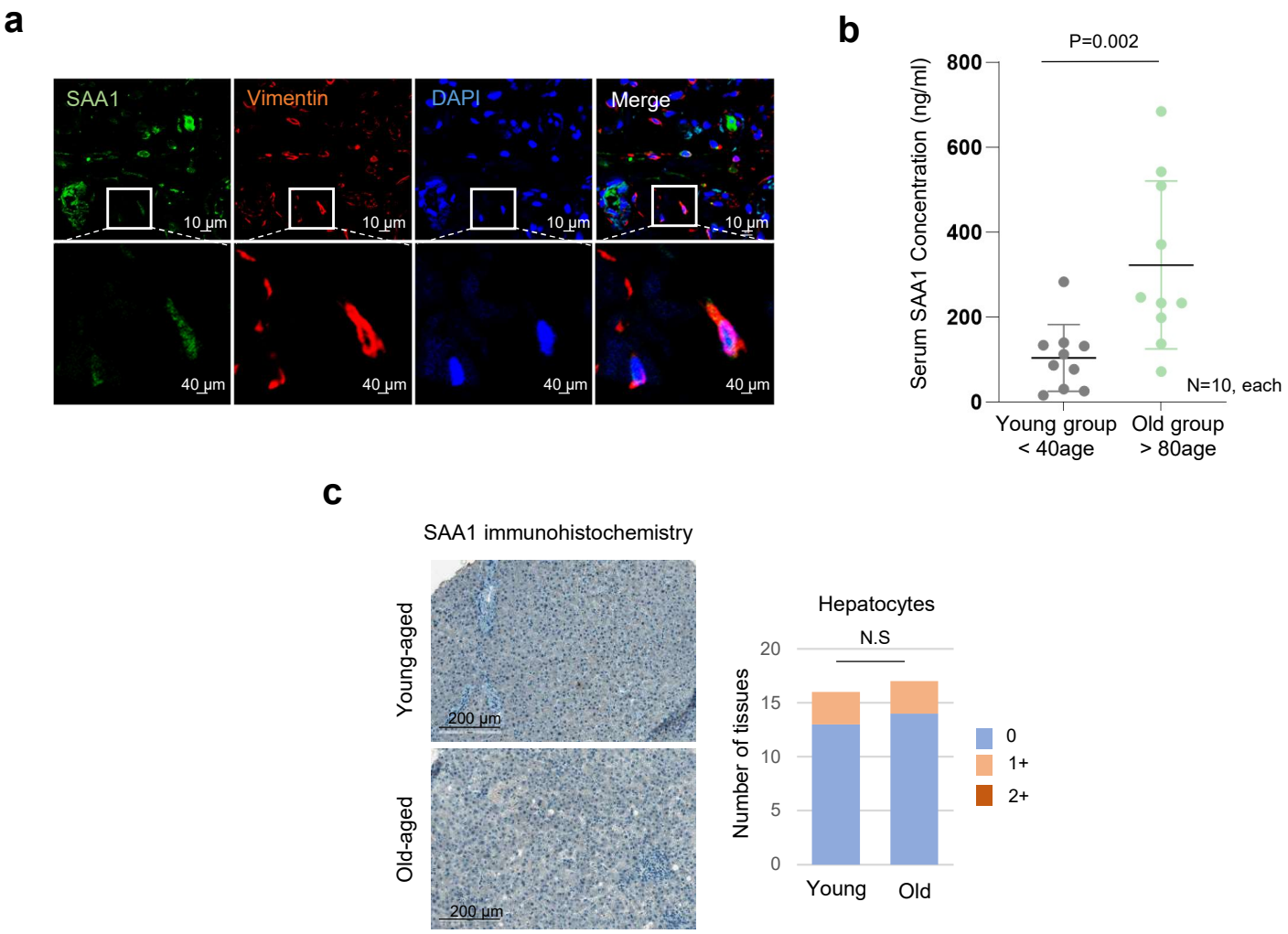
Supplementary Fig. 5 Representative Erk1/2-KTR-mClover translocation C/N curves for each cell type. Cells were starved for 24 hrs with serum free media prior to beginning experiments. Fluorescent images were acquired every 15 sec for 30 mins with 100 msec exposure time after serum (20%) stimulation.

Supplementary Fig. 6



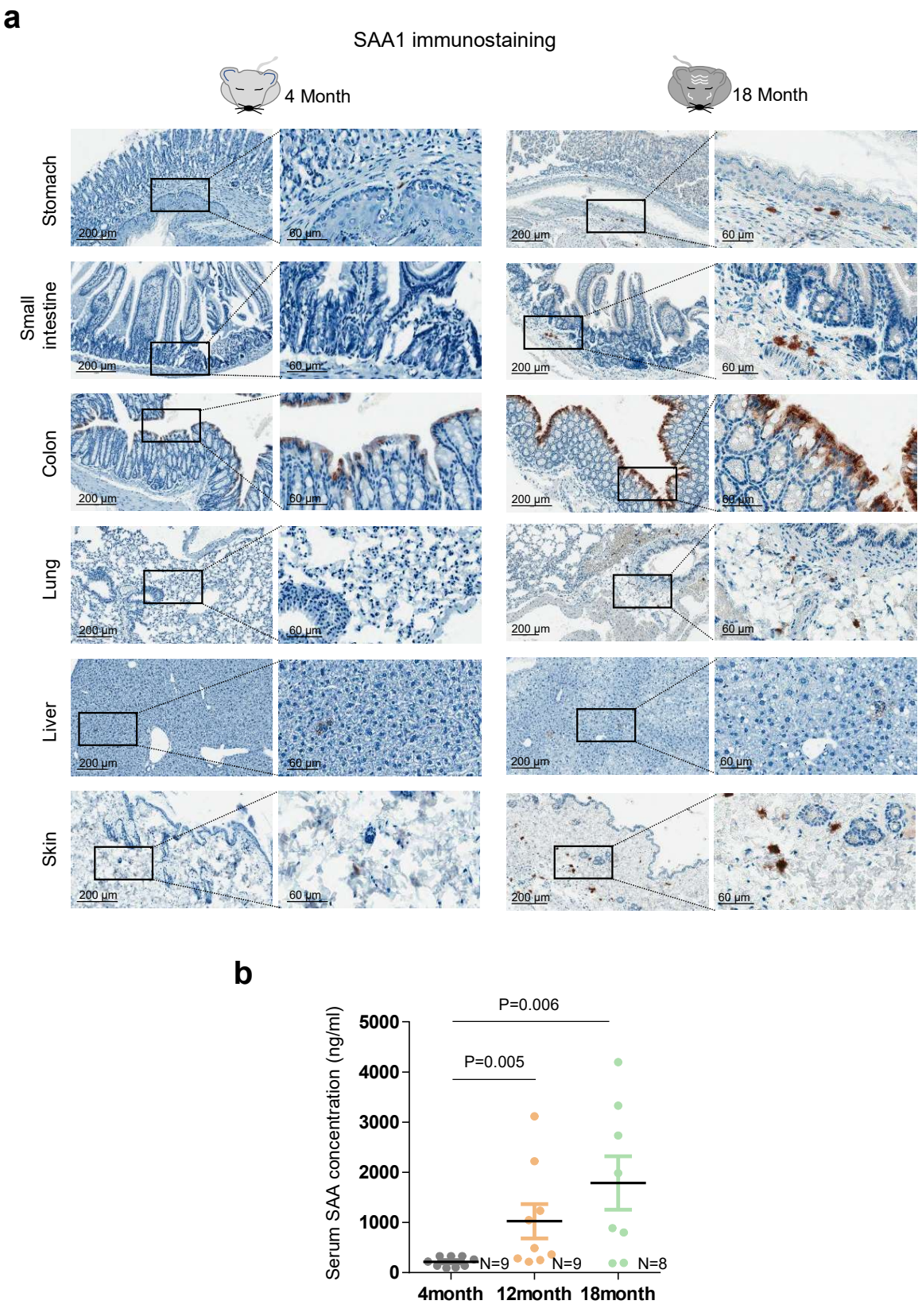
Supplementary Fig. 6 CCL5 protein expression in colon tissue from elderly subjects. CCL5 immunostaining in young and aged human colon tissues. “1” and “2” indicate high magnification views of the original figure. Image “1” primarily depicts the epithelium and image “2” depicts a stroma-rich region.

Supplementary Fig. 7



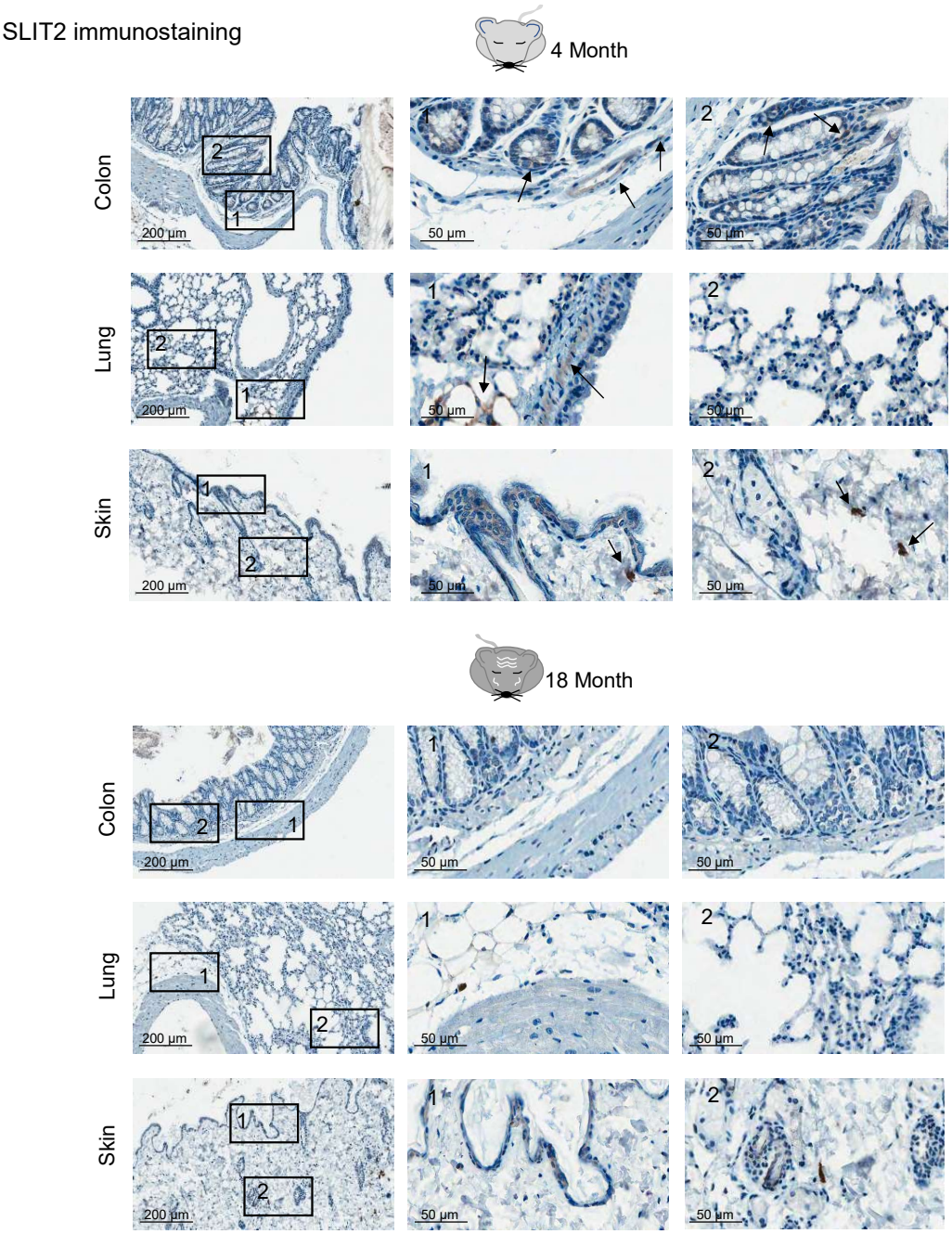
Supplementary Fig. 7 SAA1 expression in human tissues and blood. a Immunofluorescence staining of SAA1 (green) and vimentin (red) was performed in tissues from elderly subjects. DAPI (blue) was used to label nuclei. **b** Determination of SAA1 concentration in human serum from patients with benign or malignant diseases. The *p* value was calculated using the Student *t*-test. **c** Human liver tissue was immunostained with SAA1 antibody. SAA1 expression status in hepatocytes was graded as 0, +1, or +2 according to expression level. The *p* value was calculated using a one-way ANOVA.

Supplementary Fig. 8



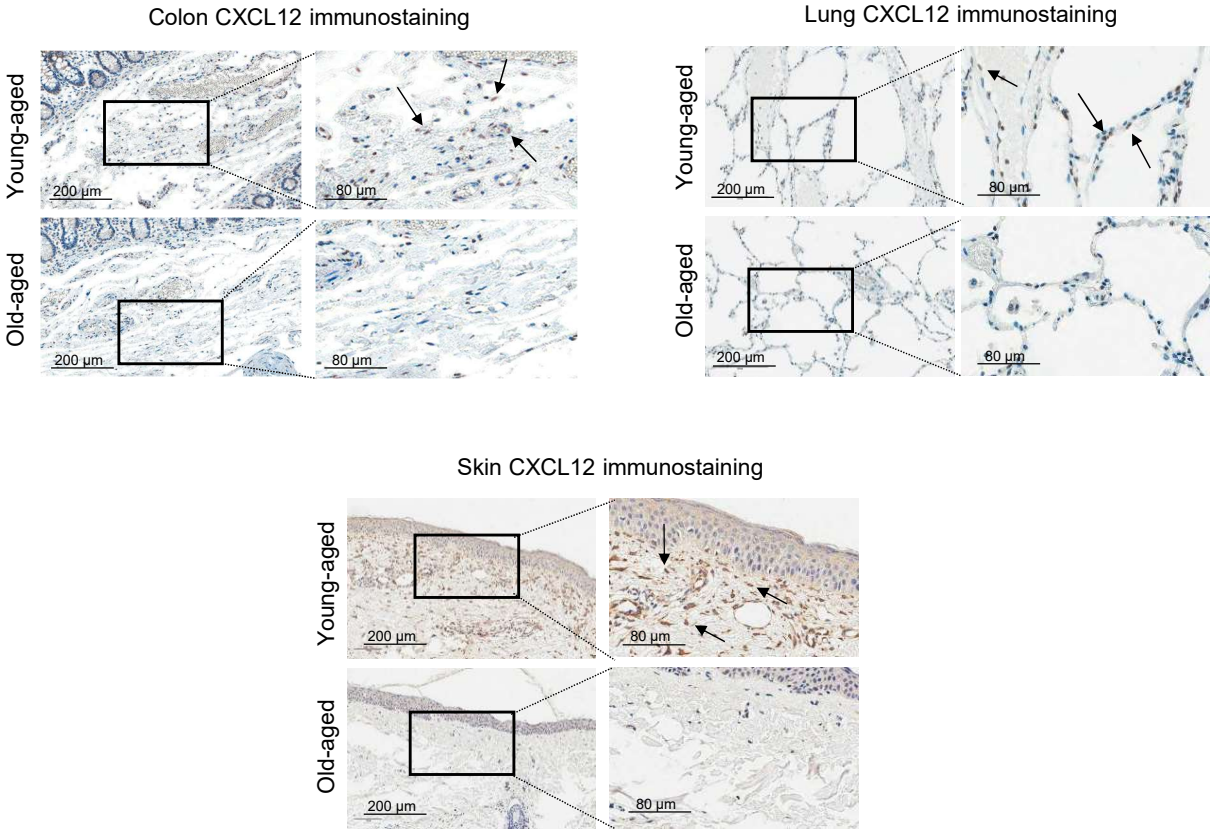
Supplementary Fig. 8 SAA1 expression in old-aged mice. a SAA1 immunostaining in the specified tissues from young (4-month-old) and old (18-month-old) male mice. **b** Mouse serum SAA protein was measured in 4-,12-, and 18-month-old male and female mice using an ELISA. The *p* value was calculated using the Mann-Whitney U test.

Supplementary Fig. 9



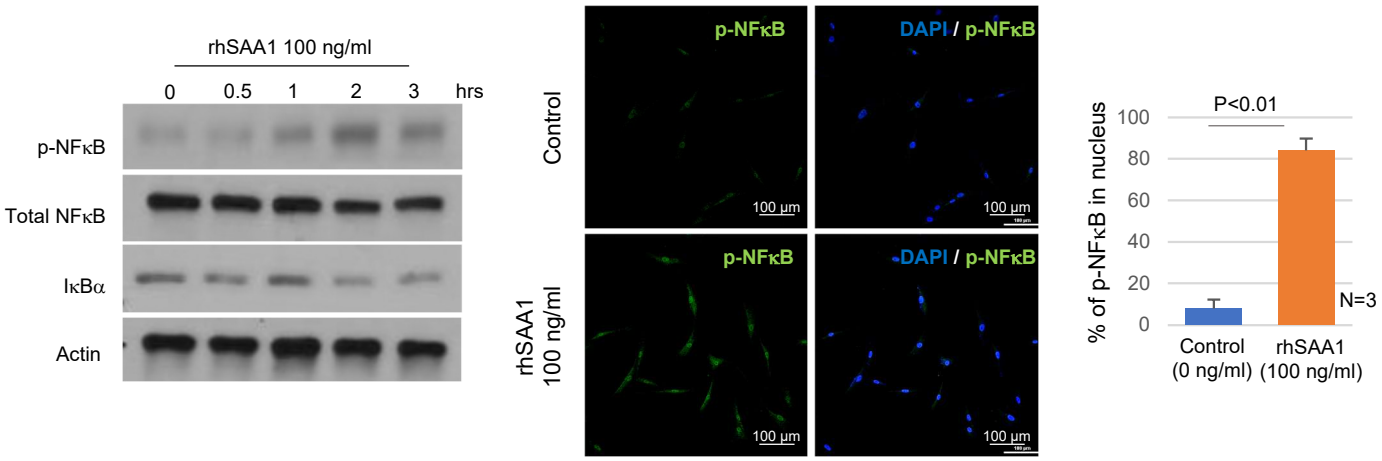
Supplementary Fig. 9 SLIT2 expression in old-aged mouse tissues. SLIT2 immunostaining in the specified tissues from young (4-month-old) and old (18-month-old) male mice. Arrow indicates SLIT2 expressing cells.

Supplementary Fig. 10



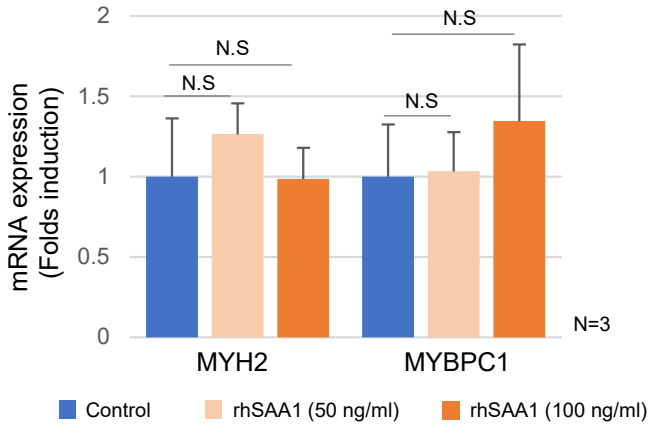
Supplementary Fig. 10 CXCL12 expression in tissue from elderly subjects. CXCL12 immunostaining in the stromal region of colon, lung, and skin tissues from elderly and young subjects. Arrow indicates CXCL12 expressing cells.

Supplementary Fig. 11



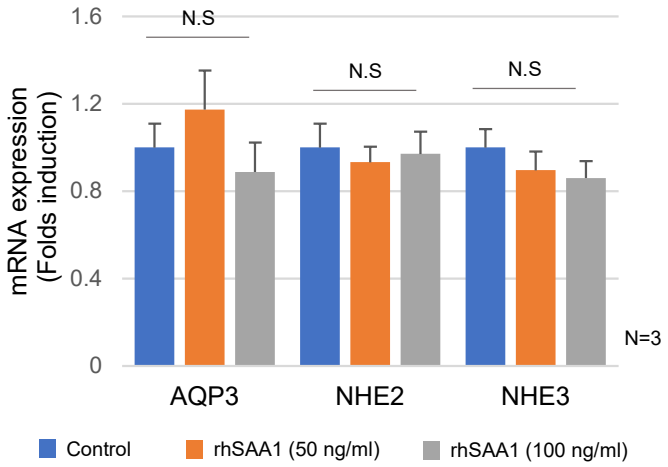
Supplementary Fig. 11 SAA1 activation of NFκB signaling. Young fibroblasts were treated with 100 ng/ml rhSAA1 for the indicated times, and immunoblotting was performed to assess activation of NFκB signaling (left). To examine the localization of phosphorylated NFκB, fibroblasts were treated with 100 ng/ml rhSLIT2 for 30 mins, and ICC analysis was performed (middle). The nuclear p-NFκB positive cells were quantified (control n = 321, 100 ng/ml n = 285) and are presented as bar graph (right). The *p* value was calculated using Student *t*-test.

Supplementary Fig. 12



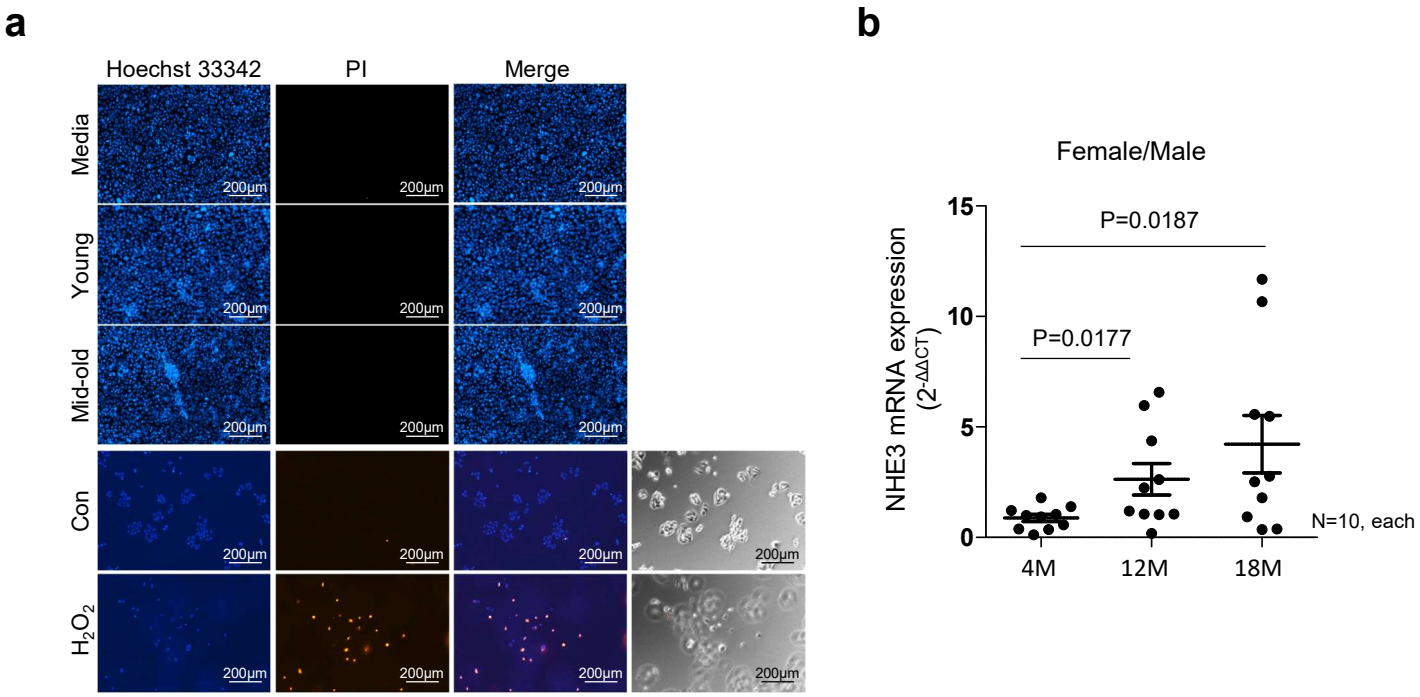
Supplementary Fig. 12 SAA1 does not affect expression of muscle contraction-related genes in PASMCs. PASMCs were treated with rhSAA1 at the indicated concentrations for 1 day. Muscle contraction-related gene (*MYH2; Myosin heavy chain 2* and *MYBPC1; Myosin binding protein C1*) expression was determined using real-time PCR, and results are representative of three independent experiments. The *p* values were calculated using the Mann-Whitney U test.

Supplementary Fig. 13



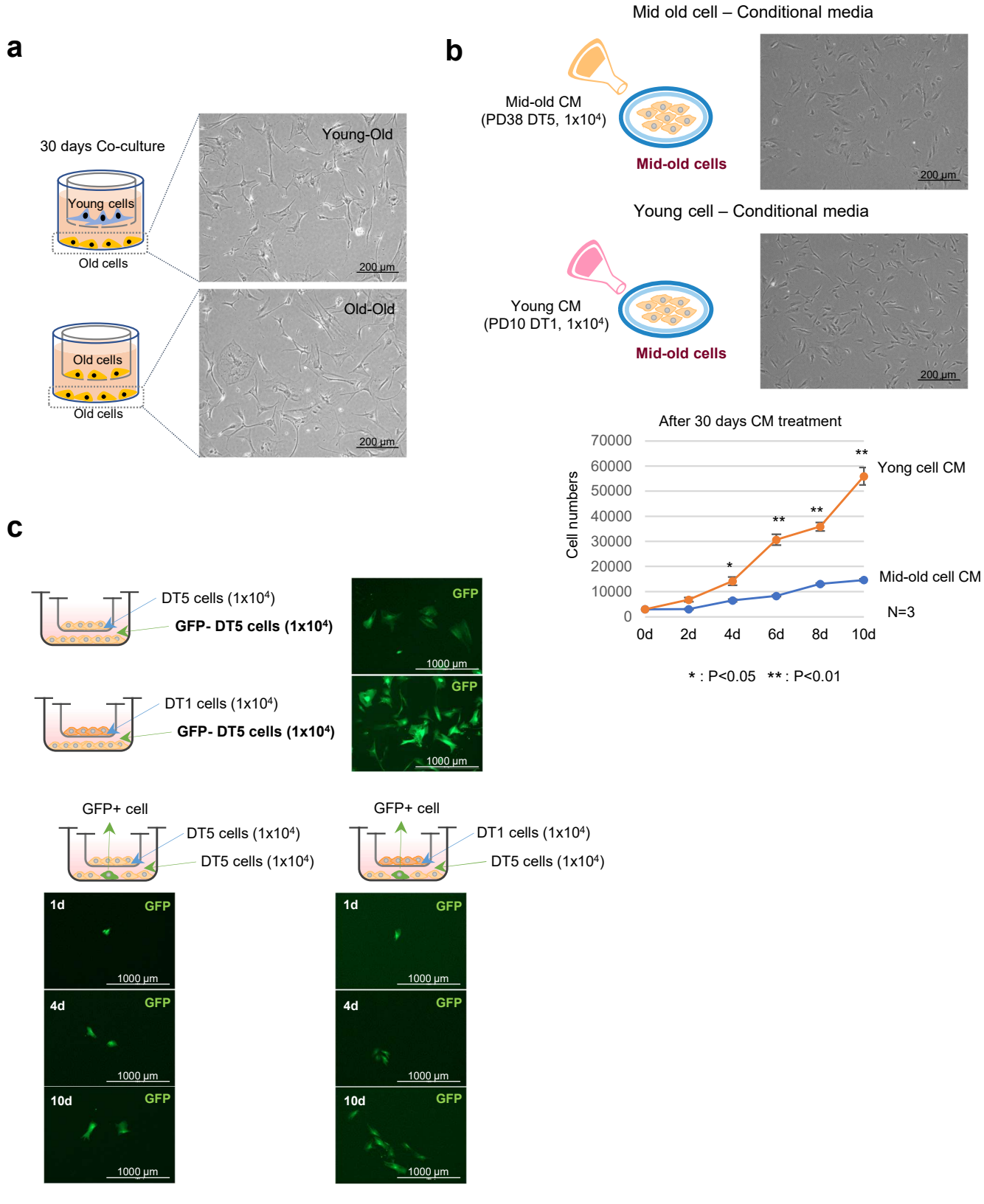
Supplementary Fig. 13 SAA1 does not affect *NHE3* or *AQP3* expression in colon epithelial cells. SW480 cells were treated with rhSAA1 at indicated concentration for 48 hrs and expression of colon functional markers (*AQP3*, *NHE2,* and *NHE3*) was measured by real-time PCR. Results are representative of three independent experiments. The *p* values were calculated using the Mann-Whitney U test.

Supplementary Fig. 14



Supplementary Fig. 14 BM degradation by mid-old cells does not affect epithelial cell viability. a A cell death assay was performed using SW480 cells stained with PI and Hoechst 33342 dye, sequentially. As a positive control for induction of cell death, SW480 cells were treated with 10 mM H_2O_2 for 40 mins. Blue fluorescence indicates Hoechst 33342-stained nuclei and red fluorescence indicates PI- stained cells undergoing cell death. **b** Expression of *NHE3* in mouse colon tissue. Total RNA was isolated from colon tissue (4-,12-, and 18- month-old male and female mice) and real-time PCR was performed to measure *NHE3* expression. The *p* value was calculated by the Mann-Whitney U test.

Supplementary Fig. 15

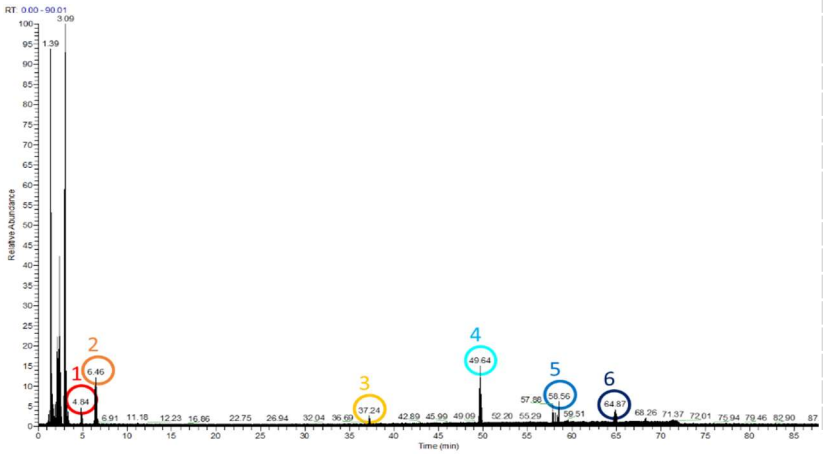


Supplementary Fig. 15 Young cell factors rejuvenated mid-old cells. a Old fibroblasts were co-cultured with old or young fibroblasts for 30 days. A representative image is shown. **b** Mid-old fibroblasts were treated with CM extracted from mid-old or young fibroblasts for 30 days, with CM changed every 3 days. At the experimental endpoint, cell morphology was analyzed (upper). After 30 days of treatment with CM, cell growth rate was determined every 2 days for 10 days by counting the number of cells (lower). Cell growth data are representative of three independent experiments. The *p* values were calculated using the Mann-Whitney U test. **c** GFP-expressing mid-old fibroblasts were co-cultured with young or mid-old fibroblasts for 30 days. At the end point, GFP fluorescence was analyzed in mid-old fibroblasts (upper). Single mid-old cells were labeled by GFP and tracked for 30 days in the co-culture system (bottom). The number of cells were increased in young cells co-cultured with mid-old cells for 10 days compared with 4 days (bottom right) but no differences were detected between 4 day and 10 day time points in mid-old cells co-cultured with mid-old cells (bottom left). The image was visualized with a Lionheart FX automated microscope, and results are representative of three independent experiments.

Supplementary Fig. 16

a

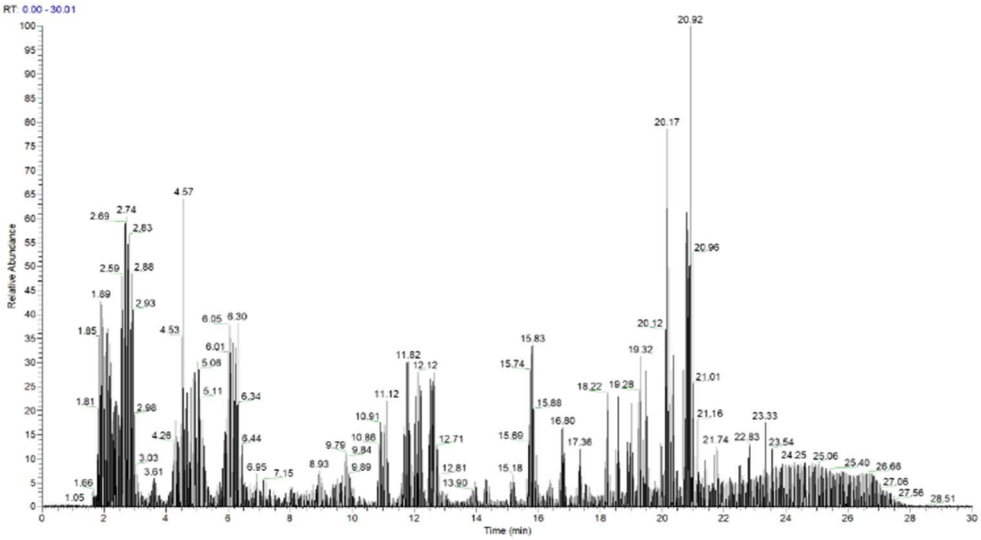
Metabolites from young cell conditioned media



1	Methocarbamol	C11 H15 N O5
1	Thian-4-one 4-[6-(trifluoromethyl)-2-pyridyl]hydrazone	C11 H12 F3 N3 S
2	2-methyl-2,3,4,5-tetrahydro-1,5-benzoxazepin-4-one	C10 H11 N O2
2	1,5-Naphthalenediamine	C10 H10 N2
2	DL-Tryptophan	C11 H12 N2 O2
2	XY5850570	C6 H10 Cl N5
3	Folic acid	C19 H19 N7 O6
4	2-[2-(2,3-dihydro-1,4-benzodioxin-6-yl)-1,3-thiazol-4-yl]-1,3-benzothiazole	C18 H12 N2 O2 S2
5	Cyprodenate	C13 H25 N O2
5	L-lanthionine	C6 H12 N2 O4 S
5	cis-1,2-dihydroxy-1,2-dihydrodibenzothiophene	C12 H10 O2 S
5	4253	C12 H22 O2
5	(R)-3-Hydroxy myristic acid	C14 H28 O3
6	prostaglandin G2 2-glyceryl ester	C23 H38 O8
6	1-Stearoylglycerol	C21 H42 O4
6	1-Linoleoyl-2-Hydroxy-sn-glycero-3-PC	C26 H50 N O7 P

b

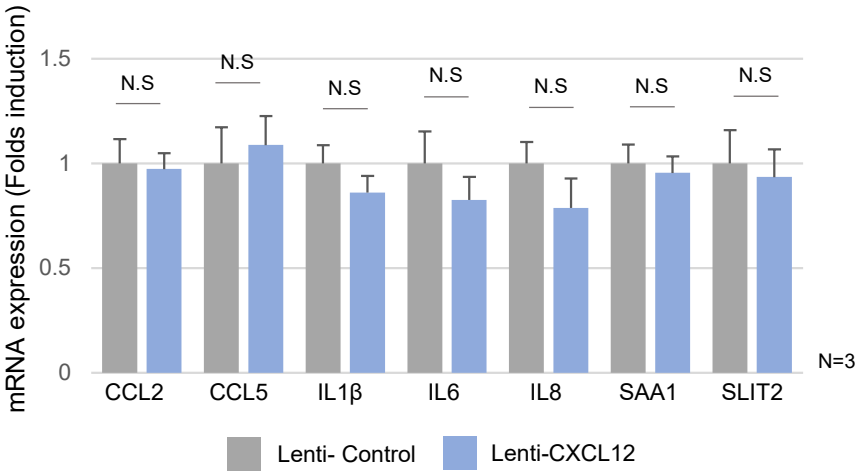
Lipids from young cell conditioned media



WE(3:0_18:0)+H	WE	DG(28:0_6:0)+NH4	DG	TG(20:1_10:1_22:0)+NH4	TG
MG(18:2)+H	MG	TG(12:0e_6:0_16:0)+NH4	TG	TG(14:0_14:0_24:0)+NH4	TG
MG(18:0)+H	MG	DG(18:0_16:0)+NH4	DG	TG(20:2e_11:4_24:2)+Na	TG
DG(6:0_12:0)+H	DG	DG(20:0_16:0)+NH4	DG	TG(18:1_18:1_18:1)+Na	TG
DG(8:0_10:0)+H	DG	DG(18:0_18:0)+NH4	DG	TG(26:1_6:0_23:1)+NH4	TG
DG(9:0_9:0)+H	DG	TG(18:0_8:0_10:3)+NH4	TG	TG(18:0_16:1_22:6)+NH4	TG
MG(19:0)+H	MG	TG(11:0_10:0_15:0)+NH4	TG	TG(27:1_6:0_24:2)+NH4	TG
DG(11:0_6:0)+NH4	DG	DG(20:1e_18:1)+Na	DG	TG(29:1_6:0_24:2)+NH4	TG
MG(18:0)+NH4	MG	SiE(18:0)+NH4	SiE	TG(30:0_16:1_22:4)+NH4	TG
DG(6:0_13:0)+NH4	DG	SiE(20:5)+H	SiE		
BisMePA(6:0_8:0)+NH4	BisMePA	CmE(20:5)+NH4	CmE		
DG(18:3e_6:0)+Na	DG	StE(20:5)+NH4	StE		
MG(24:0)+NH4	MG	TG(16:0_6:0_20:3)+NH4	TG		
TG(12:1e_6:0_6:0)+NH4	TG	StE(22:4)+NH4	StE		
TG(12:0e_6:0_6:0)+NH4	TG	SiE(22:1)+NH4	SiE		
TG(6:0_6:0_12:1)+NH4	TG	SiE(24:1)+NH4	SiE		
MG(26:0)+NH4	MG	PC(18:1_18:2)+H	PC		
DG(15:0_10:2)+Na	DG	PC(18:0_18:2)+H	PC		
TG(12:0e_6:0_8:0)+NH4	TG	DG(33:1_14:0)+NH4	DG		
BisMePA(4:0_15:0)+NH4	BisMePA	MePC(17:1_18:2)+Na	MePC		
DG(16:2e_14:0)+H	DG	TG(15:0_16:0_16:1)+NH4	TG		
TG(12:0e_8:0_8:0)+NH4	TG	PC(18:0_20:4)+H	PC		
CmE(8:0)+NH4	CmE	MePC(19:1_18:2)+Na	MePC		
DG(20:0_10:3)+NH4	DG	PC(20:0_20:4)+H	PC		
PE(8:0e_16:0)+Na	PE	TG(16:0_16:1_17:1)+Na	TG		
DG(28:0_6:0)+H	DG	TG(18:0_16:1_17:1)+NH4	TG		
TG(4:0_12:0_16:0)+NH4	TG	TG(18:0_10:1_24:1)+NH4	TG		

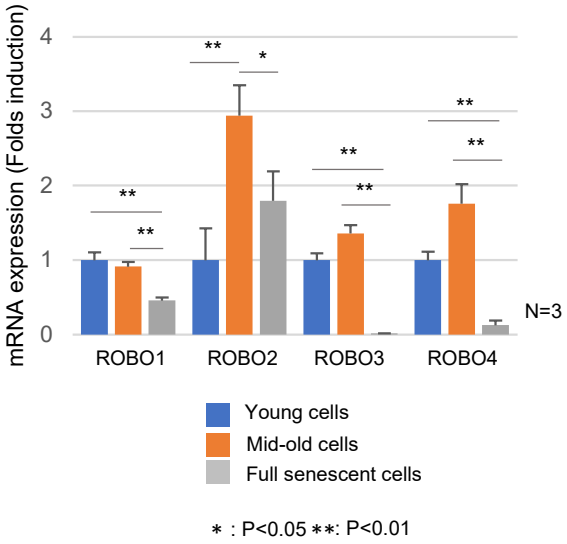
Supplementary Fig. 16 Analysis of metabolites and lipids from young cell CM. Metabolites (**a**) and lipids (**b**) were measured using an LC-MS chromatogram. WE: Fatty esters wax monoexters 4, MG: Monoacylglycerols, DG: Diacylglycerols, TG; Triacylglycerols, BisMePA: bis-methyl phosphatidic acid, CmE: Cholesteryl methyl ester, PE: Phosphatidylethanolamines, SiE: Sitosterol ester, StE: Stigmasterol ester, MePC: Methyl phosphatidylcholine, PC: Phosphatidylcholine.

Supplementary Fig. 17



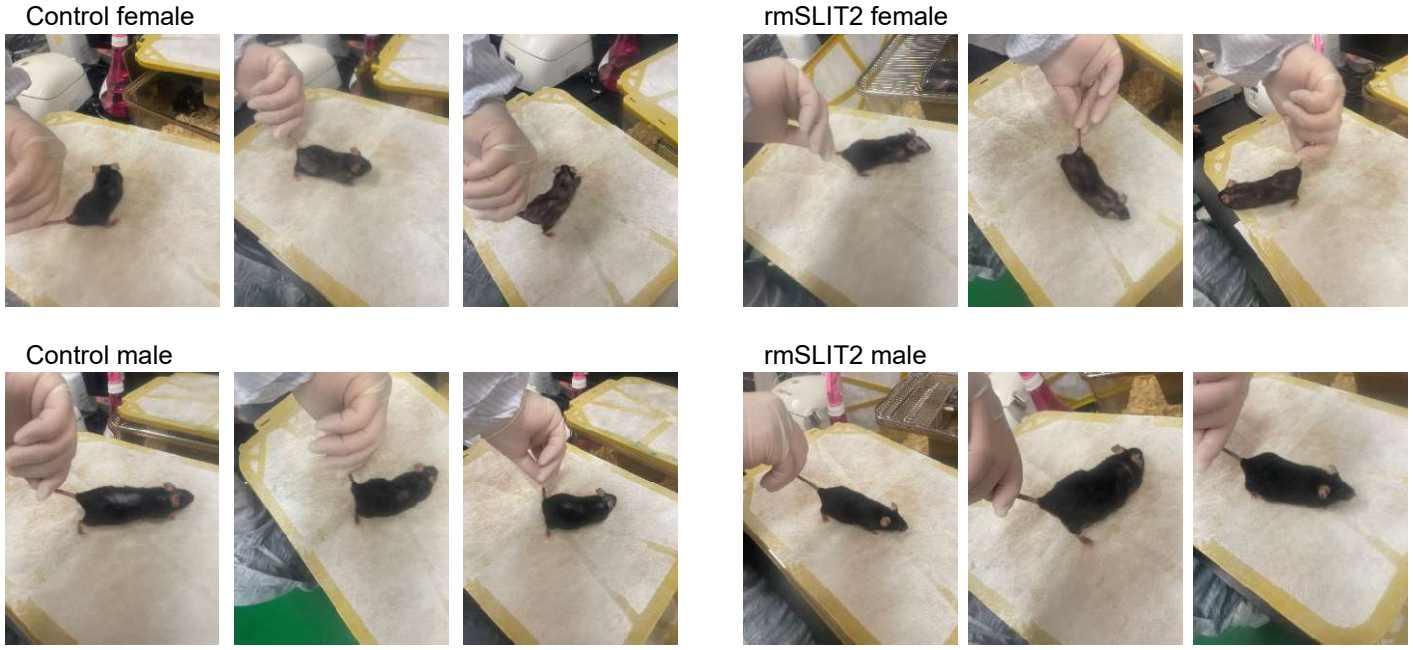
Supplementary Fig. 17 CXCL12 expression is not related to SASP expression. Mid-old fibroblasts were infected with CXCL12-expressing lentivirus and expression of SASP was measured using real-time PCR. The *p* values were calculated using the Mann-Whitney U test.

Supplementary Fig. 18



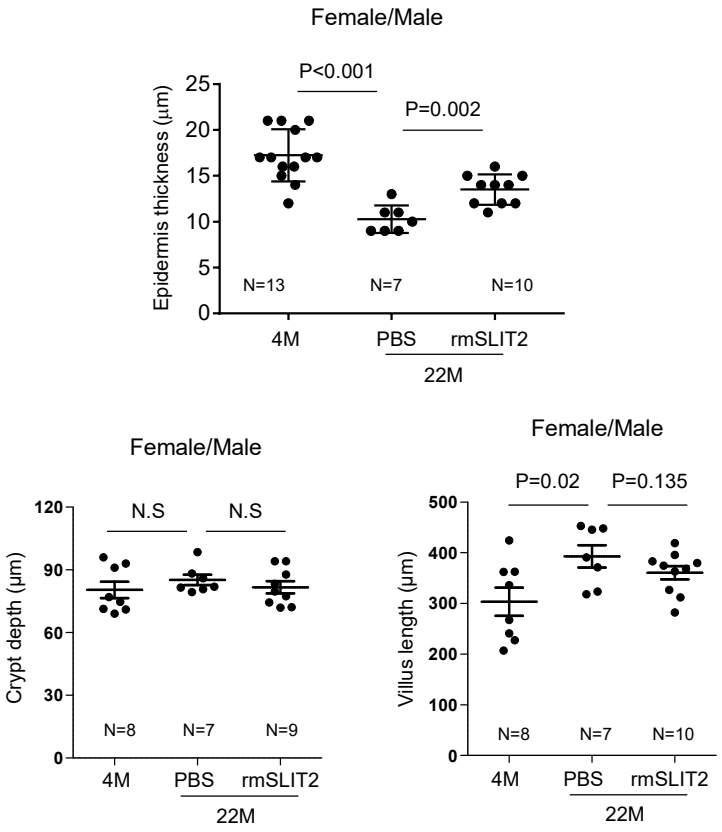
Supplementary Fig. 18 ROBO receptor expression in young, mid-old and full senescent cells. ROBO receptor expression was analyzed in young, mid-old, and full senescent fibroblasts using real-time PCR. The *p* value was calculated using the Mann-Whitney U test.

Supplementary Fig. 19



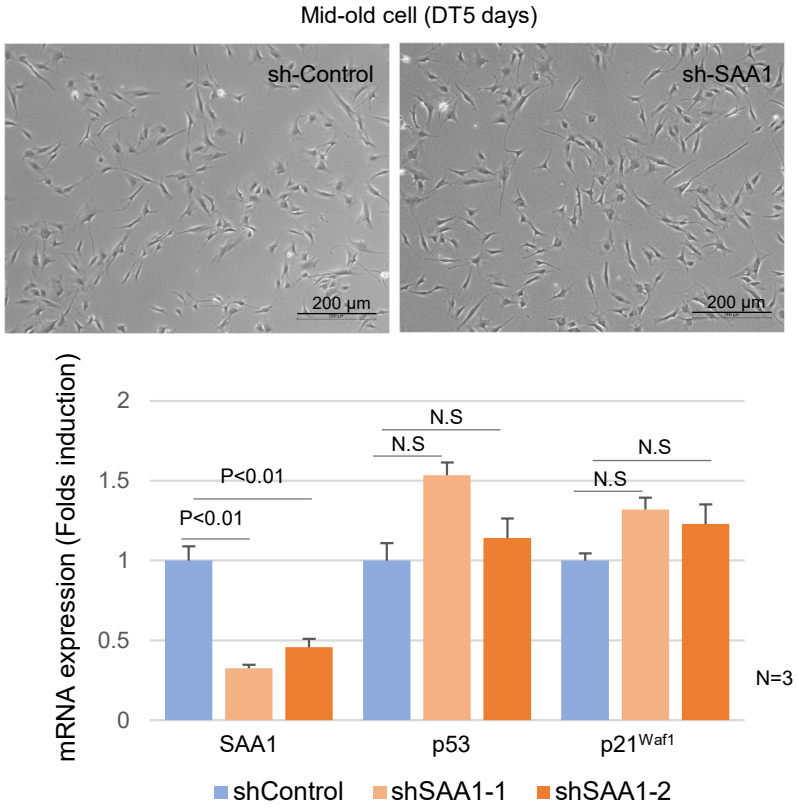
Supplementary Fig. 19 rmSLIT2 protein treatment improves hair coat condition in old-aged mice. PBS or rmSLIT2 protein were intraperitoneally injected for three times in 22-month-old mice. The representative images show hair coat condition for each experimental group.

Supplementary Fig. 20



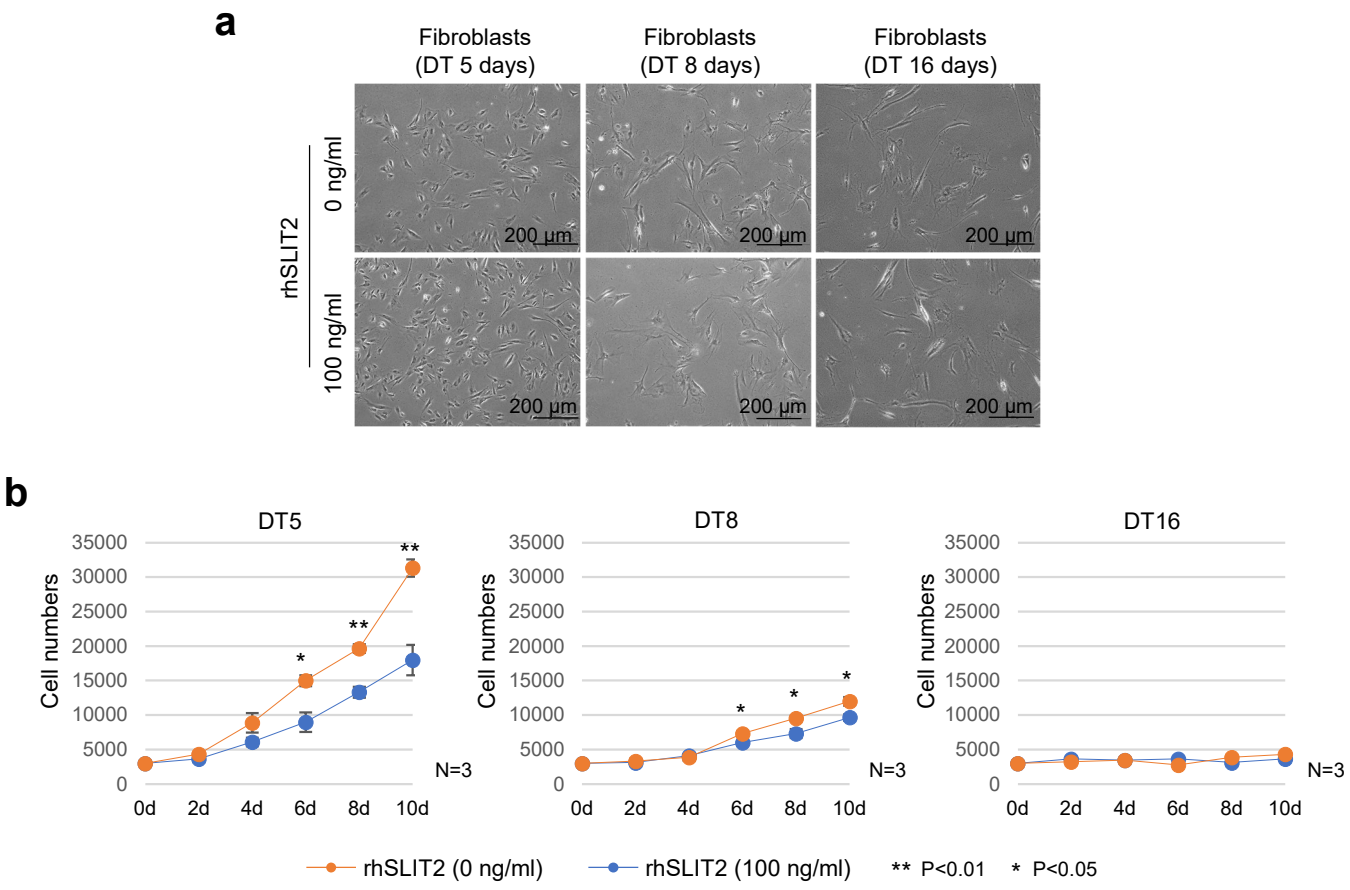
Supplementary Fig. 20 Epidermis thickness and small intestine villus length in rmSLIT2-treated mice. PBS or rmSLIT2 protein were injected intraperitoneally twice weekly for 3 weeks in 22-month-old mice. The thickness of the skin epidermis region was measured, as villus length and crypt height in small intestine. Five randomly selected histological images were analyzed. Histological analysis of skin and small intestine of 22-month-old male and female mice with and without rmSLIT2 protein administration. The *p* value was calculated using the Mann-Whitney U test.

Supplementary Fig. 21



Supplementary Fig. 21 SAA1 downregulation did not increase cell proliferation. Mid-old fibroblasts were infected with Lenti-SAA1 for 56 h and mRNA expression of p53, $p21^{Waf1}$, and SAA1 was measured, and cell morphology assessed. Results are representative of three independent experiments. The *p* values were calculated using the Mann-Whitney U test.

Supplementary Fig. 22



Supplementary Fig. 22 SLIT2 stimulates mid-old cell growth. The fibroblasts was treated with rhSLIT2 (100 ng/ml) in DT5, DT8 and DT16 for 10 days. Cells morphology (**a**) and growth rate (**b**) were analyzed. The *p* values were calculated using the Mann-Whitney U test.