SCAMP4 enhances the senescent cell secretome

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Supplemental Materials and Methods

Cell culture, establishment of senescence, transfection, and SA-β-galactosidase activity

Human diploid fibroblasts (HDFs) WI-38 and IMR-90 (Coriell Cell Repositories) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco), antibiotics, antimycotics, and non-essential amino acids (Invitrogen). Human neuroblastoma SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco), antibiotics, and antimycotics. Human umbilical vein endothelial cells (HUVECs) were cultured in endothelial basal medium (EBM) supplemented with EGM SingleQuots™ Kit with growth factors and supplements (Lonza). Human aortic endothelial cells (HAECs) were cultured in EBM-2 supplemented with EGM-2 SingleQuots™ Kit with growth factors and supplements (Lonza).

Interventions to silence gene expression using siRNAs and overexpression using lentiviruses were performed as follows. WI-38 cells at PDL39-PDL42 and senescent WI-38 cells by exposing to ionizing radiation were transfected with 50 nM of *in vitro*-synthesized Ctrl siRNA or SCAMP4 siRNA. Proliferating WI-38 cells were transduced with lentiviruses that expressed either SCAMP4-Myc or Myc (GeneCopoeia, Inc) for 3 days, or selected using puromycin (1 μg/mL) for 20 days before harvest. SA-β-galactosidase (SA-β-gal) activity was assessed using a kit (Cell Signaling).

Senescence induced by exposure to ionizing radiation, by expression of by HRAS^{G12V} (oncogene-induced senescence, OIS), and by treatment with doxorubicin

Proliferating WI-38 cells (~PDL25) and IMR-90 cells (~PDL25) were rendered senescent by exposure to 10 Gy of ionizing radiation (IR) and HAECs and HUVECs were exposed to 4 Gy of IR; cells were harvested 10 days later. For OIS experiments, proliferating WI-38 cells were transduced using a control lentivirus or a lentivirus expressing HRAS^{G12V}. Transduced cells were selected using puromycin (1 μg/mL) for 5 days, and harvested for analysis. WI-38 cells (PDL25) were treated with Doxorubicin (2 μg/mL) for 24 h; 7 days later, they were harvested for analysis.

Flow cytometry

Flow cytometry analysis was performed as described (Kim et al. 2017). Briefly, proliferating and senescent WI-38 cells were counted, washed using FACS buffer (0.5% BSA in PBS). Dead cells were stained with Zombie Aqua™ Fixable Viability Kit (BioLegend); after washing, Human TruStain FcX™ (BioLegend) was added to block the Fc receptor and cells were labeled with SCAMP4-biotin antibody (LifeSpan BioSciences, Inc.) or Human IgG H&L (Biotin) antibody (Abcam) for 15 min at 4°C, then incubated for an additional 15 min at 4°C in the dark with Allophycocyanin (APC)-Streptavidin antibody

(BioLegend). FACS analysis was performed on a Canto II flow cytometer (BD Biosciences) using FlowJo software (FlowJo v10.2).

Thymidine incorporation assay

WI-38 cells overexpressing SCAMP4-Myc or Myc tag alone were incubated with [methyl-^{3}H]-thymidine (NET027250UC, Perkin Elmer) for 16 h at 37°C. After washes with 1× PBS and harvesting the cells, the radioactivity incorporated was measured using liquid scintillation counting and normalized to total protein amounts in each sample.

Protein isolation, and Western blot and immunoprecipitation analyses

To extract membrane proteins and cell surface proteins, cell lysates were prepared and processed from proliferating (PDL23) and senescent (PDL59) WI-38 fibroblasts using the Mem-PER™ Plus Membrane Protein Extraction Kit (Thermo Scientific) or the Pierce™ Cell Surface Protein Isolation Kit (Thermo Scientific). Whole-cell lysates were prepared using M-PER™ Mammalian Protein Extraction Reagent (Thermo Scientific) supplemented with phosphatase and protease inhibitor cocktail (Thermo Scientific). Protein lysates were electrophoresed in SDS-PAGE, transferred to nitrocellulose membrane (iBlot Stack, Thermo Scientific) and detected using specific primary antibodies: SCAMP4 (Abcam), SCAMP1 (Thermo Scientific), SCAMP2 (Thermo Scientific), SCAMP3 (Antibodies-online Inc), SCAMP5 (Novus Biologicals, Abcam), Myc (Santa Cruz), IL1A (MyBioSource), EGFR (Santa Cruz), CAV1 (Caveolin-1; Santa Cruz), HSP90 (Santa Cruz), SIRT1 (Abcam), GAPDH (Santa Cruz), p21 (Millipore), ACTB (Santa Cruz), p53 (Santa Cruz), HRAS (Calbiochem), Ubiquitin (Santa Cruz), LC3-I/II (Abcam), IL1B (Proteintech Group Inc) and p16 (BD Biosciences). After incubation with secondary antibodies (GE Healthcare), Western blotting signals were detected by enhanced chemiluminescence (Millipore) using a KwikQuant Imager (Kindle Biosciences, LLC).

To assess protein stability, WI-38 HDFs proliferating (PDL21-23) and senescent cells (PDL50) were incubated with 100 μg/ml cycloheximide (Calbiochem), then harvested at 0, 1, 2, 4 h. WI-38 proliferating (PDL22-PDL25) and senescent cells (PDL50-51) were incubated with 10 μM of MG132 for 2, 4, 6, and 24 h or 100 nM of Bafilomycin A1 (Calbiochem) for 24 h, then lysed for analysis.

For immunoprecipitation, WI-38 proliferating cells were treated with 10 μM of MG132 for 3 h, then lysed with PEB (polysome extraction buffer, 20 mM Tris-HCl [pH 7.5], 100 mM NaCl, 5 mM $MgCl_2$ and 0.3% NP-40). The lysate was incubated with Protein A-agarose beads (GE Healthcare) for 1 h at 4°C to clear nonspecific binding. The lysate was incubated with Protein A-agarose beads coated with antibodies that recognized SCAMP4 (Abcam) or with rabbit IgG (Santa Cruz) for 2 h at 4°C. The beads

were washed with NT2 buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM $MgCl_2$ and 0.05% NP-40) five times, then NuPAGE™ LDS Sample Buffer (Thermo Scientific) was added to the beads.

Analysis of secreted proteins: cytokine arrays and ELISA (enzyme-linked immunosorbent assay)

To prepare conditioned media, cells were washed with 1× PBS twice, then fresh DMEM with 0.2% FBS was added; after incubation for 24-36 h, this conditioned media was collected for further analysis. IL6, IL8, GDF-15, CXCL1, CCL7, IL7, Angiogenin, MIF, IL1B (IL1β), and CCL2 secreted into the culture medium were measured using a Human Quantikine ELISA Kit (R&D systems). For cytokine arrays, conditioned media were incubated with cytokine arrays from RayBiotech at 4°C for 16 h, biotinylated antibody at 4°C for 16 h, and streptavidin-HRP for 2 h at 25°C. Signals were detected using enhanced Chemiluminescence (Millipore) by KwikQuant Imager (Kindle Biosciences, LLC).

Immunofluorescence microscopy

Immunofluorescence was performed as previously described (Kim et al. 2017). Proliferating and senescent WI-38 cells were fixed with 100% methanol (Fisher Scientific) for 10 min at -20°C. Cells were incubated with 10% normal goat serum (Thermo Scientific) for 1 h at room temperature. The anti-SCAMP4 primary antibody (Sigma Aldrich) was added to the cells and kept overnight at 4°C and for an additional 1 h incubation at 37°C with Alexa Fluor 568-conjugated secondary antibodies (Thermo Scientific). Nuclei were stained with ProLong® Gold Antifade Mountant with DAPI (Thermo Scientific). Signals were observed using a confocal microscope (ZEISS 710 LSM).

RNA isolation and reverse transcription (RT) followed by real-time quantitative (q)PCR analysis

Total RNA was extracted using TriPure Isolation Reagent (Roche), and cDNA was synthesized using random hexamers and reverse transcriptase (Invitrogen). The gene specific primers were used for qPCR and SYBR Green master mix (Kapa Biosystems) using Applied Biosystems 7300 instrument. The following gene-specific primers were used (sense and antisense respectively): CTGTCGGCAATTGGATTCTT and CGGTAGATCCTGTGCACCTT for *SCAMP4* mRNA, TACGAGGACCTGCTAACCA and GATCCCAGCCGCACTTC for *GDF-15* mRNA, AGAACCGCTCCTACAGCAAG and GAGTTGTTCCAGCCCACATT for *MIF* mRNA, GAAAGCTTGCCTCAATCCTG and CACCAGTGAGCTTCCTCCTC for *CXCL1* mRNA, and CTCCCCTGATCCTTGTTCTG and CCAATTTCTTTCATGCTGTCC for *IL7* mRNA. Primers for *ACTB* mRNA, *p16* mRNA, *IL1A* mRNA, *IL1B* mRNA, *IL6* mRNA, *IL8* mRNA, *CCL13* mRNA, *p21* mRNA, and *18S* rRNA were previously reported (Kim et al. 2012; Kim et al. 2017; Herranz et al. 2015; Noh et al. 2016).

Polysome analysis

Polysome analysis was performed as previously described (Panda et al. 2017; Tominaga-Yamanaka et al. 2012). Briefly, WI-38 fibroblasts were treated with cycloheximide (Calbiochem; 100 μg/ml) for 10 min and lysed in PEB (polysome extraction buffer). After the lysate was separated through 10% to 50% sucrose gradients, 12 fractions were collected for further analysis. The distribution of mRNAs was quantified by RT-qPCR analysis and plotted as a percentage of the specific mRNA in each fraction relative to the total amount of that mRNA in the gradient.

Supplemental Figure Legends

Figure S1. In WI-38 fibroblasts, SCAMP5 is not expressed, while SCAMP1, SCAMP2, and SCAMP3 increase moderately with senescence. (*A-C*) Western blot analysis of SCAMP1-3 levels in whole-cell lysates (*A*), membrane/cytosolic fractions (*B*), and cell-surface fractions (*C*) of proliferating and senescent WI-38 fibroblasts. **(*D*)** SCAMP5 was assessed by Western blot analysis in SH-SY5Y (human neuroblastoma cells) and in proliferating (P) and senescent (S) WI-38 fibroblasts. ACTB, loading control; HSP90, cytosolic marker; EGFR, plasma membrane protein marker. P, proliferating; S, senescent.

Figure S2. SCAMP4 is highly expressed in cells rendered senescent by exposure to IR, treatment with doxorubicin, and expression of oncogene HRAS^{G12V}. (*A-C*) Ten days after exposure to ionizing radiation (IR) of proliferating WI-38 HDFs (10 Gy) (A), IMR-90 HDFs (10 Gy) (B), and HAECs (4 Gy) (C), SA-β-galactosidase activity, Western blot analysis of SCAMP4 levels, and RT-qPCR analysis of *SCAMP4* mRNA levels were carried out. **(*D*)** Ten days after IR of HUVECs (4 Gy), Western blot and RT-qPCR analyses were performed to assess the levels of SCAMP4 protein (*left*) and *SCAMP4* mRNA (*right*), respectively. **(*E*)** Proliferating WI-38 fibroblasts were incubated with doxorubicin for 24 h; after removing Doxo, cells were cultured for an additional 10 days before analysis. Western blot and RT-qPCR analyses were performed to assess the levels of SCAMP4 protein (*left*) and *SCAMP4* mRNA (*right*), in control and Doxorubicin (Doxo)-induced senescent WI-38 HDFs. **(*F*)** Ten days after infection with lentivirus to express HRAS^{G12V} and trigger oncogene-induced senescence in WI-38 HDFs cells, SA-β-galactosidase activity and Western blot analysis was performed to assess the levels of SCAMP4, HRAS, p16, and ACTB; RT-qPCR analysis was performed to assess *SCAMP* mRNA levels. In (*A-F*), ACTB and GAPDH are protein loading controls; p21 and p16 are protein markers of senescence; *p21* mRNA is a transcript marker of senescence.

Figure S3. Treatment with proteasome inhibitor MG132 enhances SCAMP4 accumulation, promotes senescence. (*A*) The levels of SCAMP4, senescent markers p21, p53, p16, and SIRT1, and loading control ACTB were measured by Western blot analysis in proliferating (PDL25) and senescent (PDL50) WI-38 fibroblasts that were either left untreated or treated with MG132 for 24 h. **(*B*)** The levels of IL6, IL8, GDF-15, and MIF were measured by ELISA in the conditioned media of cells prepared as described in panel (*A*). **(*C*)** Western blot analysis of SCAMP4 protein levels in proliferating (PDL25) and senescent (PDL51) WI-38 fibroblasts upon addition of Bafilomycin A1 (Baf A1; 100 nM for 24 h) to inhibit autophagy. LC3-I/II, autophagy marker; p21, negative control; ACTB, loading control. **(*D*)** ELISA to quantify the levels of secreted IL8, GDF-15, and IL1B in cells in fibroblasts in which SCAMP4 had been

silenced 3 days after transfection with SCAMP4 siRNA; control cells were transfected with Ctrl siRNA in pre-senescent cells. Graphs in (*B*,*D*) represent the means ± S.E.M. from three independent experiments; **, *P*-value<0.01; *, *P*-value<0.05.

Figure S4. SCAMP4 overexpression increases IL1A translation, enhances SASP factor secretion. (*A*) Analysis of polysomes prepared from 20 days after expression of SCAMP4-Myc or Myc in proliferating WI-38 fibroblasts as described in Fig. 4. WI-38 cell lysates were loaded onto 10-50% linear sucrose gradients and the relative distributions of *IL1A* mRNA and (control) *ACTB* mRNA were calculated after RT-qPCR analysis of RNA extracted from each fraction. **(*B-D*)** Three days after overexpression of either SCAMP4-Myc or Myc in proliferating WI-38 fibroblasts (PDL26), cells were harvested for analysis. Western blot analysis was performed to measure the levels of SCAMP4 and senescent marker proteins (*B*), and RT-qPCR analysis was performed to measure level of mRNAs encoding SASP factors (*C*). The levels of secreted IL6, IL8, GDF-15, CXCL1, and MIF were quantified by ELISA (*D*). Graphs in (*C*,*D*) represent the means ± S.E.M. from three independent experiments; **, *P*-value<0.01; *, *P*-value<0.05.

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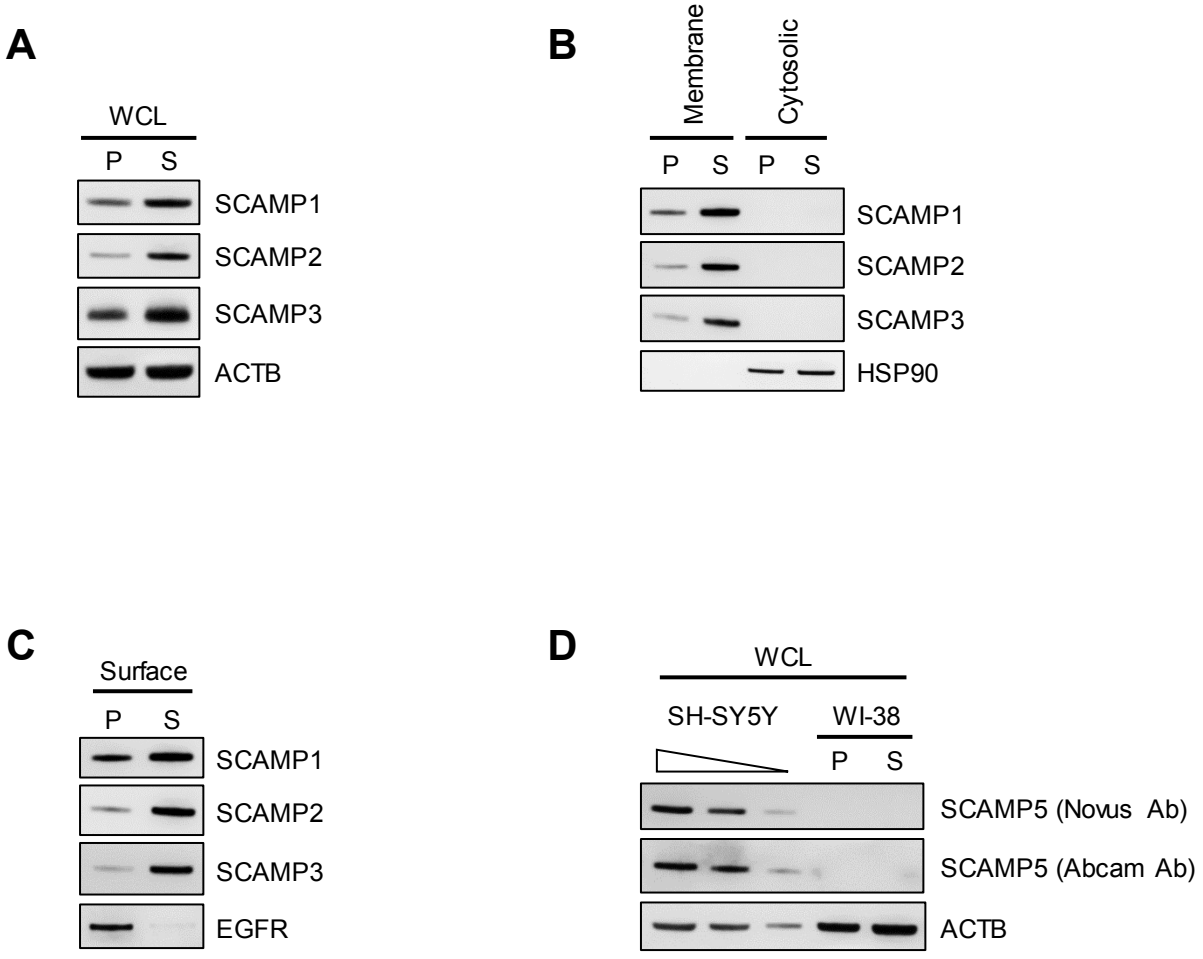
A
WCL
P S
SCAMP1
SCAMP2
SCAMP3
ACTB
B
Membrane
Cytosolic
P S P S
SCAMP1
SCAMP2
SCAMP3
HSP90
C
Surface
P S
SCAMP1
SCAMP2
SCAMP3
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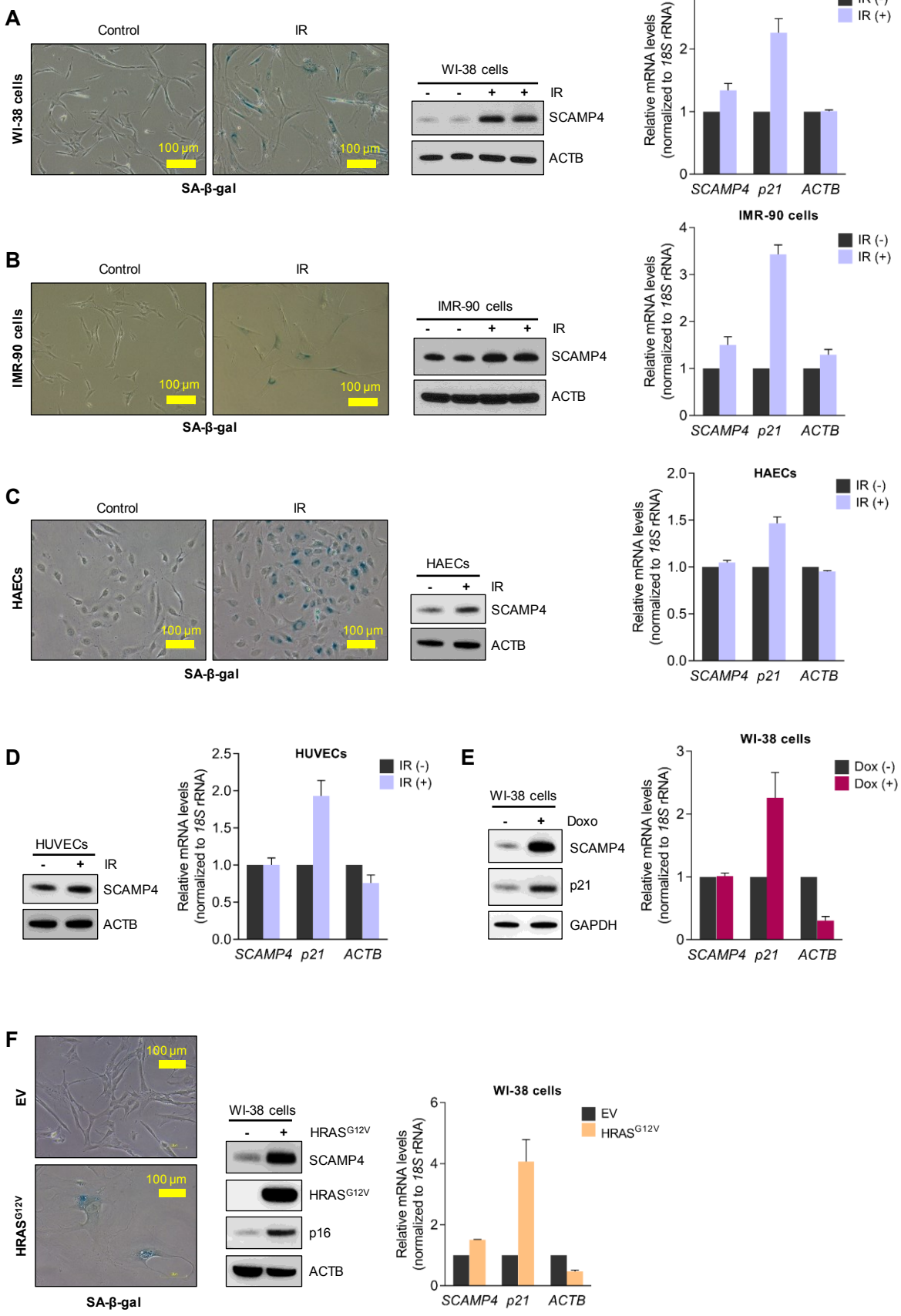
SCAMP5 (Abcam Ab)
ACTB

Kim_FigS2


A
Control
IR
WI-38 cells
100 μm
SA-β-gal
WI-38 cells
IR
SCAMP4
ACTB
WI-38 cells
IR (-)
IR (+)
Relative mRNA levels (normalized to 18S rRNA)
SCAMP4 p21 ACTB
B
IMR-90 cells
IMR-90 cells
C
HAECs
HAECs
D
HUVECs
HUVECs
E
WI-38 cells
Doxo
SCAMP4
p21
GAPDH
Dox (-)
Dox (+)
F
EV
HRASG12V
SA-β-gal
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SCAMP4
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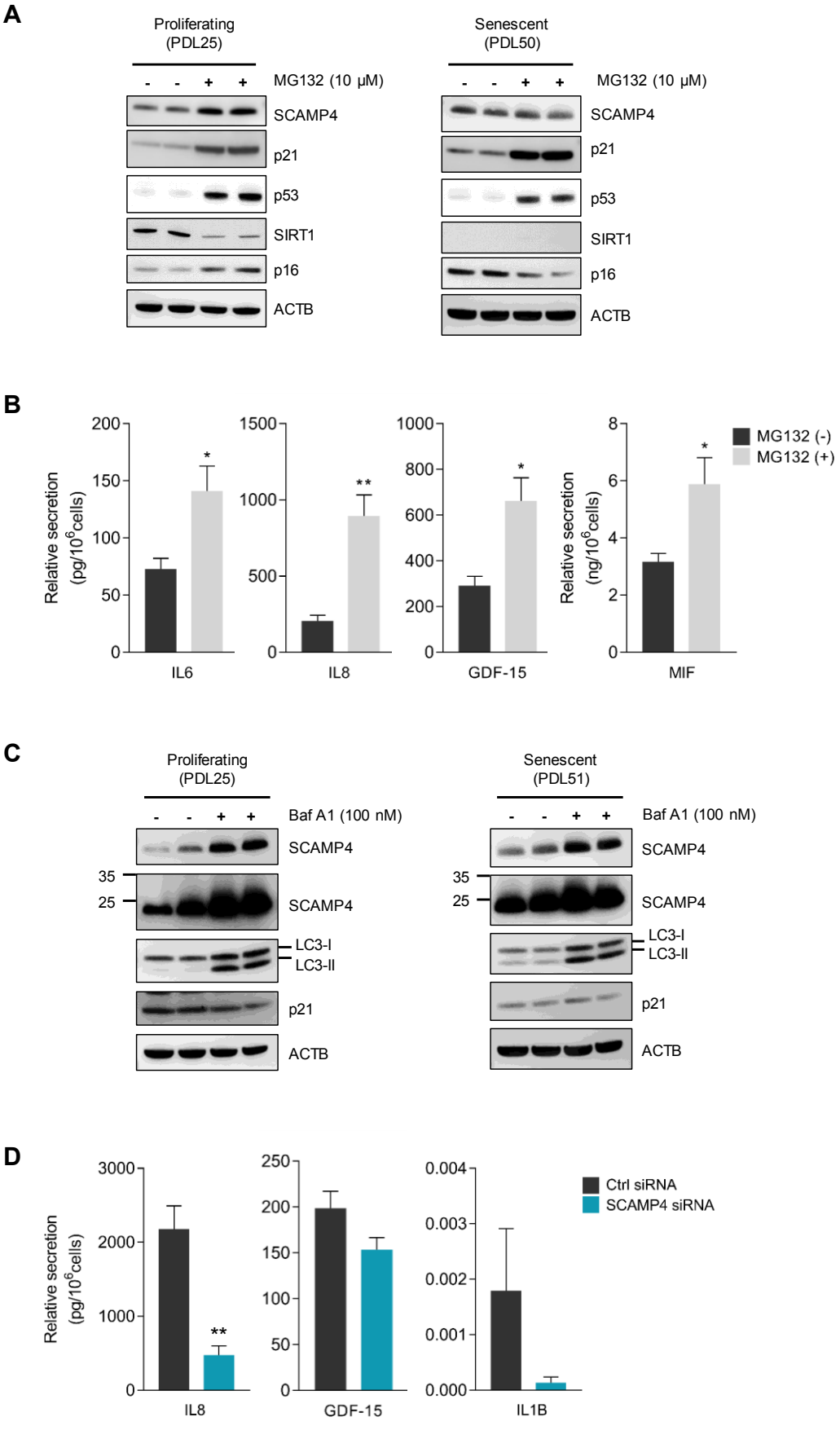
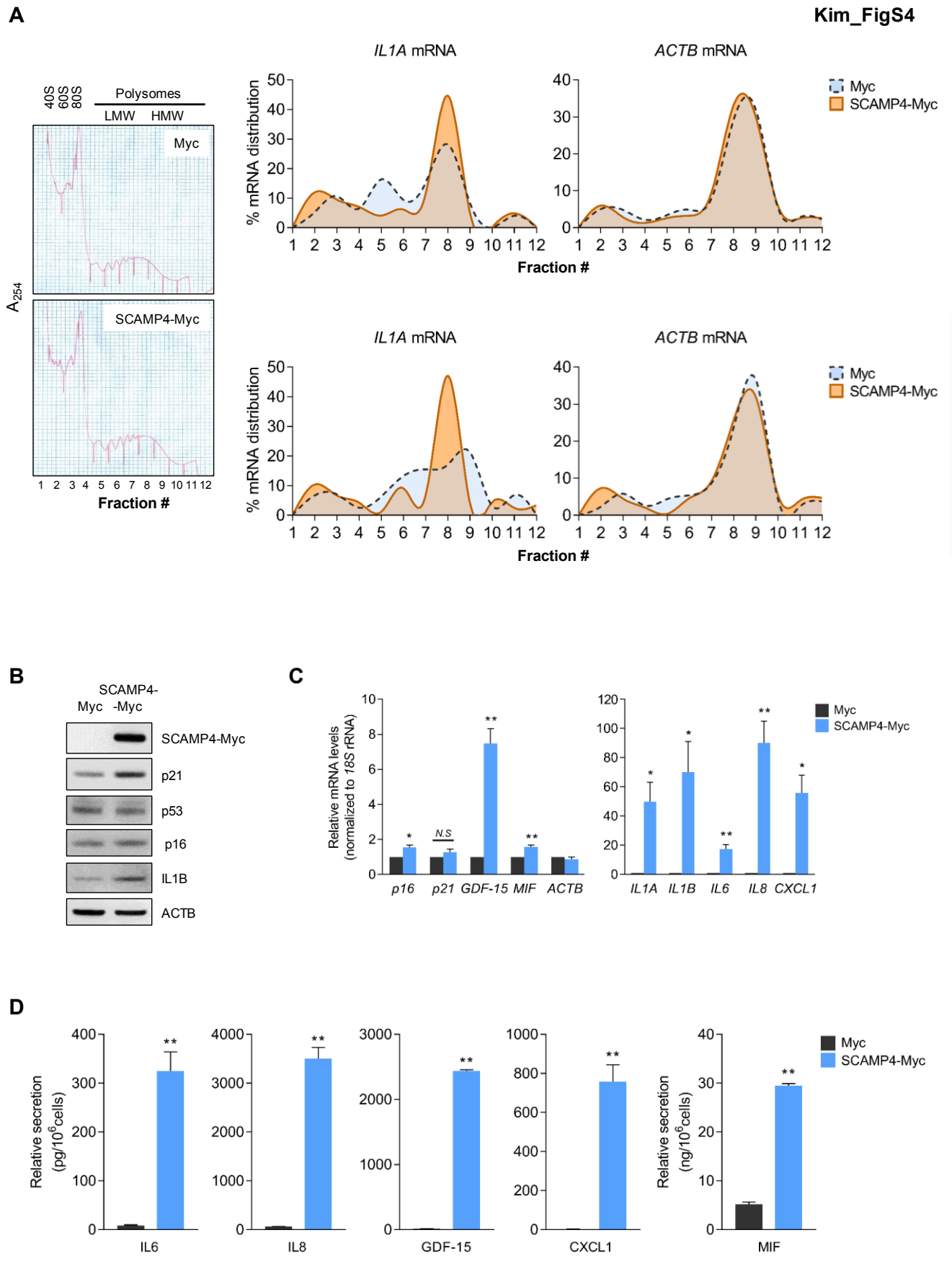
p16
ACTB
WI-38 cells
EV
HRASG12V

Kim_FigS3


A
Proliferating (PDL25)
Senescent (PDL50)
MG132 (10 μM)
SCAMP4
p21
p53
SIRT1
p16
ACTB
B
Relative secretion (pg/10^6cells)
Relative secretion (ng/10^6cells)
MG132 (-)
MG132 (+)
IL6
IL8
GDF-15
MIF
C
Proliferating (PDL25)
Senescent (PDL51)
Baf A1 (100 nM)
SCAMP4
LC3-I
LC3-II
p21
ACTB
35
25
D
Relative secretion (pg/10^6cells)
Ctrl siRNA
SCAMP4 siRNA
IL8
GDF-15
IL1B


A
Kim_FigS4
40S
60S
80S
Polysomes
LMW
HMW
Myc
SCAMP4-Myc
A254
Fraction #
IL1A mRNA
ACTB mRNA
% mRNA distribution
Myc
SCAMP4-Myc
B
SCAMP4-
Myc
-Myc
SCAMP4-Myc
p21
p53
p16
IL1B
ACTB
C
Relative mRNA levels
(normalized to 18S rRNA)
N.S
p16
p21
GDF-15
MIF
ACTB
IL1A
IL1B
IL6
IL8
CXCL1
D
Relative secretion
(pg/10^6cells)
(ng/10^6cells)
IL6
IL8
GDF-15
CXCL1
MIF