Senescence is a Spi1-induced anti-proliferative mechanism in primary hematopoietic cells

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SUPPLEMENTARY INFORMATION

Senescence is a Spi1/PU.1-induced anti-proliferative mechanism in primary hematopoietic cells

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Supplementary information includes Methods and 11 Figures.

Supplementary Methods

Mice, cell culture and chemicals

Experiments using mice were conducted with the ethical approval of Institut Gustave Roussy Area Standing Commitee on Animals. Population doubling was calculated as $\log_2 (N_{t+1}/N_t) = 3.322*(\lg N_{t+1} - \lg N_t)$. N_{t+1} is the number of living cells at 't+1' day; N_t is the number of the cells at 't' day. Living fibroblastic and hematopoietic cells were evaluated by trypan blue exclusion test.

SB203580 (Sigma-Aldrich) was diluted in DMSO and used at 20μM.

Plasmids

The cDNA of the murine wild-type (WT) Spi1 or the Δβ4-Spi1 mutant containing a deletion of the amino acids 250–254 (1) was cloned into the retroviral vector PLPC for infection of fibroblasts or MSCV-IRES-GFP for infection of LSK, MEP and GMP progenitor cells. MSCV-$HRAS^{V12}$-IRES-GFP was obtained from Addgene.

Antibodies

The primary antibodies directed against phospho-p53 (Ser15) (Abcam, ab1431), p53 (Santa Cruz, sc-6243), phospho-p38MAPK14 (Thr180/Thr182) (Cell Signaling, 9211), p38MAPK14 (Cell Signaling, 9217), Spi1 (Santa Cruz, sc-352), β-actin (Sigma, A5441), α-adaptin 1/2

(Santa Cruz, sc-10761), CDKN1A (Abcam, ab7960), CDKN2A (Santa Cruz, sc-468), Dec1 (Abcam, ab23797), γ-H2AX (Upstate 05-636), H2AX (Santa Cruz, sc-54606), RAS (Thermo Scientific, Active Ras Pull-Down and Detection Kit) were used for immunostaining. Specific peroxidase-conjugated secondary antibodies or IRDye® 800CW (or 600CW)-conjugated goat anti-mouse (or rabbit) IgG and IRDye® 800CW-conjugated Donkey anti-Goat IgG were used to detect protein expression by LAS-3000 imager (Fujifilm) using an enhanced chemiluminescence kit (Pierce) or by Odyssey infrared imaging system (LI-COR Biosciences), respectively. Images were cropped using Photoshop software (Adobe Systems France, Paris, France).

Western blotting

Analysis of cell extracts by Western blotting was performed as described. (2)

RNA extraction and quantification by real time quantitative PCR (qPCR)

Total RNA was isolated from cells using RNeasy Plus Mini Kit or RNeasy Plus Micro Kit (Qiagen) and 150 ng of cDNA were prepared using Superscript II Reverse Transcriptase (LifeTechnologies). Quantitative PCR was performed on 5 ng of cDNA three times in duplicate on the 7500 RT-PCR System (Applied Biosystems) using TaqMan Gene Expression Assays (Applied Biosystems) (Spi1, Mm00488142_m1; Polr2α, Mm00839493_m1; GAPDH, Dec1, Mm00478593-m1; IL1α, Mm00439620_m1; CDKN1A, Mm04205640_g1 ; and CDKN2A, Mm00494449_m1). RNA extraction of sorted MEP and GMP cells was performed using Direct-zol RNA microprep R2060 (ZymioResearch) and cDNA prepared using Superscript IV Reverse Transcriptase (LifeTechnologies). Fold changes in mRNA expression levels were calculated relative to wt mice and normalized to Polr2α and GAPDH mRNA level by the comparative Ct method using the formula $2^{-\Delta\Delta Ct}$.

Luciferase assay

50000 HeLa cells were seeded in 0.5mL of medium and co-transfected 24hrs later with 5 ng of pFes-3X-LucF, 1ng of pGL4-74 lucR (Promega) and PLPC (control), PLPC-Spi1, PLPC-Δβ4-Spi1, MSCV (control), MSCV-Spi1 or MSCV-Δβ4-Spi1 using Jetprime™ (PolyPlus). pFes-3X-LucF vector contains 3 copies of the *Fes* gene promoter sequence including the Spi1 binding sites as described in (1). The Renilla luciferase vector was used to normalize for the transfection efficiencies variation. Forty-eight hours later, cells were lysed and the Firefly (LucF) and Renilla (LucR) luciferase activities were measured with the dual luciferase kit (Promega) according to the manufacturer's instructions. Luminescence was recorded with a Fluostar Optima microplate reader (BMG Labtech). The fold induction of LucF relative to the fold induction of vector control was calculated after normalisation to LucR activities. The experiments were performed in triplicate.

Preparation of LSK, MEP and GMP progenitor cells from mouse bone marrow

Bone marrow cells were removed from the femurs, tibias, iliac crests and humeri of 6- to 10-week-old female C57BL/6 mice (Harlan). Erythrocytes were eliminated via the isotonic ammonium chloride shock procedure in ice and a subsequent centrifugation. The isolation of lineage depleted (Lin^-) bone marrow cells was performed with a mouse hematopoietic progenitor cell enrichment kit (BD Biosciences) according to the manufacturer's protocol. Lin^- cells were incubated either with APC-labelled streptavidin (BD Biosciences), APC-eFluor®780-conjugated rat anti-c-kit (clone ACK2, eBioscience) and PE-Cy™7-labelled rat anti-Sca1 (clone D7, BD Pharmingen) for LSK cell sorting, or with APC-labelled streptavidin, APC-eFluor®780-conjugated rat anti-c-kit, PE-Cy™7-labelled rat anti-Sca1, FITC-labelled rat anti-CD34 (clone RAM34, eBioscience) and PerCP-Cy™5.5-labelled rat anti-CD16/CD32 (clone 93, eBioscience) antibodies for MEP and GMP cell sorting. Progenitor cells of bone marrow were purified with a FACSAria instrument (Becton Dickinson) as follows: LSK ($Lin^-c\text{-}kit^+Sca1^+$),

MEP ($Lin^-c\text{-}kit^+Sca1^-CD34^-CD16/CD32^-$) and GMP ($Lin^-c\text{-}kit^+Sca1^-CD34^+CD16/CD32^+$). Fluorescence-minus-one controls were developed to optimize the identification of LSK, MEP or GMP progenitor cells. Subsequently, sorted cells were incubated in HSC expansion medium (StemSpan™ SFEM) supplemented with 10% FBS (Stem Cell), 50ng/ml of SCF and 10ng/ml of murine recombinant TPO, IL3, IL6, Flt3L at 37°C and 5% CO_2 for 24 hours until infection. For MEP progenitor cells, 2U/mL of EPO were also added.

Bone marrow immunophenotyping by flow cytometry and blood sample analysis of TgSpi1 mice

Single cell suspensions of freshly collected bone marrow cells were immuno-stained either with APC-eFluor®780-conjugated rat anti-c-kit (clone ACK2, eBioscience), FITC-conjugated rat anti-CD71, APC-conjugated rat anti-Ter119 and PE-conjugated streptavidin/biotin conjugated rat anti-CD123 antibodies for detection of erythroid progenitor cells (CFU-E), as previously described,(3) or with PerCP-Cy™5.5-conjugated rat anti-CD11b (clone M1/70, eBioscience), PE-Cy™7-conjugated rat anti-Gr1 (Ly-6C and Ly-6G, clone RB6-8C5, eBioscience), antibodies for detection of myeloid hematopoietic cells. Stained cells were analyzed with a LSRII flow cytometer (BD Biosciences) or a Fortessa analyzer (BD Biosciences), and FlowJo (Tree Star) was used for data acquisition and analysis.

Peripheral blood was collected from mice via their submandibular vein into EDTA-coated tubes, and cells were counted on a MS9-5 hematology counter (Melet Schloesing Laboratory).

Virus preparation and infection of primary fibroblasts

Retroviral production was performed by co-transfection of 3.3μg of each VSVg, Gag/Pol and empty vectors, PLPC, PLPC- $HRAS^{V12}$ or PLPC-Spi1 into sub-confluent HEK293EBNA cells using Jetprime™ (PolyPlus). The viral supernatants were used to infect human primary

fibroblasts with 4μg/mL of polybrene for 24 hours before adding puromycin at 4μg/mL. Two days later, cells were seeded at 10^4 cells/mL and counted at different times. SA-βgal activity assays and protein extractions were performed at day 14.

Virus preparation and infection of LSK, MEP and GMP progenitor cells

The viruses were prepared by co-transfection of 3.3μg of each VSVg, Gag/Pol and MSCV-IRES-GFP empty, MSCV-Spi1-IRES-GFP or MSCV-HRAS^{V12}-IRES-GFP vectors into sub-confluent HEK293EBNA cells using Jetprime™ (PolyPlus). Fresh viral supernatants supplemented with 4μg/mL of polybrene were used twice to infect primary murine LSK cells over 48 hours. Cells were harvested, washed with PBS and resuspended in HSC expansion medium (StemSpan™ SFEM, Stem Cell) with 10% FBS (Stem Cell) and 50ng/ml of SCF and 10ng/ml of murine recombinant TPO, IL3, IL6, Flt3L at 37°C (corresponding to day 0).

The percentage of GFP-positive cells was evaluated for each condition at 4 time points (Days 0, 3, 5 and 7) with a LSRII flow cytometer (BD Biosciences), using non-infected cells as control. GFP analyses were performed using FlowJo software (Tree Star). The numbers of living cell were determined on days 0, 3, 5 and 7 using trypan. On day 6, the GFP-positive cells in each group were sorted using a FACSAria instrument (BD Biosciences) and were kept for 24 hours before being used to evaluate the SA-βgal activity and for protein extraction.

When used, SB203580 (Sigma) was added at days 0 and 5 post-infection.

To infect primary murine MEP and GMP progenitor cells, similar protocols were used except that infection was performed once over 24 hours. For MEP progenitor cells, 2U/mL of EPO were also added. The SA-βgal activity and RNA expression was evaluated at days 4 or 6 post-infection on GFP$^+$ sorted MEP and GMP, respectively.

Senescence-associated β–galactosidase staining assay (SA-βgal) using cytochemical staining

The senescence response of the fibroblasts, LSK, MEP and GMP progenitor cells was confirmed by determining the expression of SA-βgal activity as described previously. (4) The images were captured using a 40X objective lens on a Nikon Eclipse TE300 microscope (Nikon) equipped with a Nikon DS-Fi1 camera or with an AxioCam MRc color video camera (Zeiss), digitally saved using Axiovision version 4 software (Zeiss), and further processed using Photoshop software (Adobe Systems France, Paris, France). More than 1000 positive cells showing SA-βgal staining were counted in 9 randomly selected fields for each group of fibroblasts and LSK cells.

SA-βgal staining assay using flow cytometry combined or not with immunophenotyping

We followed a previously described protocol (5) using 5-dodecanoylaminofluorescein di-beta-D-galactopyranoside (C_{12}FDG) for fibroblasts or dodecylresorufin β-D-galactopyranoside (C_{12}RG) (Invitrogen) for hematopoietic cells as the fluorogenic substrate and combined or not with immunophenotyping. The isolated cells were incubated 2h with the C_{12}RG probe at 60μM. Next, cells were washed with PBS containing 2% FBS and labeled either with antibodies against c-kit (eBioscience, APC-eFluor®780-conjugated) and Sca1 (BD Pharmingen, PE- Cy™7-conjugated), or with antibodies against CD11b (eBioscience, PerCP-Cy™5.5-conjugated), Gr1 (eBioscience, PE-Cy™7-conjugated) and F4/80 (eBioscience, clone BM8, APC™-conjugated). To exclude dead cells from analysis, DAPI was used. Stained cells were analyzed with a Fortessa analyzer (BD Biosciences), collecting 10000-50000 events per sample. FlowJo (Tree Star) was used for data acquisition and analysis.

The detection of SA-β-gal activity in MEP and GMP progenitor cells of TgSpi1 mice was performed as described above for LSK on Lin^- bone marrow cells. Isolated Lin^- bone marrow cells were stained 2h with the C_{12}RG probe at 60μM. Next, cell suspensions were washed with PBS containing 2% FBS and labelled with antibodies against c-kit (eBioscience, APC-

eFluor®780-conjugated), Sca1 (BD Pharmingen, PE- Cy™7-conjugated), CD34 (eBioscience, FITC-conjugated) and against CD16-CD32 (eBioscience, PerCP- Cy™5.5-labelled). Stained cells were analyzed with a Fortessa analyzer (BD Biosciences), and FlowJo (Tree Star) was used for data acquisition and analysis.

SA-βgal staining assay of bone marrow sections

The SA-βgal staining of bone marrow sections was performed using a previously described protocol (5) with modifications. Briefly, femurs of mice were flash frozen in liquid nitrogen, sectioned (7-10 sections/tissue sample), and the sections were fixed with 1% formaldehyde for 1 min before being washed twice with PBS for 3 min. The fixed sections were incubated with the staining solution as described above for 16h at 37°C in dark. The sections were washed with PBS and fixed in 4% paraformaldehyde for 10 min and stained with a nuclear fast red solution (0.1% nuclear fast red in 5% aluminum sulfate). The sections were dried and sealed for imaging.

Peripheral blood smears

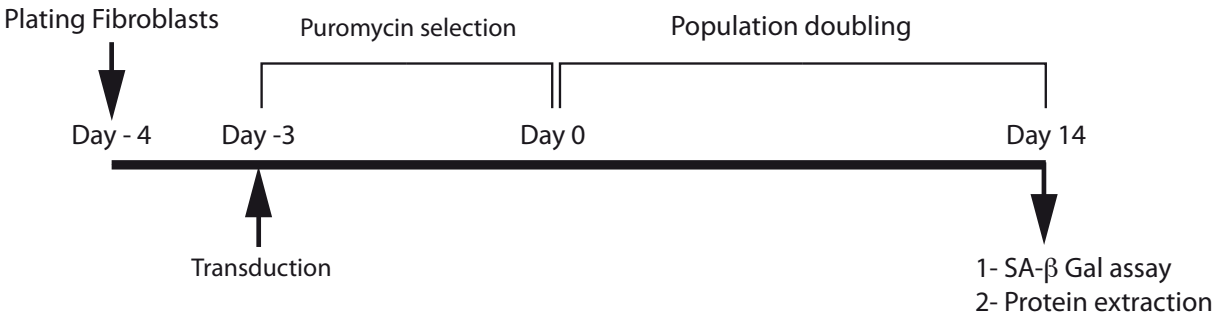
Peripheral blood smears were prepared using May-Grunwald Giemsa staining and examined by light microscopy**.** The images were captured with a 40X objective lens on the Olympus Provis 50 microscope (Olympus Optical) equiped with a charge-coupled device color video camera (Hamamatsu), digitally saved using Archimed-Pro software (Microvision Instruments), and further processed using Photoshop software (Adobe Systems France).

Immunohistochemistry

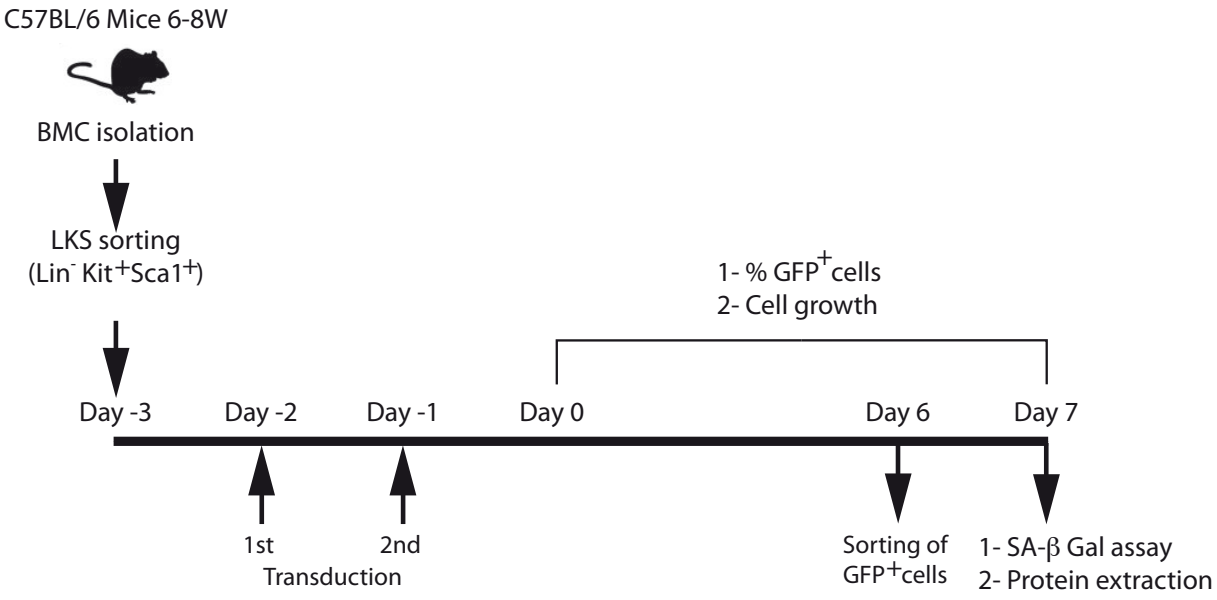
Following fixation of the mouse femurs in 4% paraformaldehyde, paraffin sections (4 μm thick) were xylene treated then rehydrated. Heat-induced epitope retrieval was achieved with pH6 citrate buffer at 98°C for 30 min. Endogenous peroxidase activity was quenched by

3% H_2O_2 for 10 min. The sections were placed in coverplates (Shandon) and incubated with blocking serum then with a rabbit anti-Spi1 antibody (Cell Signaling, clone 9G7, dilution 1/75) in blocking serum (1/10) for 1 hour. The detection was performed using the Rabbit PowerVision kit (ImmunoVision Technologies) and the signal was revealed with DAB peroxidase substrate solution. Cell nuclei were counterstained with Mayer's hematoxylin solution and finally, the sections were dehydrated and mounted (Diamount). Bone marrow sections from each animal were scanned using a virtual slide system (VS120, Olympus) with a 20X objective lens.

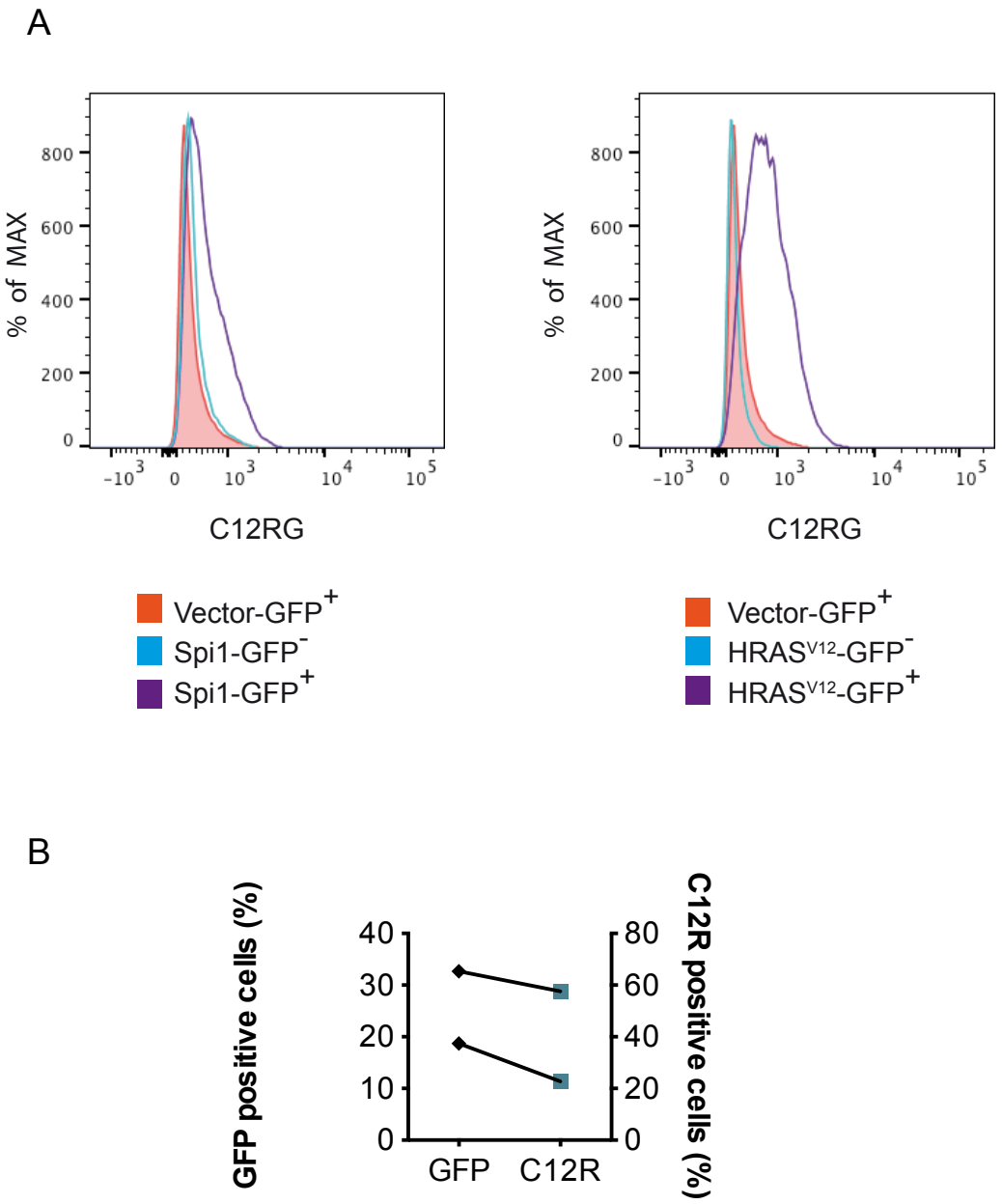
Supplementary Figures



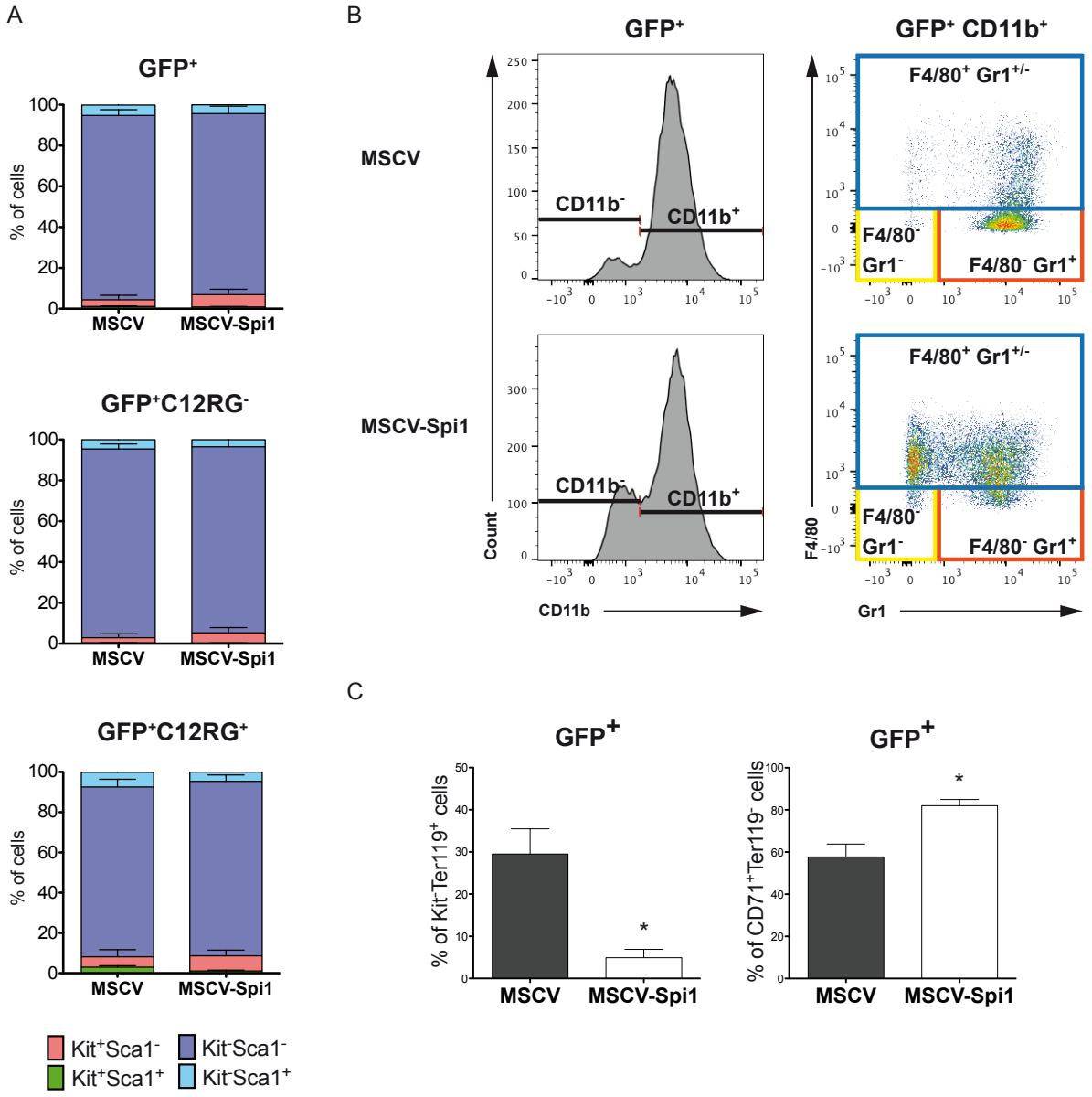
Supplementary Figure S1. Design of experiments and time frame of analysis in primary fibroblasts, BJ and WI38. At day 0, cells were plated at 10^4 cells/mL. Population doubling was calculated from day 0 to day 14. SA-βgal assay and protein extracts were prepared at day 14.



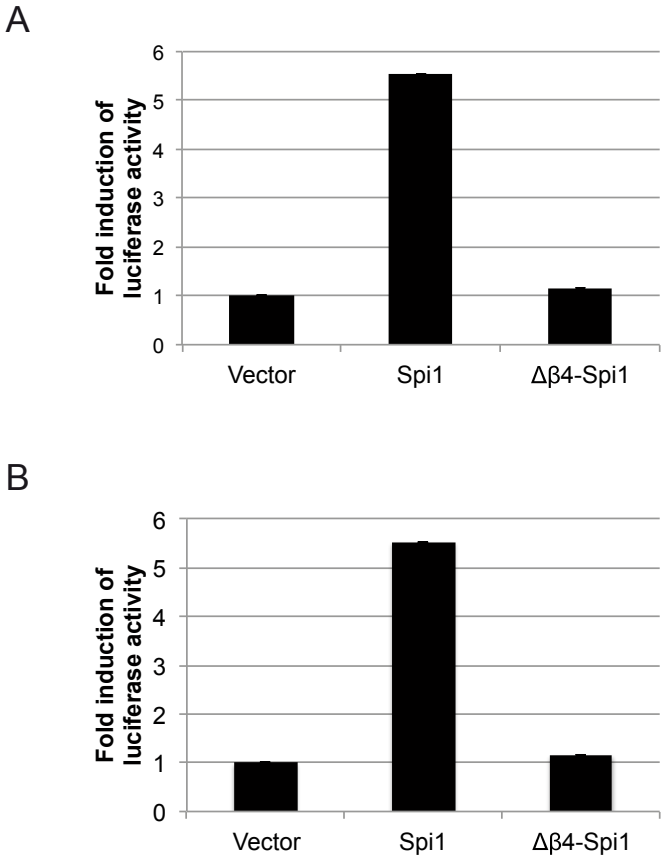
Supplementary Figure S2. Design of experiments and time frame of analysis in murine LSK cells. At day 0, transduced cells were plated at $3x10^4$ cells/mL. The cell growth and percentage of GFP-positive cells were measured from day 0 to day 7. At day 6, GFP-positive cells were FACS-sorted and kept for 24 hours before being used to evaluate the SA-βgal activity and extract total proteins.



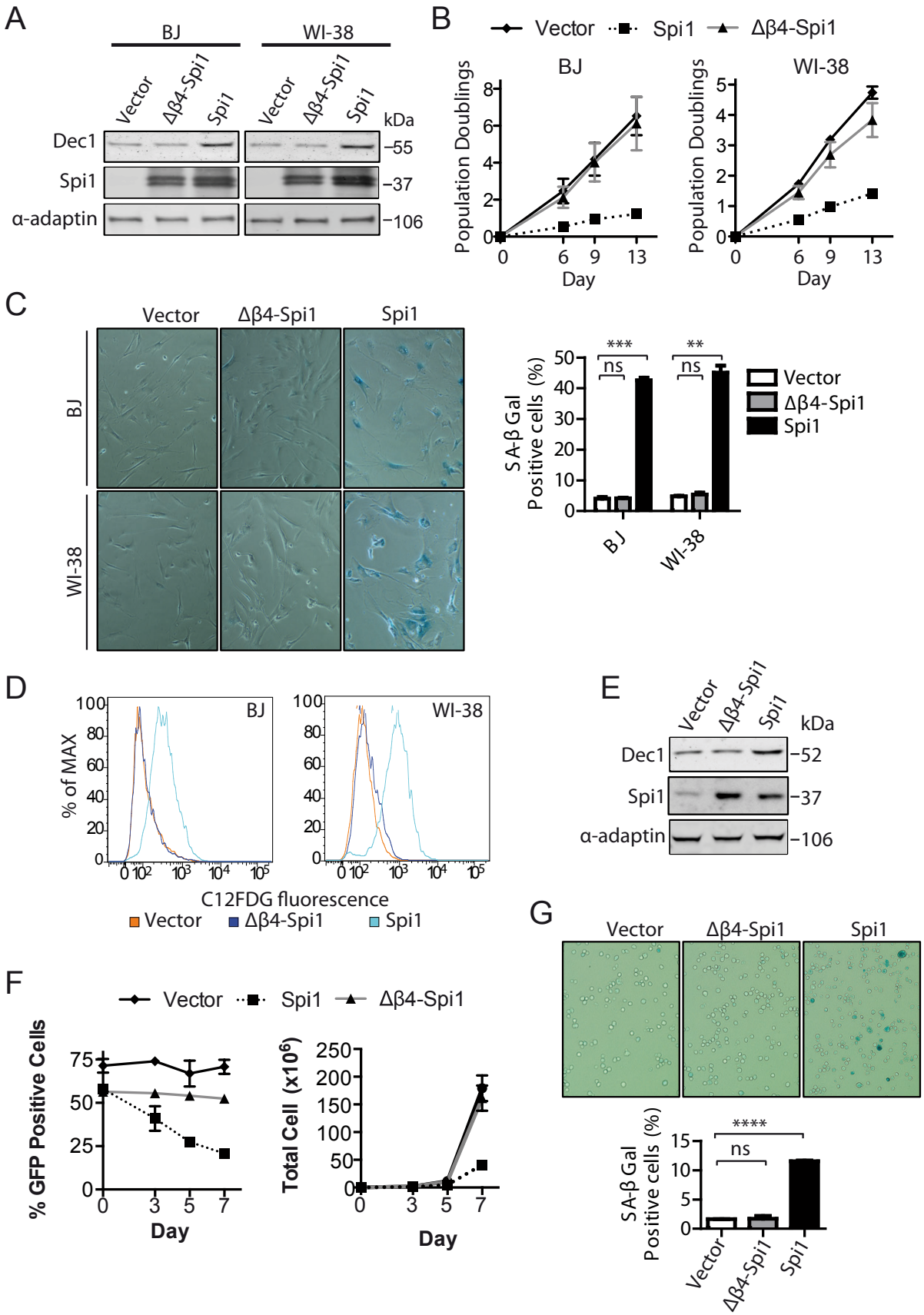
Supplementary Figure S3. (A) Representative flow cytometric detection of SA-βgal activity of cells subjected to retroviral-mediated expression of Spi1, $HRAS^{V12}$ or empty vector as a control. SA-βgal assays of GFP-positive and -negative gated cells were performed 7 days post infection using $C_{12}RG$ as a fluorogenic substrate. Only the GFP-positive cells of the control (MSCV-infected cells, Vector) is shown because there were a too small number of GFP-negative cells in that sample. (B) Percentage of senescent cells was measured using $C_{12}RG$ as a fluorogenic substrate and presented with the percentage of GFP-positive cells.



Supplementary Figure S4. Characterization of Spi1-induced senescent cells according to hematopoietic markers (A) Distribution of cells according to c-kit and Sca1 markers and SA-βgal activity using $C_{12}RG$ as fluorogenic substrate by flow cytometry among total GFP^+, $GFP^+C_{12}RG^-$ or $GFP^+C_{12}RG^+$, seven days after transduction of LSK cells with MSCV-Spi1 or MSCV control vectors. The means ± SEM of at least 3 independent experiments are shown. (B) Representative flow cytometry profiles based on the surface markers CD11b, Gr1 and F4/80, in cells overexpressing or not Spi1, seven days after transduction of LSK cells with MSCV-Spi1 or MSCV control vectors. Percentages are presented in the Figure 3A. (C) The histograms represent the % of engaged erythroid cells (Kit^- $Ter119^+$) and CFU-E ($CD71^+Ter119^-$) analysed by flow cytometry among GFP^+ sorted cells, four days after transduction of MEP with MSCV-Spi1 or MSCV control vectors. The means ± SEM of 2 independent experiments performed in duplicate are shown. **P*<0.05 from unpaired two-tailed Student's t tests.



Supplementary Figure S5. Dual luciferase reporter assay of the *Fes* gene promoter in HeLa cells co-transfected with Spi1 expression vector, pfes-LucF and LucR normalisation vector. The effect of PLPC (A) or MSCV (B) WT Spi1 or Δβ4-Spi1 protein expression vectors on transcription as measured by dual luciferase assay. The activity of luciferase was measured by the relative light unit, normalized to the transfection efficiency (LucR). The fold induction presented by the histograms is relative to the luciferase activity obtained in the control cells transfected with the empty expression vector.


A
BJ
WI-38
Vector
Δβ4-Spi1
Spi1
kDa
Dec1
Spi1
α-adaptin
-55
-37
-106
B
Vector
Spi1
Δβ4-Spi1
BJ
WI-38
Population Doublings
Day
C
Vector
Δβ4-Spi1
Spi1
BJ
WI-38
SA-β Gal Positive cells (%)
***
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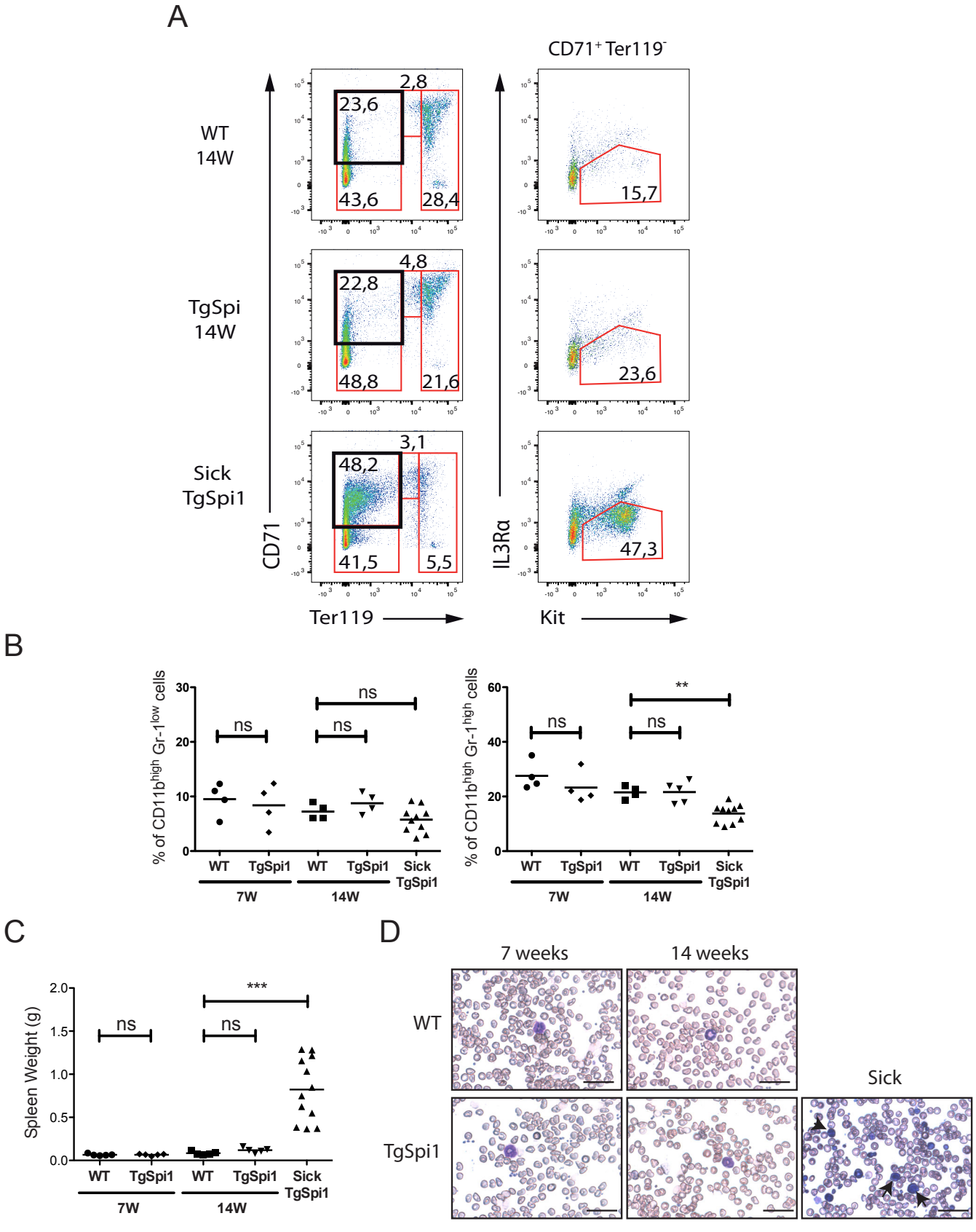
ns
D
% of MAX
C12FDG fluorescence
E
Dec1
Spi1
α-adaptin
-52
-37
-106
F
% GFP Positive Cells
Total Cell (x10^6)
Day
G
SA-β Gal Positive cells (%)
****

Supplementary Figure S6. Spi1 requires its DNA binding function to trigger premature senescence.

(A) Western blot analysis of Spi1 and Dec1 in BJ and WI-38 cells subjected to the retroviral-mediated expression of Spi1, Δβ4-Spi1 or an empty vector as a control 10 days after puromycin selection. α-Adaptin served as the loading control. (B) Population doublings (PDs) of BJ (left panel) and WI38 (right panel) cells transduced as described in (A) over the indicated periods of time. Day 0 was the first day after puromycin selection. PDs for each time point are the mean value of triplicates experiments. (C, G) Mean percentages of SA-βgal positive cells subjected to the retroviral-mediated expression of Spi1, Δβ4-Spi1 or an empty vector, 10 days after puromycin selection in fibroblasts and 7 days post-infection in GFP-positive sorted hematopoietic cells, respectively. Magnification of images, 200X. The means ± SD of at least 3 independent experiments are shown. ***P*<0.005; ****P*<0.0005; *****P*<0.0005 from two-tailed Student's t tests. (D) Flow cytometric detection of SA-βgal activity using C_{12}FDG as a fluorogenic substrate in fibroblast cells. (E) Western blot analysis of Spi1 and Dec1 in hematopoietic cells subjected to the retroviral-mediated expression of Spi1, Δβ4-Spi1 or an empty vector after 7 days of infection. α–Adaptin served as the loading control. (F) Percent of GFP-positive cells and the number of total living cells retrovirally transduced with Spi1 and Δβ4-Spi1 or an empty vector (control) at the indicated periods of time. The means ± SD of at least 3 independent experiments are shown.


A
CD71+ Ter119-
WT 14W
TgSpi 14W
Sick TgSpi1
2,8
23,6
43,6
28,4
15,7
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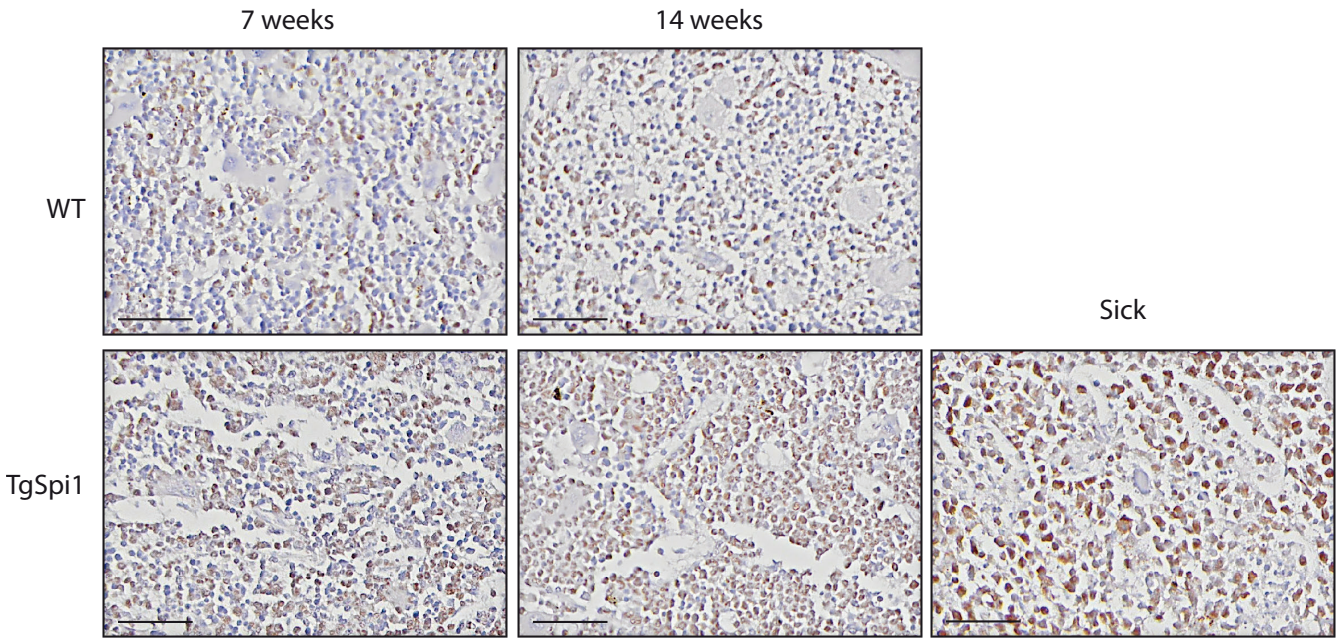
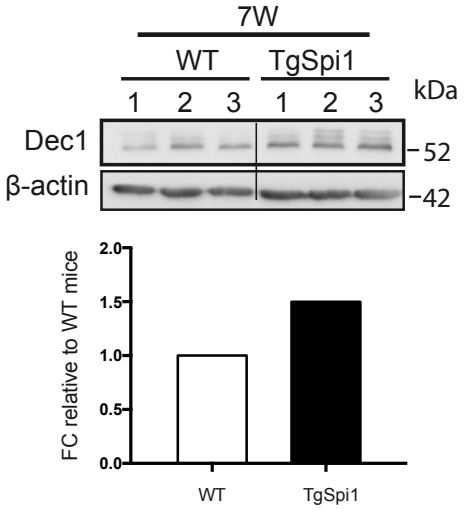
22,8
48,8
21,6
23,6
3,1
48,2
41,5
5,5
47,3
CD71
Ter119
IL3Rα
Kit
B
% of CD11b^high Gr-1^low cells
% of CD11b^high Gr-1^high cells
ns
**
WT
TgSpi1
Sick TgSpi1
7W
14W
C
Spleen Weight (g)
***
D
7 weeks
14 weeks
WT
TgSpi1
Sick

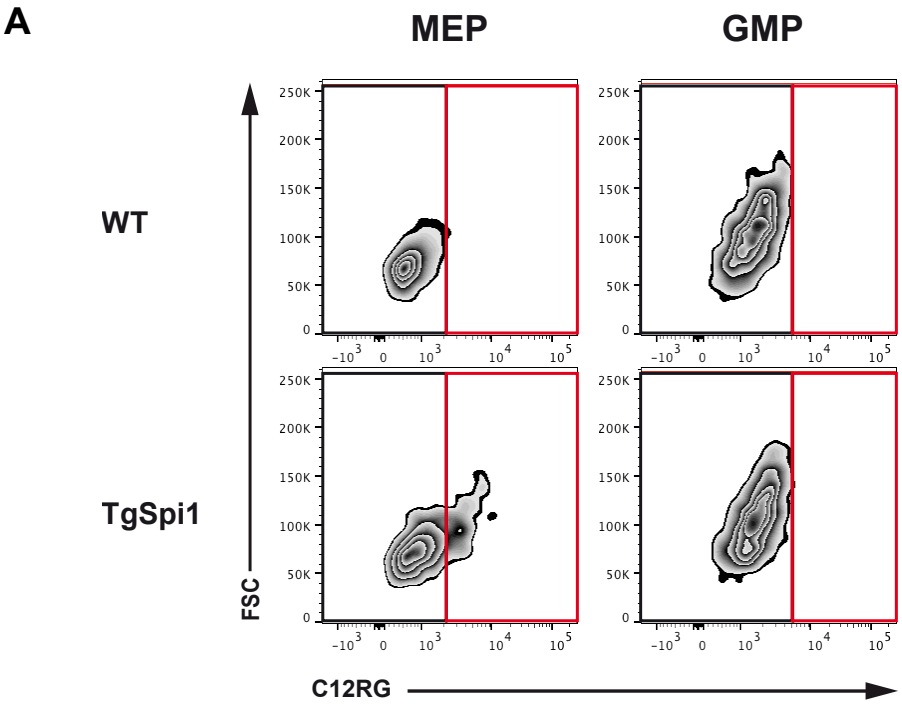
Supplementary Figure S7. Characterization of bone marrow and blood cells of WT and TgSpi1 mice. (A) Representative flow cytometry density plots showing the $CD71^{+}Ter119^{-}Kit^{+}IL3R\alpha^{-}$ (CFU-E) staining in bone marrow cells of 14-weeks-old WT and TgSpi1 mice and sick TgSpi1 mice. Bone marrow cells expressing high levels of CD71 and low level of Ter119 (left panels, black box) were investigated for IL3Rα and Kit expression (right panels, red box). Percentages of each population within total alive bone marrow cells (left panels) or within $CD71^{+}Ter119^{-}$ population (right panels) are shown (B) Scatter dot plots represent the flow cytometry analysis of markers for population enriched in monocytes ($CD11b^{high}Gr1^{low}$) or granulocytes ($CD11b^{high}Gr1^{high}$) in whole bone marrow of WT and TgSpi1 mice at the indicated ages. Each symbol represents one animal. Bars indicated the mean values. Ns: non significant; *P<0.05; ** P <0.001; **** P <0.0001 from two-tailed Student's t tests. (C) Scatter dot plots show the means of the spleen weight of 7- and 14-weeks-old WT and TgSpi1 mice and sick TgSpi1 mice. Each symbol represents one animal. *** P<0,001 ; ns, non significant from two-tailed Student's t tests. (D) Representative pictures of peripheral blood smeared slides, stained with May-Grünwald Giemsa. Scale bars represent 25μm.



Supplementary Figure S8. Expression of Spi1 in the bone marrow cells. Representative views of immunohistochemical staining of Spi1 (brown staining) in the bone marrow sections of WT and non sick TgSpi1 mice of 7- or 14-weeks-old and sick TgSpi1 mice. Cell nuclei were counterstained with hematoxylin (blue staining). Scale bars represent 50 μm.



Supplementary Figure S9. Dec1 protein level in bone marrow cells from WT mice and non sick TgSpi1 mice aged of 7 weeks analyzed by Western blotting. The histograms represent the quantified results, using ImageJ, relative to β-actin and to values for WT mice.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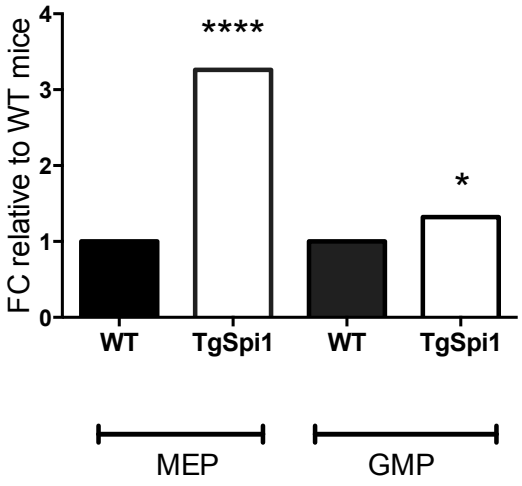
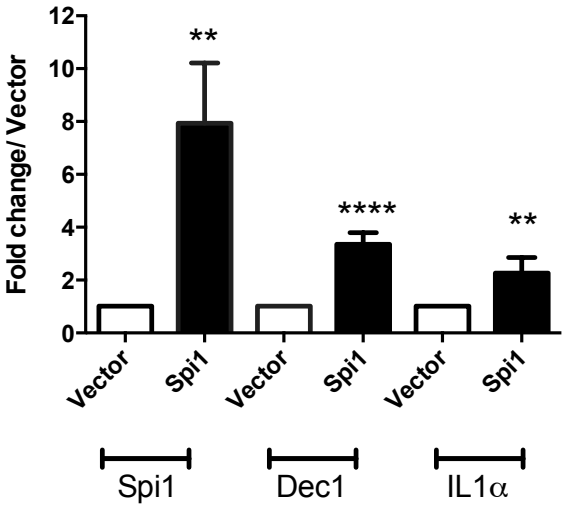
MEP
GMP
WT
TgSpi1
FSC
C12RG


B
FC relative to WT mice
****
*
WT
TgSpi1
WT
TgSpi1
MEP
GMP


C
Fold change/ Vector
**
****
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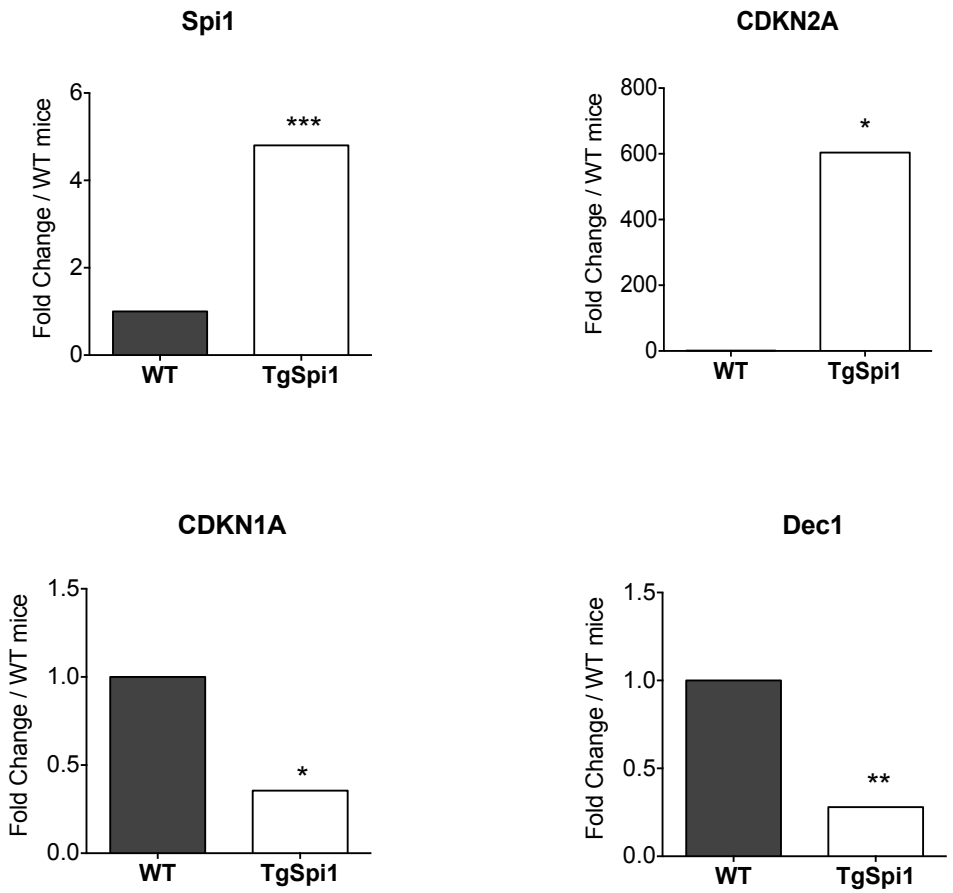
Vector
Spi1
Vector
Spi1
Vector
Spi1
Spi1
Dec1
IL1α

Supplementary Figure S10. (A) Representative flow cytometry density plots showing the $C_{12}RG^-$ (black square) and $C_{12}RG^+$ (red square) labeling among MEP ($Lin^-Sca^-Kit^+CD34^-CD16/32^-$) and GMP ($Lin^-Sca^-Kit^+CD34^+CD16/32^+$) cells from bone marrow of 7-week-old WT and non sicked TgSpi1 mice. The WT mice were used to define $C_{12}RG^-$. (B) *Spi1* mRNA levels in sorted MEP and GMP cells from bone marrow from 7-week-old WT (N=2) and non sicked (N=2) TgSpi1 mice. Bars represent the fold change relative to levels in WT mice, as calculated from the $2^{-\Delta\Delta Ct}$ values (ΔCt, Ct target gene-CtPolr2α). Statistical analysis of the $2^{-\Delta Ct}$ values was carried out using Student's *t* test; * $P<0.05$, **** $P<0.0001$. (C) *Spi1, Dec1 and IL1α* mRNA levels in MEP and in GMP progenitor cells subjected to the retroviral-mediated expression of Spi1 (MSCV-Spi1) or empty vector as a control (MSCV) were analysed 4 days post-infection. mRNA levels were quantified using real-time qPCR and normalized to the *Polr2α* and GAPDH mRNA level (FC of the $2^{-\Delta Ct}$, Ct target gene-Ct housekeeping gene, relative to MSCV). Statistical analysis was carried out on the $2^{-\Delta Ct}$ values using Student's *t* test; ** $P<0.01$; **** $P<0.0001$.



Supplementary Figure S11. *Spi1*, *CDKN2A, CDKN1A and Dec1* mRNA levels in sorted CFU-E ($CD71^{+}Ter119^{-}Kit^{+}IL3R\alpha^{-}$) cells of bone marrow from 14-week-old WT (N=9) and sick TgSpi1 (N=8) mice. mRNA levels were quantified using real-time qPCR and normalized to the *Polr2α* mRNA level. Bars represent the fold change relative to levels in WT mice, as calculated from the $2^{-\Delta\Delta Ct}$ values (ΔCt, Ctgene-CtPolr2α). Statistical analysis of the $2^{-\Delta Ct}$ values was carried out using Student's *t* test; * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

REFERENCES

1. Guillouf C, Gallais I, Moreau-Gachelin F. Spi-1/PU.1 oncoprotein affects splicing decisions in a promoter binding-dependent manner. J Biol Chem. 2006 Jul 14;281(28):19145-55.
2. Rimmele P, Kosmider O, Mayeux P, Moreau-Gachelin F, Guillouf C. Spi-1/PU.1 participates in erythroleukemogenesis by inhibiting apoptosis in cooperation with Epo signaling and by blocking erythroid differentiation. Blood. 2007 Apr 1;109(7):3007-14.
3. Ridinger-Saison M, Evanno E, Gallais I, Rimmele P, Selimoglu-Buet D, Sapharikas E, et al. Epigenetic silencing of Bim transcription by Spi-1/PU.1 promotes apoptosis resistance in leukaemia. Cell death and differentiation. 2013 Sep;20(9):1268-78.
4. Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, et al. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proceedings of the National Academy of Sciences of the United States of America. 1995 Sep 26;92(20):9363-7.
5. Debacq-Chainiaux F, Erusalimsky JD, Campisi J, Toussaint O. Protocols to detect senescence-associated beta-galactosidase (SA-betagal) activity, a biomarker of senescent cells in culture and in vivo. Nature protocols. 2009;4(12):1798-806.