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Supplemental Information

$p19^{INK4d}$ Controls Hematopoietic Stem Cells in a Cell-Autonomous Manner during Genotoxic Stress and through the Microenvironment during Aging

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Supplemental figures and legends

Figure S1

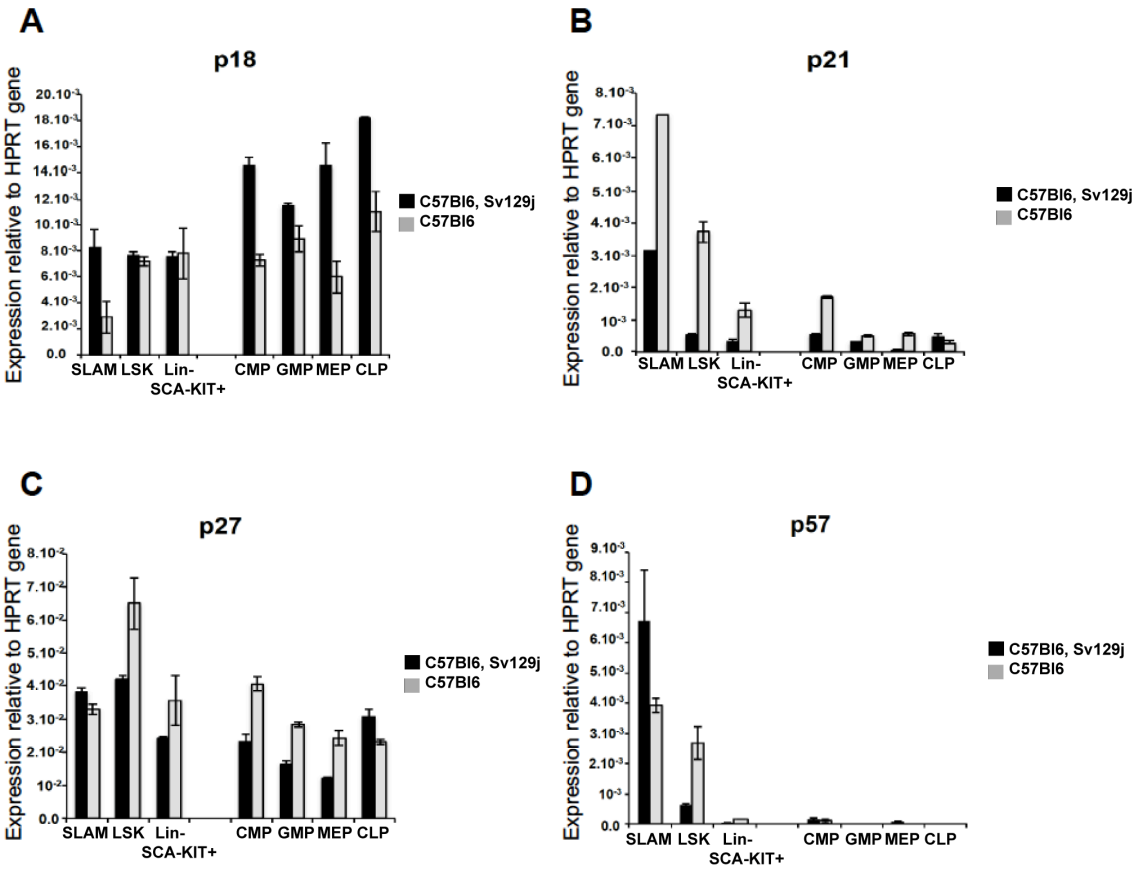

Figure S1, related to Figure 1. Expression of different CDKIs in C57Bl6 and C57Bl6/Sv129J mice.

(A-D) mRNA expression levels of *p18*^{Ink4c} **(A)**, *p21*^{CIP1} **(B)**, *p27*^{KIP1} **(C)** and *p57*^{KIP2} **(D)** measured by qRT-PCR in different progenitor cell populations of C57BL/6 and mixed C57BL/6-Sv129J mice. SLAM ($Lin^-SCA1^+C\text{-}KIT^+CD48^-CD150^+$), LSK ($Lin^-SCA1^+C\text{-}KIT^+$), myeloid progenitors ($Lin^-SCA1^-C\text{-}KIT^+$), CLP ($Lin^-SCA1^{low}C\text{-}KIT^{low}CD127^+THY\text{-}1^-$), CMP ($Lin^-SCA1^-C\text{-}KIT^+FC\gamma R^-CD34^+$), GMP ($Lin^-SCA1^-C\text{-}KITt^+FC\gamma R^+CD34^+$) and MEP ($Lin^-SCA1^-C\text{-}KIT^+FC\gamma R^-CD34^-$) populations were sorted by flow cytometry. Each population

represents a pool derived from 10 mice. Data are normalized to *HPRT* transcript levels and represent the mean ± SEM of biological triplicate experiments.

Figure S2

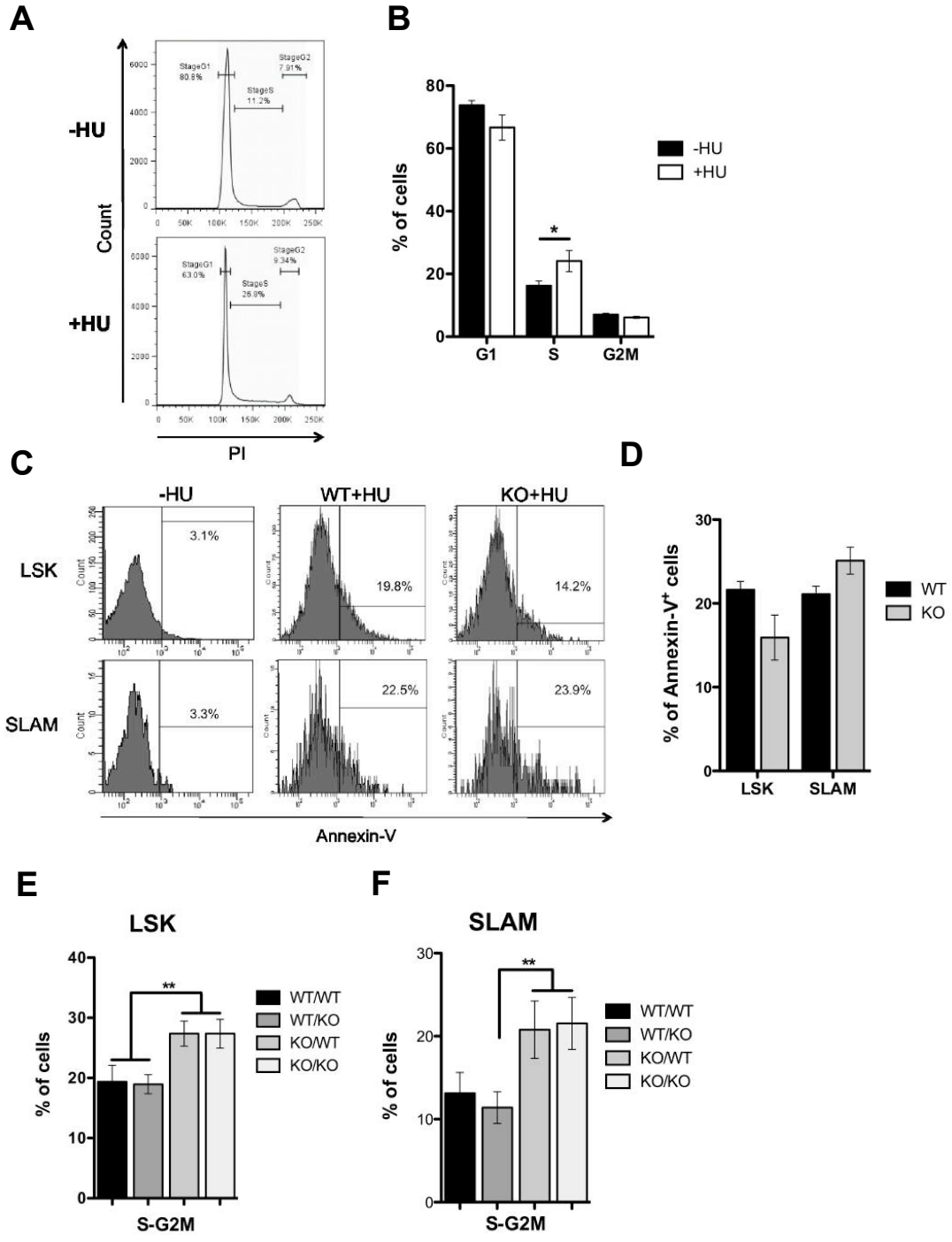

Figure S2, related to Figure 3. Cell cycle analysis during genotoxic and replicative stress.

(A, B) Cell cycle analysis by PI staining of Lin$^-$ cells in mice with or without HU treatment. Representative cell cycle histogram **(A)** and frequencies of cells in different phases of the cell

cycle **(B)**. Data represent mean ± SEM (n=7). **(C, D)** Immunophenotype **(C)** and apoptosis (annexin-V-positive cells) **(D)** in LSK and SLAM populations of WT and KO mice after HU treatment. **(E, F)** Cell cycle analysis by Ki-67/Hoechst co-staining two days after 5-FU injection. Frequencies of S-G2M phases in LSK **(E)** and SLAM **(F)** populations in 2 month-old transplanted WT and KO mice. Data represent mean ± SEM (n=6). *p<0.05, **p<0.01. WT: wild type, KO: $p19^{INK4d-/-}$, unpaired *t* test.

Figure S3

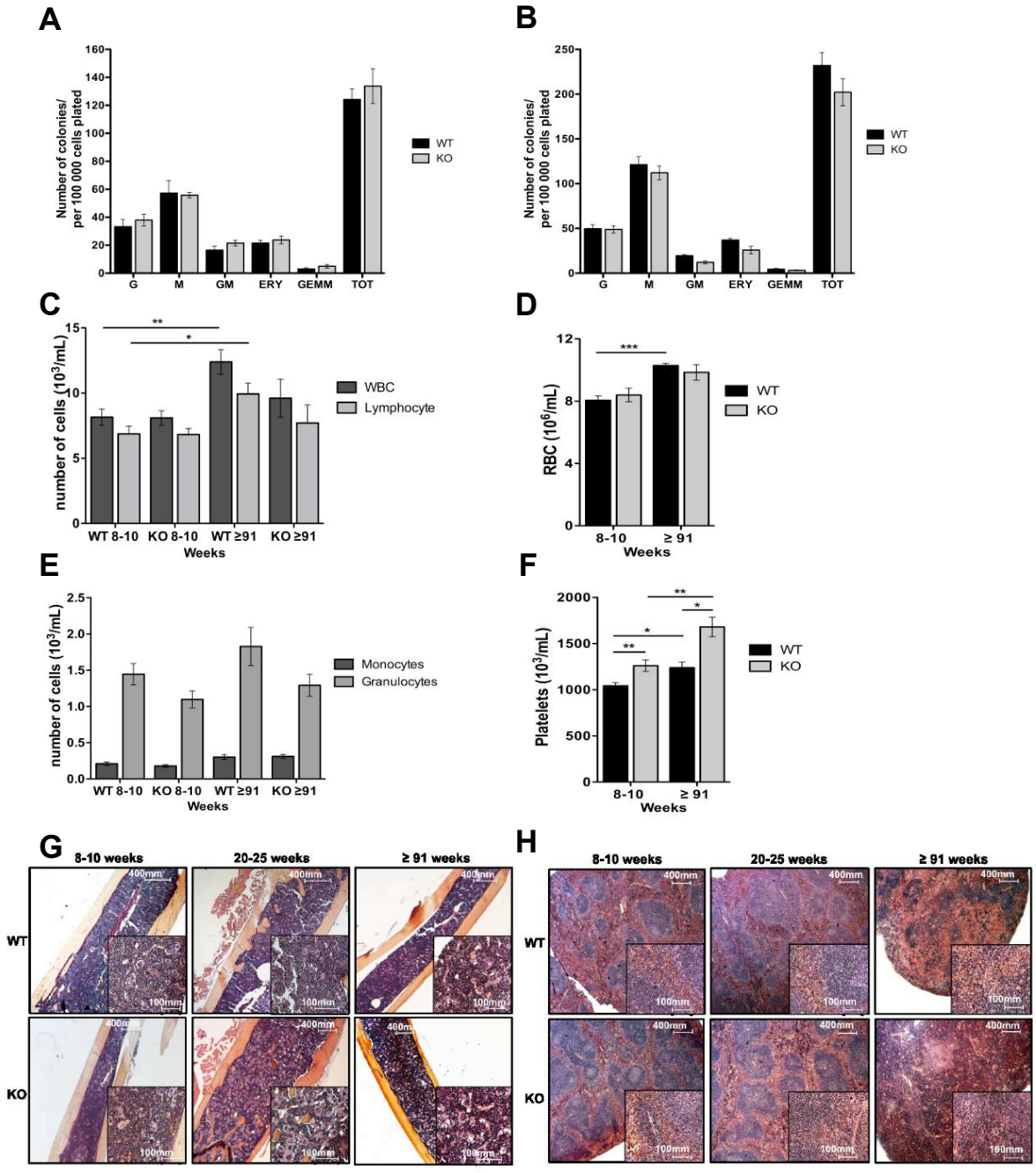

Figure S3, related to Figure 5. Assessment of myeloid progenitors and whole blood cell populations in WT and *p19*$^{INK4d-/-}$ mice during aging.

(A, B) Progenitor assays in methylcellulose cultures of BM cells from 8-10 week-old **(A)** and > 48 week-old **(B)** WT and KO mice. Experiments were performed in triplicate for each biological replicate. Error bars represent mean ± SEM, n=4. G= Granulocyte colony, M=

Monocytes/Macrophage colony, GM= Granulo-Macrophage colony, ERY= Erythroid colony, GEMM= Granulocyte, Erythroid, Megakaryocyte, Monocyte/Macrophage colony, TOT= total colony number. **(C-F)** Whole blood cell analysis in 8-10 (n=10) and > 91 (n=4) week-old WT and KO mice. Data represent mean ± SEM **(C)** White blood cell (WBC) and lymphocyte counts, **(D)** Red blood cell (RBC) counts, **(E)** Monocyte and granulocyte counts, **(F)** Platelet counts, **(G, H)** HES staining of BM **(G)** and spleen **(H)** from 8-10 (n=4), 20-25 (n=5) and > 91 (n=3 for WT and n=8 for KO) week-old WT and KO mice. Representative picture of BM and spleen of one mouse is shown.

WT: wild type, KO: $p19^{INK4d-/-}$,*p<0.05, **p<0.01, ***p<0.001, unpaired *t* test.

Figure S4

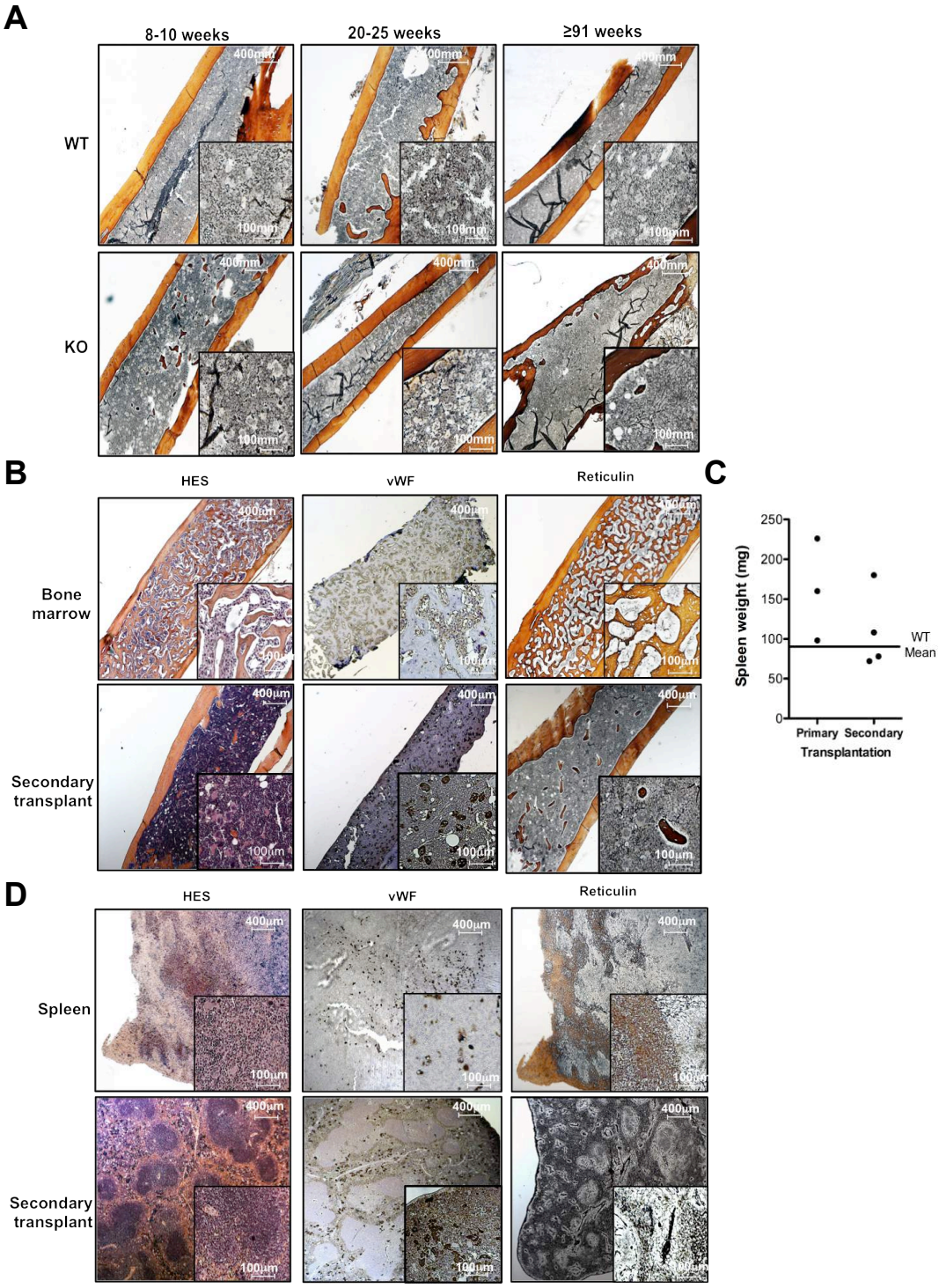

Figure S4, related to Figure 6. Progressive fibrosis development with age in the absence of p19^{INK4d}

(A) Reticulin staining of BM in 8-10, 20-25 and > 91 week-old mice. Representative picture of reticulin staining in BM of one mouse is shown, **(B-D)** Engraftment of a 4g fibrotic spleen in primary and secondary recipients. Representative pictures of HES, vWF and reticulin staining in BM **(B)** and spleen **(D)** of a KO mouse with a 4g fibrotic spleen (upper pictures) and of one secondary recipient (lower pictures). Spleen weights in primary (n=3) and secondary recipients (n=4) **(C)**. Each dot represents one mouse. The bar indicates the mean weight (WT).

Supplemental Experimental Procedures

Mice experimentation

Eight to twelve week-old WT or *p19*$^{INK4d-/-}$ mice were used as recipients for transplantation assays. Recipient mice were irradiated at lethal (11 Gy) or sublethal (10.5 Gy) doses. Cells were injected in a volume of 100 or 200 μL into the retro-orbital plexus 24 h after irradiation. 5-Fluorouracil (5-FU) or bromodeoxyuridine (BrdU) were injected intra-peritoneally in a volume of 100 μl/10 g body weight. Peripheral blood (100 μL) was collected under anesthesia with 5% isofluorane from the retro-orbital plexus into heparin-coated capillaries and suspended in 3% citrate. Nucleated cells, differential cell counts, hematocrit levels and platelet counts were determined using an automated blood counter (MS9, Melet Schloessing, Cergy-Pontoise, France). Bone marrow (BM) and spleen cells were collected post-euthanasia by carbon dioxide and processed immediately. BM cells were collected by flushing two femurs and two tibias of individual mice with PBS (1X) supplemented with 5% fetal calf serum (FCS) using a 28G needle. Cellularity was calculated using the four bones of mice. Spleen cells were obtained after grinding half a spleen and cellularity was calculated by reporting the half spleen measure to the entire spleen.

Flow Cytometry

To evaluate the distribution or to sort cells within the LSK compartment, BM cells were resuspended at 10^8 cells/mL and incubated with a lineage Lin$^-$ cocktail «biotin mouse lineage panel» against B220, GR-1, MAC-1, CD3, and TER119 (CAS number: 559971, Pharmingen, San Diego, California). Cell were subsequently stained with biotin streptavidin-APC Cy7 (CAS number: 405208, BioLegend, UK) or a cocktail of B220 APC (Clone: RA3.6B2, CAS number: 103212, BioLegend, UK), GR-1 APC (Clone: RB6.8C5, CAS number: 108412, BioLegend, UK), MAC-1 APC (Clone: M1/70, CAS number: 101212, BioLegend, UK), CD3 APC (Clone: 17A2, CAS number: 100235, BioLegend, UK) and TER119 APC (CAS

number: 116212, BioLegend, UK) with anti-mouse C-KIT-PerCP5.5 (Clone: 2B8, CAS number: 105824, BioLegend, UK), SCA-PE Cy7 (Clone: D7, CAS number: 115904, BioLegend, UK), CD150-PE (Clone: TC15-12F12.2, CAS number: 103212, BioLegend, UK), CD48-APC (Clone: HM48-1, CAS number: 103412, BioLegend, UK) or pacific blue (Clone: HM48-1, CAS number: 103418, BioLegend, UK). Hematopoietic progenitor cells were stained with streptavidin-APC Cy7, SCA-PE-Cy7, C-KIT-PE Cy5.5, CD150-APC (Clone: TC15-12F12.2, CAS number: 115910, BioLegend, UK), CD105-Pacific blue (Clone: MJ7/18, CAS number: 120412, BioLegend, UK), FCγR-PE (Clone: 93, CAS number: 101308, BioLegend, UK). Apoptotic cells were stained with Annexin V-Pacific blue antibody (CAS number: 640918, BioLegend, UK). For all experiments, specific IgG related to each antibodies were used as negative control. Flow cytometry was performed using a FACS LSR II or FACS CANTO II (BD, Mountain View, CA) and data analysis was performed with DIVA software. To sort cells, MoFlo or Influx flow cytometers (BD, Mountain View, CA) were used.

For SP staining, BM cells were resuspended at 10^8 cells/mL and stained with the APC conjugated Lin^- cocktail, PE-Cy7 conjugated SCA, and PerCP5.5 conjugated C-KIT antibodies for 30 min at 4°C. Subsequently cells were washed in cold PBS (1X) and resuspended at 10^6 cells/mL in Dulbecco modified Eagle medium (DMEM) supplemented with 5% FCS, 10 mM HEPES and stained with 5 µg/mL Hoechst 33342 (CAS number: 23491-52-3, Sigma-Aldrich, St Louis, MO) for 90 minutes at 37°C. Cells were then resuspended in cold Hanks balanced salt solution (HBSS) containing 5% FCS. Analysis of SP cells was performed on a FACS LSR II (BD, Mountain View, CA).

Ploidy of MK was performed on flushed BM cells resuspended in 2 mL of 1/1 CATCH-PBS FCS 5% media and labeled with FITC–anti-CD41 mAb after preincubation with anti-CD16/CD32 Fc(III/II) antibody. Cells were then washed and incubated in a hypotonic citrate

solution containing 50 μg/mL propidium iodide (PI) (CAS number: 25535-16-4, Sigma-Aldrich, St Louis, MO) for at least 4 h at 4°C. Ploidy level was measured on a LSR II (BD, Mountain View, CA) cytometer.

For cell cycle analysis after HU treatment, Lin^- cells were isolated and resuspended in lysis buffer (Triton 0.1%, 1 mg/mL of sodium citrate) containing RNAse (20 mg/mL) and PI (20 mg/mL).

For cell cycle analysis of HSC, total BM cells were flushed in PBS (1X), resuspended at 10^8 cells/mL and stained for 30 min on ice with a Lin^- cocktail. Cells were washed and incubated with PE-Cy7 conjugated anti-SCA1, PerCP5.5 conjugated anti-C-KIT, PE conjugated anti-CD150, APC conjugated anti-CD48 and APC-Cy7 conjugated anti-streptavidin antibodies. Cells were then washed, incubated with a cytofix-cytoperm buffer for 15 min on ice and washed twice in 1X Permwash buffer (BD Bioscience, Strasbourg, France). Cells were then incubated with FITC conjugated anti-Ki-67 (CAS number: 556026, BD Bioscience, Strasbourg, France) diluted in 1X Permwash buffer for 45 min on ice. Following this, cells were washed in 1X Permwash buffer and stained for 30 min with Hoechst 33342 at room temperature (RT) and analyzed with a MoFlo cell sorter or a LSR II cytometer (BD, Mountain View, CA). For long-term BrdU kinetic experiments, mice were injected intra-peritoneally with BrdU (1 mg/6 g body weight, Sigma-Aldrich, St Louis, MO) and were allowed to freely drink water containing BrdU (1 mg/mL) for 21 days. At days 1, 4, 8, 11 and 21, mice were sacrificed and BM was flushed and LSK and SLAM cells were stained with previously described antibodies. BrdU incorporation was evaluated by using BrdU-FITC (BrdU intracellular staining kit, BD Bioscience, Strasbourg, France) antibody and Hoechst 33342 (10 mg/mL, Sigma-Aldrich, St Louis, MO) as DNA content.

Competitive transplantation

As the genetic background of our mice (C57BL/6 x Sv129J) is not associated with a polymorphism in Ly5, the donor cells were distinguished by using lentiviruses encoding GFP or Cherry. Lin^- cells from 8 week-old mice were isolated as previously described and transduced at day 0 in MEMα media supplemented with SCF (20 ng/mL), TPO (10 ng/mL), IL3 (10 ng/mL), IL6 (10 ng/mL) and FLT3 (20 ng/mL). Lin^- cells from WT mice were transduced with a sinPRRL lentivirus encoding GFP and cells from *p19*$^{INK4d-/-}$ mice with a sinPRRL lentivirus encoding CHERRY. At day 2, cells were sorted on the Lin^-GFP^+ (WT) or $Lin^-CHERRY^+$ (*p19*$^{INK4d-/-}$) and $5x10^4$ of both Lin^-GFP^+ and $Lin^-Cherry^+$ cells were re-injected retroorbitally into sublethally irradiated WT recipient mice. The number of injected SLAM cells per mouse was calculated based on the percentage of SLAM cells within the LSK population and of LSK cells within the Lin^- population.

Histology and immunohistochemistry

Freshly collected bones were fixed in 4% paraformaldehyde and embedded in paraffin, and sections (4 μm) were stained with hematoxylin eosin and safran. For VWF immunohistochemistry, paraffin sections were processed for heat-induced antigen retrieval (citrate buffer pH 6 for 30 min) and incubated for 1 h with VWF antibody (1/200; Dako). Staining was visualized using the peroxidase/diaminobenzidine Rabbit PowerVision kit (ImmunoVision Technologies). All slides were immunostained in cover plates the same day, ensuring a perfectly standardized intensity of staining. Argentafin reticular fibers staining was performed with a standard histological staining kit (Argentafin reticular fibers Kit; Diapath). The kit detects argyrophyl reticular fibers in connective tissue. Reticular fibers argyrophyl characteristics are given by the capacity to link argental salts, which once reduced as metal silver, have a typical black stain. Nervous and reticular fibers: black; Connective: brown; Collagen: yellow. Slides were examined using a Zeiss Axiophot microscope (Carl Zeiss, Le

Pecq, France) with 2.5×1 (magnification ×2.5) or 10×/1 (magnification ×10) numeric aperture objectives.

Quantitative real-time RT-PCR (qRT-PCR)

RNA was extracted with the RNeasy micro kit (Qiagen) and reverse transcription was performed with the superscript VILO kit (Invitrogen). Primers and internal probes for qRT-PCR were designed using primer 3 software. qRT-PCR was performed with an ABI 7500 Fast real-time PCR system using SYBR Green Universal Master mix containing specific primers. The expression level of all genes was expressed relative to *ML-32*, *HPRT* and *PPIA*. As the results were similar, only those relative to *HPRT* are presented. Probes and primers used in qRT-PCR assays are listed in **Table S**. Data were analyzed with 7500 Fast system SDS software 1.3.1. All experiments were performed in duplicate.

Table S, primer list.

Primer	Sequence
p16INK4a F	CCCAACGCCCCGAACT
p16INK4a R	GAGCAGAAGAGCTGCTACGTGAA
p15INK4b F	ATCCCAACGCCCTGAACCGCT
p15INK4b R	AGTTGGGTTCTGCTCCGTGGAG
p18INK4c F	GTCAACGCTCAAAATGGATTTGGG
p18INK4c R	GGATTAGCACCTCTGAGGAGAAG
p19INK4d F	GGAGCTGGTGCATCCTGACGC
p19INK4d R	TGGCACCTTGCTTCAGGAGCTC
p21CIP1 F	TCGCTGTCTTGCACTCTGGTGT
p21CIP1 R	CCAATCTGCGCTTGGAGTGATAG
p27KIP1 F	AGCAGTGTCCAGGGATGAGGAA
p27KIP1 R	TTCTTGGGCGTCTGCTCCACAG
p57KIP2 F	AGCTGAAGGACCAGCCTCTCTC
p57KIP2 R	ACGTCGTTCGACGCCTTGTTCT
HPRT F	GCCTAAGATGAGCGCAAGTTG
HPRT R	TACTAGGCAGATGGCCACAGG
PPIA F	GGCCGATGACGAGCCC
PPIA R	TGTCTTTGGAACTTTGTCTGCAA
mL32 F	GAAACTGGCGGAAACGCA
mL32 R	GGATCTGGCCCTTGAACCTT

Treatment for myelosuppression by administration of 5-FU

To study cell cycle of LSK and SLAM cells, one simple PI injection of 5-FU at 250 mg/kg body weight was used. To analyze the survival rate of mice after the myelosuppressive treatment, 5-FU was intra-peritoneally injected on a weekly basis (150 mg/kg body weight) until the death of mice. To rule out systemic toxicity of 5-FU, WT and *p19*$^{INK4d-/-}$ mice were

reconstituted with either WT or *p19*$^{INK4d-/-}$ Lin$^-$ cells. One month after transplantation and reconstitution of the hematopoietic system, 5-FU (150 mg/kg body weight) was injected once per week for 2 weeks and animal survival rates were followed. Analysis of cell cycle and apoptosis was performed 2 days after 5-FU injection (50 mg/kg body weight) into chimeric mice two months after hematopoietic reconstitution.

Assessment of colony-forming cell (CFC) potential

To assess clonogenic potential, total BM cells were plated at a concentration of 0.5-1x10^3 cells/mL in methylcellulose medium (Methocult M3234, Stem Cell Technologies, Vancouver, Canada). Colonies derived from CFCs were scored at days 7-12 under an inverted microscope (Nikon, Japan). To determine the proliferation rate of myeloid progenitors, Lin$^-$Sca-1$^-$c-Kit$^+$ cells were sorted at one cell/well in 96-well plates and cultured in DMEMα media supplemented with 10% FCS and 50 ng/mL SCF or 20 ng/mL G-CSF. At day 7, the number of cells per colony was counted using an inverted microscope (Nikon, Japan). To measure the clonogenic potential of SLAM in 48 week-old mice, OP9/OP9Δ1 co-culture was performed. 5 x10^4 OP9/OP9Δ1 (1:1) stromal cells were plated on 96-well plates. One day after, WT or *p19*$^{INK4d-/-}$ SLAM cells of ≤ 48 week-old mice were sorted at one cell/well in the 96-well plates containing stromal cells and incubated in DMEMα + 15% FCS. Cells were maintained in culture for 21 days by changing half of the medium once per week, and colony numbers were assessed. To assess the clonogenic potential of MK-Ps, total BM and spleen cells were plated at 75x10^3 cells/mL and 150x10^3 cells/mL, respectively, in serum-free fibrin clots. MK colonies were enumerated seven days after acetylcholinesterase staining, as previously described (Long and Williams, 1981).

Cytokine arrays

After flushing BM or crushing spleen in PBS (1X), cells were centrifuged and supernatants of BM and spleen of WT and *p19*$^{INK4d-/-}$ mice were collected. TPO levels were measured using

the Mouse Thrombopoietin Quantikine ELISA Kit (R&D system). Latent and active TGFβ1 levels were measured using the Human TGFβ1 Quantikine ELISA Kit (R&D system). Levels of 40 cytokines/chemokines were measured using the Mouse Cytokine Antibody Array, Panel A (R&D system).

Oxidative stress induction

Lin^- cells were isolated and incubated in 25 cm^3 culture bottles at a density of 1×10^6 cells/mL with or without menadione (10 mM) in DMEMα supplemented 10% FBS media. After 1 h of incubation, cells were washed in HBSS (1X) and stained with the APC conjugated Lin^- cocktail, PE-Cy7 conjugated Sca1, and PerCP5.5 conjugated c-Kit antibodies for 30 min at 4°C. Briefly, to measure ROS production by flow cytometry, LSK and SLAM cells were incubated before or after oxidative stress induction with 10 μmol/mL $DCFH_2$-DA at 37°C for 15 min. Cells were resuspended in 200 μL of HBSS (1X) and analyzed using an LSR II flow cytometer (BD, Mountain View, CA).

For measurement of DNA double stranded breaks, LSKs were cytocentrifuged at 700 rpm for 7 min on L-polylysine coated slides and fixed with PFA 4% for 20 min at RT. Cells were washed with PBS (1X), permeabilized with cold ethanol 70% for 20 min on ice and washed two times with PBS (1X). Cells were then saturated for 90 min in PBS (1X)-BSA 8% and stained with primary P-H2AX (Cell signaling, Boston, USA) and secondary ALEXA-546 antibodies (Invitrogen, Saint Aubin, France). Nuclei were stained with Dapi (Sigma-Aldrich, St Louis, MO). Cells were examined using a Leica DMI 4000, SPE, laser scanning microscope (Leica, Microsystem, France) with a 63X/1.4 numeric aperture (NA) oil objective. Images were processed using Adobe Photoshop 6.0 software.

References

Long, M.W., and Williams, N. (1981). Immature Megakaryocytes in the Mouse: Morphology and quantitation by acetylcholinesterase staining. Blood *58*, 1032-1039.