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Supplemental Information

CABLES1 Deficiency Impairs Quiescence and Stress Responses of Hematopoietic Stem Cells in Intrinsic and Extrinsic Manners

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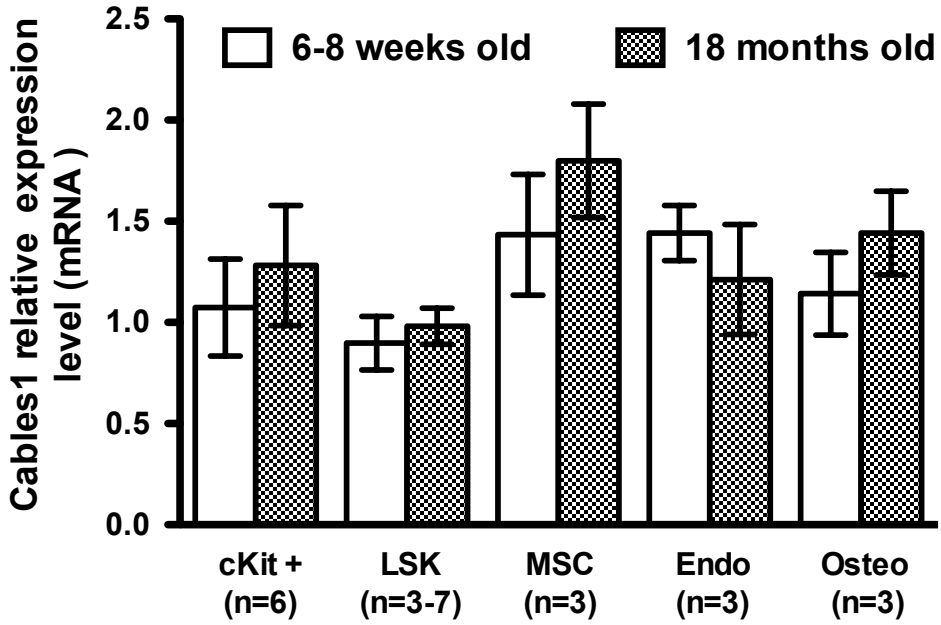

Figure S1: *Cables1* mRNA expression in murine immature hematopoietic and niche cells during aging. Young (6-8 weeks old) and 18 month-old mice were used. Lin^- (lineage markers negative cells, namely $CD3^-B220^-Ter119^-Gr\text{-}1^-$), LSK ($Lin^-c\text{-}Kit^+Sca\text{-}1^+$) and $c\text{-}kit^+$ ($Lin^-c\text{-}Kit^+Sca\text{-}1^-$) cells were sorted by fluorescence-activated cell sorting (FACS). After bone digestion, cellular components of the hematopoietic microenvironment – osteoblasts ($CD45^-Ter119^-CD31^-Sca\text{-}1^-CD51^+$), endothelial cells ($CD45^-Ter119^-CD31^+$), and MSC (mesenchymal stem cells ($CD45^-Ter119^-/CD31^-Sca^+CD51^+$) were also sorted by FACS. Expression levels were normalized to Hprt transcript levels. FACS purified c-kit positive cells served as reference to standardize for relative expression. Data are expressed as mean ± SEM, Student's two-tailed unpaired t-test was performed for statistical analysis.

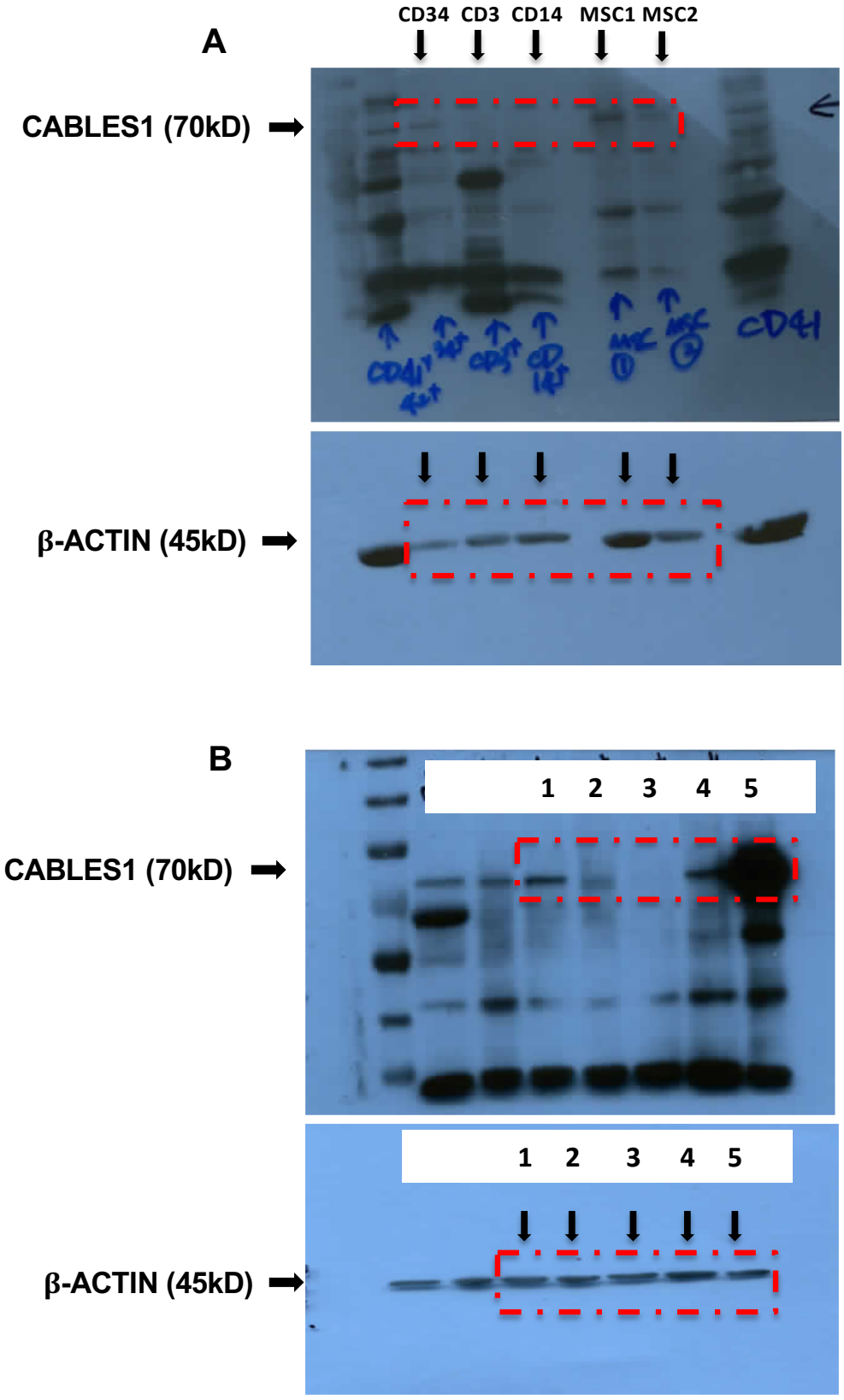

Figure S2. CABLES1 expression in hematopoietic and niche BM cells. (A) Uncropped Western blots with the indicated areas of selection shown in Figure 1C. Upper panel corresponds to CABLES1 and lower panel to **β**-ACTIN. **(B)** Uncropped blots for confirmation of CABLES1 antibody specificity using overexpression and shRNA mediated *Cables1* knockdown. 1: Scramble shRNA, 2: *Cables1* shRNA N1, 3: *Cables1* shRNA N2, 4: *GFP*-overexpressing, 5: CABLES1-GFP overexpressing cells. The signal corresponding to the expected 70kD band (corresponding to CABLES1 protein) was sharply diminished by shRNA treatment. Upper panel corresponds to CABLES1 and lower panel to **β**-ACTIN.

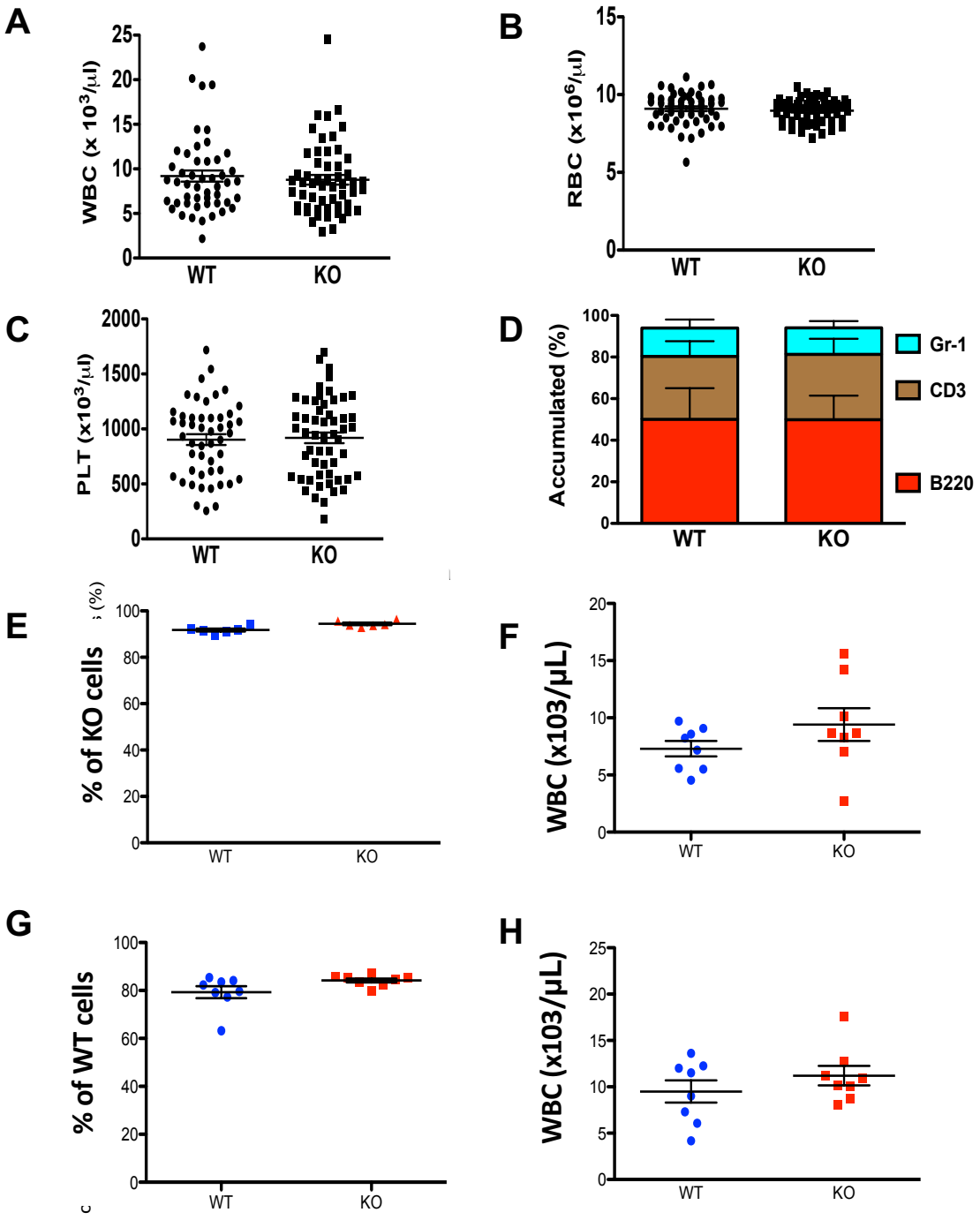

Figure S3: 10- to 12-week old *Cables1*$^{-/-}$ mice do not harbor obvious abnormalities in the hematopoietic compartment under steady state conditions. **(A)** WBC (white blood cells), **(B)** Red blood cells, **(C)** platelet (PLTs) counts, **(D)** percentages of B-lymphocytes, T-lymphocytes and myeloid cells within the peripheral blood in 5 mice per group. All values are expressed as mean ± SEM. **(E, F, G, H)** 5x10^6 BM cells from *Cables1* KO or WT mice were injected into *Cables1* WT or KO 9.5 Gy irradiated mice (n=8 per group). Percentage and white blood cells of KO (**E, F**) or WT (**G, H**) donor cells three months after injection. All values are expressed as mean ± SEM, no statistic differences were found using Student's unpaired two-tailed t-test.

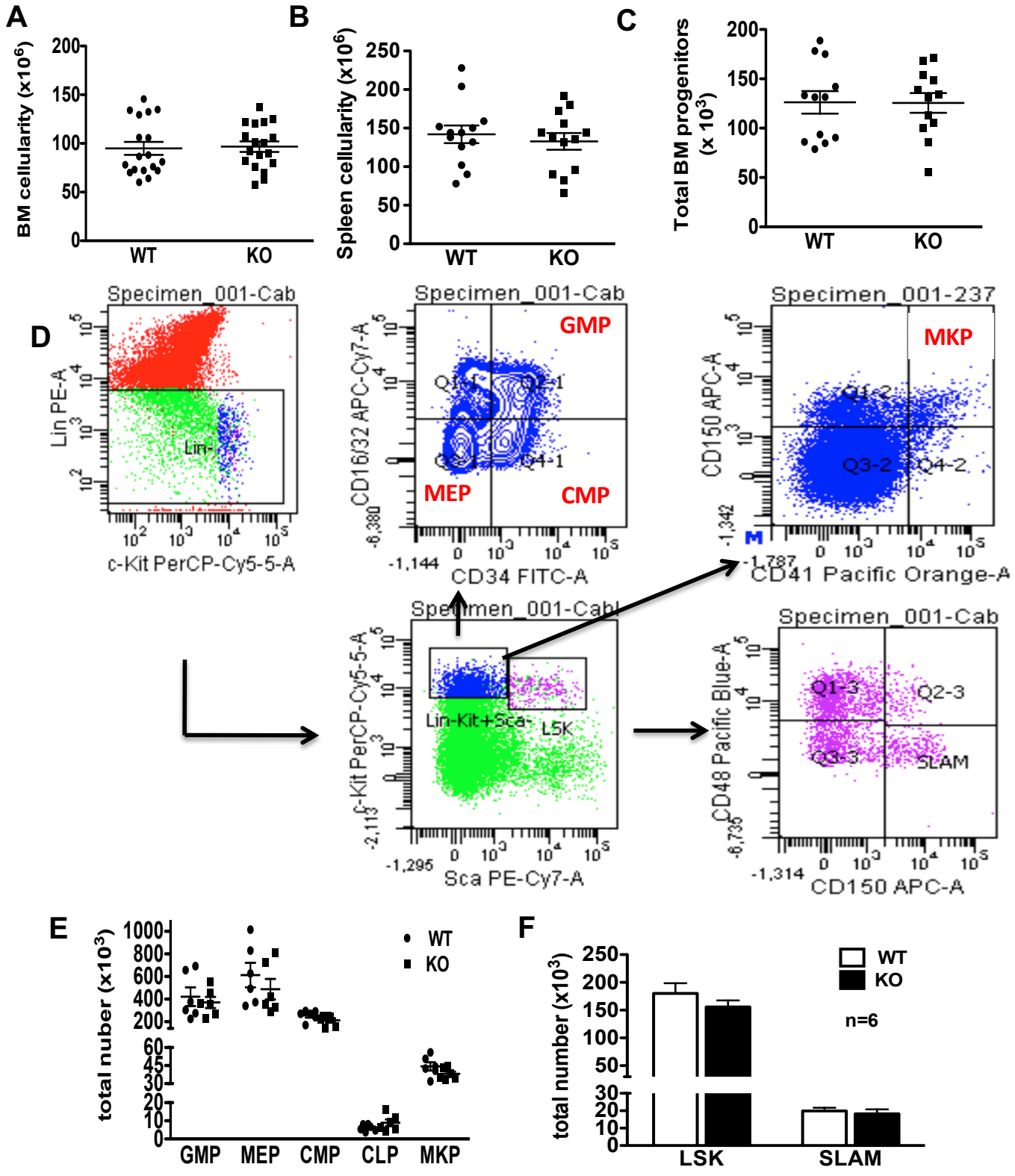

Figure S4: Analysis of HSPC within the BM in 10- to 12-week old *Cables1*$^{+/+}$ and *Cables1*$^{-/-}$ mice. (A) BM cellularity, **(B)** Spleen cellularity, **(C)** Total numbers of BM CFUs in *Cables1*$^{-/-}$ and *Cables1*$^{+/+}$ mice. **(D)** Full gating strategy with actual staining plots for immunophenotypic analysis of HSCs and progenitor cells. **(E)** Numbers of phenotypically defined GMP, CMP, CLP, MEP, MKP and **(F)** LSK and SLAM cells. GMP = granulomonocytic progenitors, CMP = common myeloid progenitors, CLP = common lymphoid progenitors, MEP = mega-erythroid progenitors, MKP = megakaryocytic progenitors. All values are expressed as mean ± SEM, no statistic differences were found using Student's unpaired two-tailed t-test.

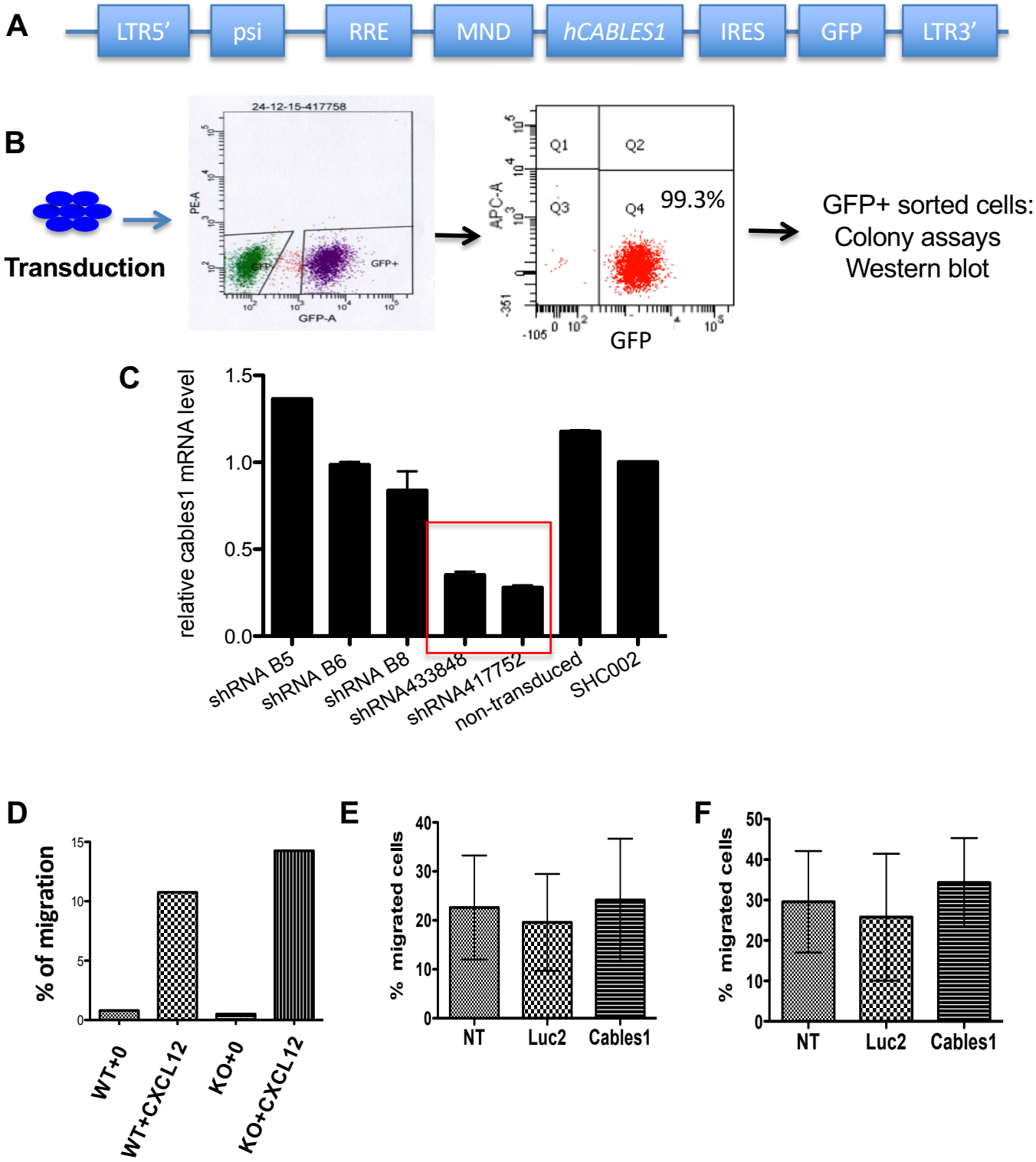

Figure S5: **Overexpression and shRNA strategy. (A)** pTrip lentiviral vectors encoding either GFP alone or human CABLES1 and GFP. **(B)** Prestimulated CB-CD34$^+$ cells were transduced and sorted on GFP expression for verification of CABLES1 expression. **(C)** *Cables1* mRNA expression was assessed by q-PCR after transduction of CD34+ cells with different human short hairpin RNAs (shRNAs) to *Cables1* or a control shRNA (shC002).**(D)** Chemotactic responses to CXCL12 of Lin$^-$ cells from BM *Cables1*$^{-/-}$ and littermate control mice (n=1). (**E**) Chemotactic responses to CXCL12 of HL-60 (n=3) and **(F)** U937 (n=3) cells either non transduced (NT) or transduced with lentiviral vectors encoding either GFP alone (Luc2) or human CABLES1 and GFP (Cables1). All values are expressed as mean ± SEM, statistic analysis was performed using Student's unpaired two-tailed t-test.

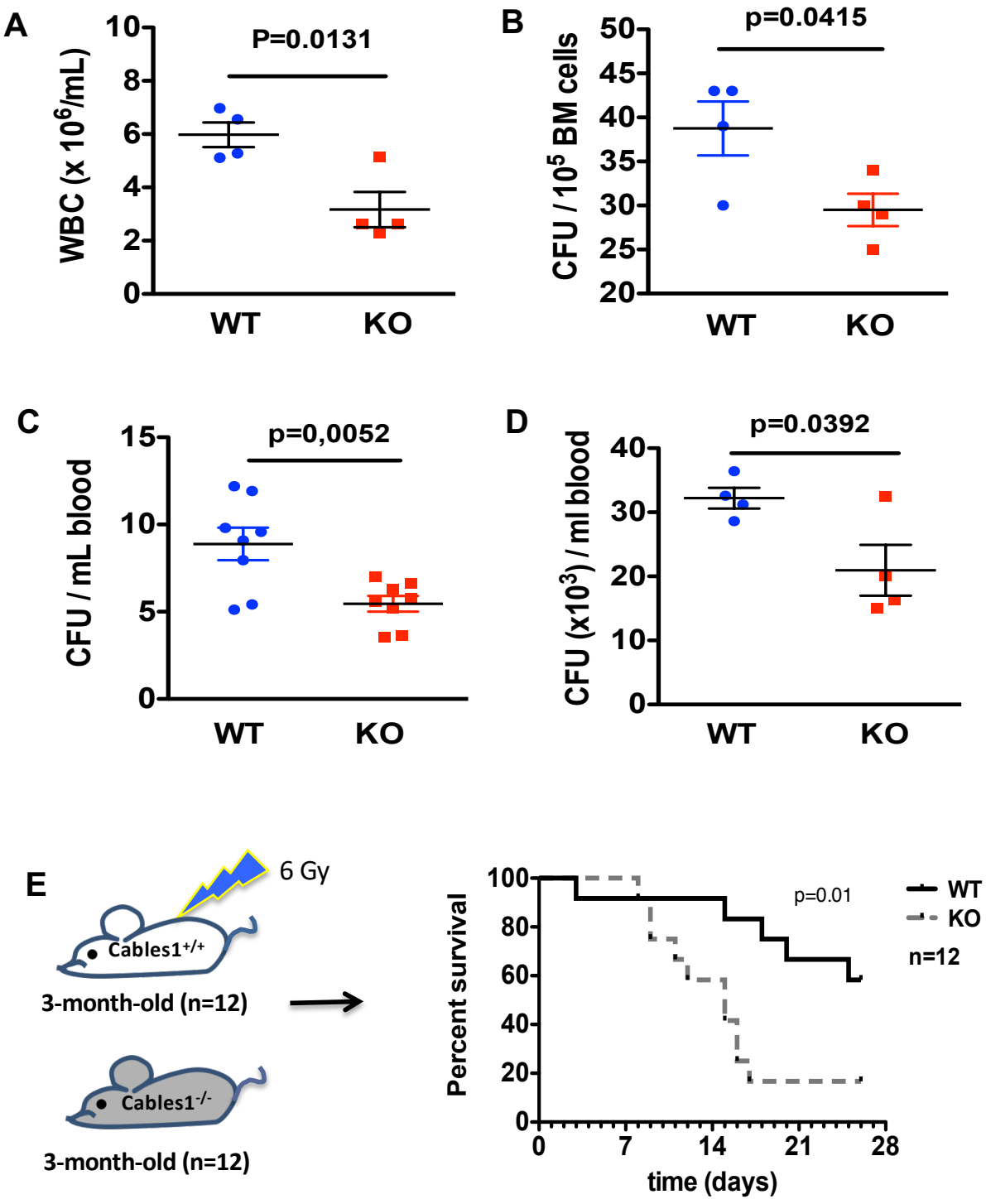

Figure S6 : **Impact of *Cables1* on hematopoietic regeneration and response to irradiation.** 3-month-old *Cables1*$^{-/-}$ and littermate control mice were injected intraperitoneally with 4 mg of CY followed by subcutaneous injection of 5 µg per day during 5 days of recombinant human G-CSF. **(A)** WBC, **(B)** BM CFU, **(C)** Blood CFU at day 4, **(D)** blood CFU at day 6. (**E)** 3-month-old *Cables1*$^{-/-}$ and littermate control mice were subjected to TBI (6 Gy) and survival was assessed (n=12). All values are expressed as mean ± SEM, statistic analysis was performed using Student's unpaired two-tailed t-test. Log-rank (Mantel-Cox) test was applied for comparing survival between WT and *Cables1*$^{-/-}$ mice.

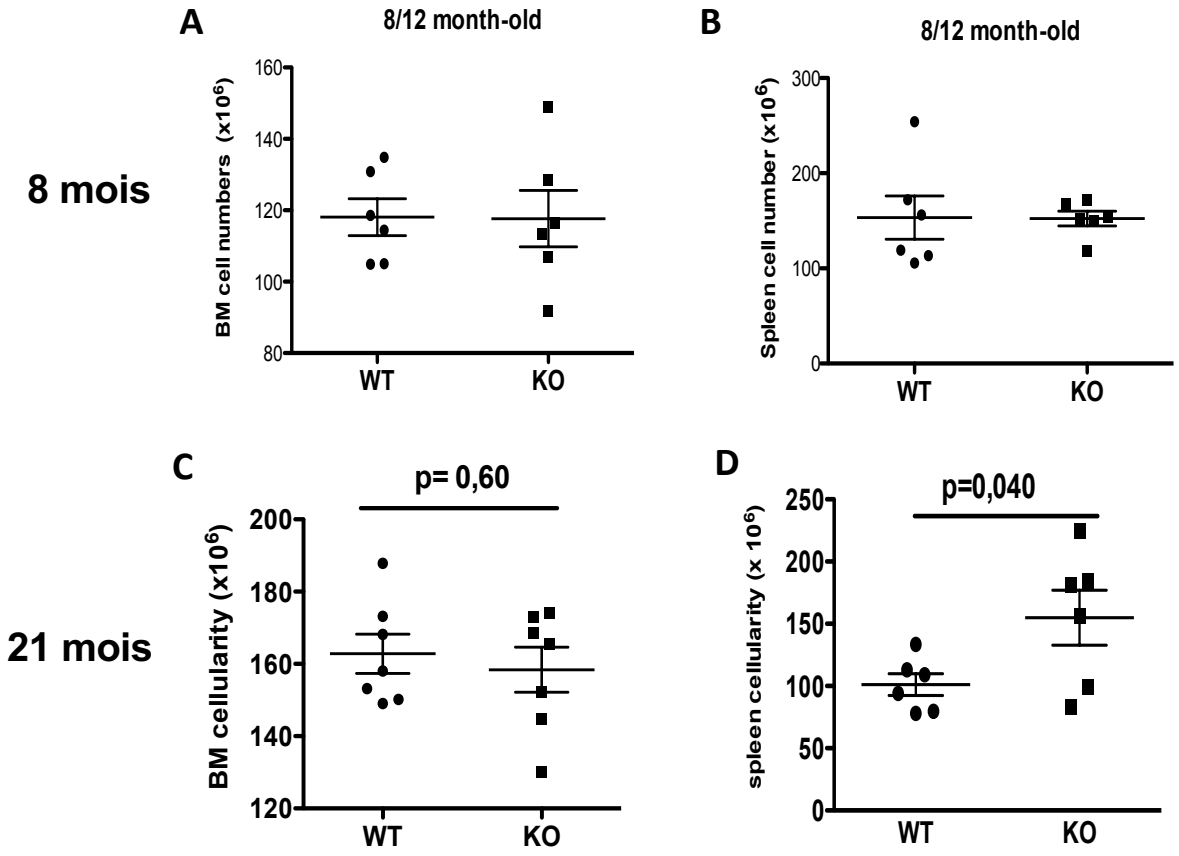

Figure S7: Bone marrow and spleen cellularities in WT and *Cables1* KO mice. (A) BM cellularity in middle-aged mice. **(B)** spleen cellularity in middle-aged mice. **(C)** BM cellularity in aged mice. **(D)** spleen cellularity in aged mice. All values are expressed as mean ± SEM, statistic analysis was performed using Student's unpaired two-tailed t-test.

Supplemental information

Murine bone marrow (BM) cell isolation

BM cells were extracted by centrifugation from intact hips, femora and tibiae, whereas spleens were gently mashed on a 70-μm nylon strainer to generate a single cell suspension. Cell collection was performed in PBS FCS 2% and filtered through a 70-μm nylon strainer. Nucleated cell numbers were measured on a Sysmex XP-300 counter (Sysmex, Lincolnshire, IL). All cell numbers were standardized as total count per two hips and legs, spleen, unless specified. Red blood cell lysis was performed using ACK buffer before staining. Cells harvested from tissues were immediately immunophenotyped.

Mouse BM progenitor cell analysis by flow cytometry

All staining analyses were performed on a BD FACSCanto™ II (BD Bioscience) flow cytometer. Hematopoietic progenitor cells were analyzed according to the following phenotypes: LSK cells ($Lin^-Kit^+Sca\text{-}1^+$), SLAM cells ($CD150^+CD48^-LSK$), granulomonocytic progenitors (GMP, $Lin^-Kit^+Sca\text{-}1^-CD34^+CD16^+/32^+$), common myeloid progenitors (CMP, $Lin^-Kit^+Sca\text{-}1^-CD34^+CD16^-/32^-$), mega-erythroid progenitors (MEP, $Lin^-Kit^+Sca\text{-}1^-CD34^-CD16^-/32^-$), common lymphoid progenitors (CLP, $Lin^-c\text{-}Kit^{int}Sca\text{-}1^{int}CD127^+CD135^+$) and megakaryocytic progenitors (MKP, $Lin^-Kit^+Sca\text{-}1^-CD41^+CD150^+$). Cells from the BM microenvironment were analyzed according to the following phenotypes: Mesenchymal stem cells (MSC, $CD45^-TER119^-CD31^-CD51^+Sca\text{-}1^+$), osteoblasts ($CD45^-TER119^-CD31^-CD51^+Sca\text{-}1^-$) and endothelial cells ($CD45^-TER119^-CD31^+$). The following mAbs were used: anti–mouse CD3 (clone 145-2C11, hamster IgG1), CD4 (clone RM4-5, rat IgG2a), CD (clone 53–6.7, rat IgG2a), CD11b (clone M1/70, rat IgG2b), CD16/32 (clone 93, rat IgG2a), CD34 (clone RAM34, rat IgG2a), CD41 (clone MWReg30, rat IgG1), CD45R/B220 (clone RA3-6B2, rat IgG2a), CD45.1 (clone A20, mouse IgG2a), CD45.2 (clone 104, mouse IgG2a), CD48 (clone HM48-1, Armenian hamster IgG), CD117 (clone 2B8, rat

IgG2b), CD127 (clone A7R34, rat IgG2a), CD135 (clone A2F10, rat IgG2a), CD150 (clone TC15-23F12,2, rat IgG2a), Ter119 (clone TER-119, rat IgG2b), Gr-1 (clone RB6-8C5, rat IgG2b), Sca-1 (clone E13-161,7, rat IgG2a). Antibodies were conjugated to biotin, BV 650, FITC, PE, APC, AF700, PE-cyanin (Cy) 5, PE-Cy7, eFluor 450, AF 647, APC-eFluor 780, peridinin chlorophyll protein PerCP-Cy5,5 or pacific blue and purchased from BD, eBioscience, or BioLegend. The lineage antibody cocktail included anti-CD3, anti-CD45R/B220, anti-CD11b, anti-TER119, and anti–Gr-1 mAbs. Secondary labeling was performed with Streptavidin–pacific orange from Thermo Fisher Scientific. Viability was assessed by adding 4′,6-diamidino-2-phenylindole (DAPI) at 0.5 μg/ml).

Fluorescence Activated Cell Sorting

For analyzing *Cables1* mRNA expression, 3-month-old wild type mice were used. Gr-1$^+$, B220$^+$, CD3$^+$, CD4$^+$ and CD8$^+$ cells were sorted from unfractionated BM or spleen, respectively. Progenitor cells were enriched using mouse hematopoietic progenitor (stem) cell enrichment set (Cat 558451; BD biosciences, USA) according to manufacturer's instructions. Depleted cells were then used for surface marker staining and sorted according to the phenotypes described above. For sorting niche component cells, femora, tibiae and hip bones were dissected and digested with collagenase I (Gibco) diluted with 1x Hanks' Balanced Salt Solution (HBSS; Gibco). Digested cells were stained with relevant antibodies according to the phenotypes described above. Cells were sorted by a BD FACSAria™ III cell sorter (BD bioscience, USA).

Immunofluorescence staining

100,000 freshly isolated CB CD34$^+$ cells were spun onto poly-lysine-coated slides. Cells were fixed with 2% paraformaldehyde/5% sucrose in PBS at room temperature (RT) for 15 min. Fixed cells were washed with PBS and permeabilized with 1% Brij in PBS for 2 min at RT. After

washing with PBS, cells were blocked for nonspecific binding sites with 1% bovine serum albumin and 1% defatted milk in PBS (BSA-milk-PBS). Then cells were incubated with mouse anti-CABLES1 (provided by Bo. R. Rueda) diluted in BSA-milk-PBS for 60 min at RT. They were then washed with PBS followed by incubation with FITC-conjugated anti-mouse secondary antibody for 30 min at RT (Molecular Probes). After washing 3 times with PBS, the slides were mounted with Vectorshield containing DAPI (Vector Labs, CA). Cells were examined under a Zeiss LSM 510 laser scanning fluorescence confocal microscope. To support the specificity of the staining, negative control for the immunostaining was done with T-lymphocytes.

In-vivo BrdU incorporation assay

Mice were injected i.p. with BrdU (1mg BrdU/g body weight) (Sigma) the first day. During the following 12 continuous days, mice were fed with BrdU-containing water (1mg/ml). BM cells were harvested, lineage depleted and cell surface was stained as described above. Cells were fixed and permeabilized with a BrdU staining kit (BD Biosciences). Samples were incubated with DNAse I for one hour at 37°C, followed by intra-cellular staining with anti-BrdU-FITC (Cat. 557891 FITC BrdU Flow Kit BD Pharmingen San Diego, CA). Cells were washed and suspended in Permwash buffer containing 10 μg/ml DAPI. Samples were analyzed by flow cytometry (BD FACSCanto™ II, BD Bioscience, USA).

Assessment of colony-forming cell (CFC) potential

To assess the clonogenic potential, BM mononucleated cells (BMMNCs) were plated at a density of 50,000 cells per ml methylcellulose medium, spleen cells were plated at a density of 100,000 cells per ml methylcellulose medium. Whole PB (25 μl blood per plate) was lysed with Ammonium-Chloride-Potassium (ACK) solution to eliminate red blood cells. After two washes with PBS containing 5% FBS (PBS-5% FBS), lysed cells were plated in methylcellulose medium

(MethoCult™ M3234, STEMCELL Technologies) (Foudi et al., 2006). The cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Colonies derived from colony forming cells (CFC) were scored at day 7-12 under an inverted microscope (Nikon, Japan).

Homing experiments

BM cells (15 $\times 10^6$/host) from *Cables1*$^{+/+}$ or *Cables1*$^{-/-}$ mice (3 month-old) were stained with the cell tracker carboxyfluorescein-succinimidyl ester (CFSE), and marked cells were injected through the retro-orbital vein into previously irradiated (9.5 Gy) mice (Foudi et al., 2006). Four or 16 hours after injection, mice were sacrificed, PB was collected and one femur and the spleen were dissected for analysis. The numbers of CFSE$^+$ cells in PB, BM and spleen were detected by flow cytometry.

Separation of cells in the G0/G1/SG2M phase by Hoechst and Pyronin Y staining

BM cells from wild type mice were used for sorting of LSK cells in the G0, G1 and S/G2/M phase. To this end unfractionated BM cells were depleted and stained for LSK phenotype. Stained cells were then incubated with 10 μg/ml Hoechst 33342 (Sigma Chemical Co, St Louis, MO) for 1.5 hours at 37°C in Hanks balanced salt solution (HBSS) medium, supplemented with 2mM Hepes, 10% FBS and 1mg/l glucose. During the last 45 min of incubation, cells were co-incubated with 0.5 μg/ml Pyronin Y (RNA dye) (Sigma Chemical Co, St Louis, MO). G0, G1 and S/G2/M phase LSK cells were sorted by BD FACSARIA III device.

Chemotactic assays

Chemotaxis assays were performed as previously described (Riviere et al., 1999). Briefly, Lin- cells (10^5) or transduced cells (Hl-60 or U937 cells) were added to the upper chambers of a 24-well plate with 5-μm-pore-size Transwell inserts (EMD Millipore), containing or not 100 ng/ml of

CXCL12 (Peprotech, France) in the lower chamber. Lin- migrated cells were enumerated by CFU-C mix assay after 4 hours of incubation at 37°C, 5% CO_2 and referred to input BM CFU-Cs to obtain a migration percentage.

Western blot

Separated cells were lysed in 2x Laemmli buffer and Protease Inhibitor Cocktail (company). Protein lysates were separated on 10% SDS-PAGE 100 V for 3 hours. Proteins were transferred onto polyvinylidene difluoride (PVDF) membrane by wet electroblotting. The membrane was blocked in blocking buffer for one hour and incubated with primary antibody overnight. PVDF membrane was washed with TBST, then incubated with secondary antibody. Membrane was washed with TBST for 50 min, incubated with chemiluminescence substrate for oner min and exposed to Kodak autoradiography film.

RNA extraction and quantitative polymerase chain reaction (qPCR)

Total RNAs were prepared using **Direct-zol™ RNA Kit** (Zymo Research Corp., Irvine, USA). First-strand cDNA was synthesized by using the SuperScript II Reverse Transcriptase (Life Technologies, Carlsbad, USA). PCR reactions were carried out in the ABI Prism GeneAmp 5700 Sequence Detection System using Takyon SYBR MasterMix (Eurogentec, France). The expression levels of *Cables1* were calculated relatively to the expression of HPRT and SDHA as endogenous housekeeping controls. Mouse brain and $CD34^+$ cells were used as reference for *Cables1* expression level in murine and human cells, respectively. Primer sequences are listed in Table1.

Table 1 : primers used in this study

Mouse *Cables1*	F	CCT-TCA-TAC-ATG-ACC-ACA-GTG-A
	R	TCA-GGC-TCC-TGA-TTT-TGC-T
Mouse *Hprt*	F	TGA-TTA-TGG-ACA-GGA-CTG-AAA-GA
	R	AGC-AGG-TCA-GCA-AAG-AAC-TTA-TAG
Mouse *Sdha*	F	AAG-TTG-AGA-TTT-GCC-GAT-GG
	R	TGG-TTC-TGC-ATC-GAC-TTC-TG
Mouse *Zfy1*	F	TGG-AGA-GCC-ACA-AGC-TAA-CCA
	R	CCC-AGC-ATG-AGA-AAG-ATT-CTT
Mouse *Bcl2*	F	AAG-CTG-TCA-CAG-AGG-GGC-TA
	R	CAG-GCT-GGA-AGG-AGA-AGA-TG
Human *CABLES1*	F	CCT-TGG-AGA-CCC-TGG-AAG-AT
	R	GCC-ATT-CCT-GGT-ATC-GTG-TT
Human *MRPL32*	F	CTG-CAG-TCT-CCT-TGC-ACA-CCT
	R	TGT-CCT-GAA-TGT-GGT-CAC-CTG-A
Human *SDHA*	F	TGG-TTG-TCT-TTG-GTC-GGG
	R	GCG-TTT-GGT-TTA-ATT-GGA-GGG