Stem Cell Reports, Volume *3*

Supplemental Information

Cytokine-Regulated GADD45G Induces Differentiation and Lineage Selection in Hematopoietic Stem Cells

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Supplemental Data

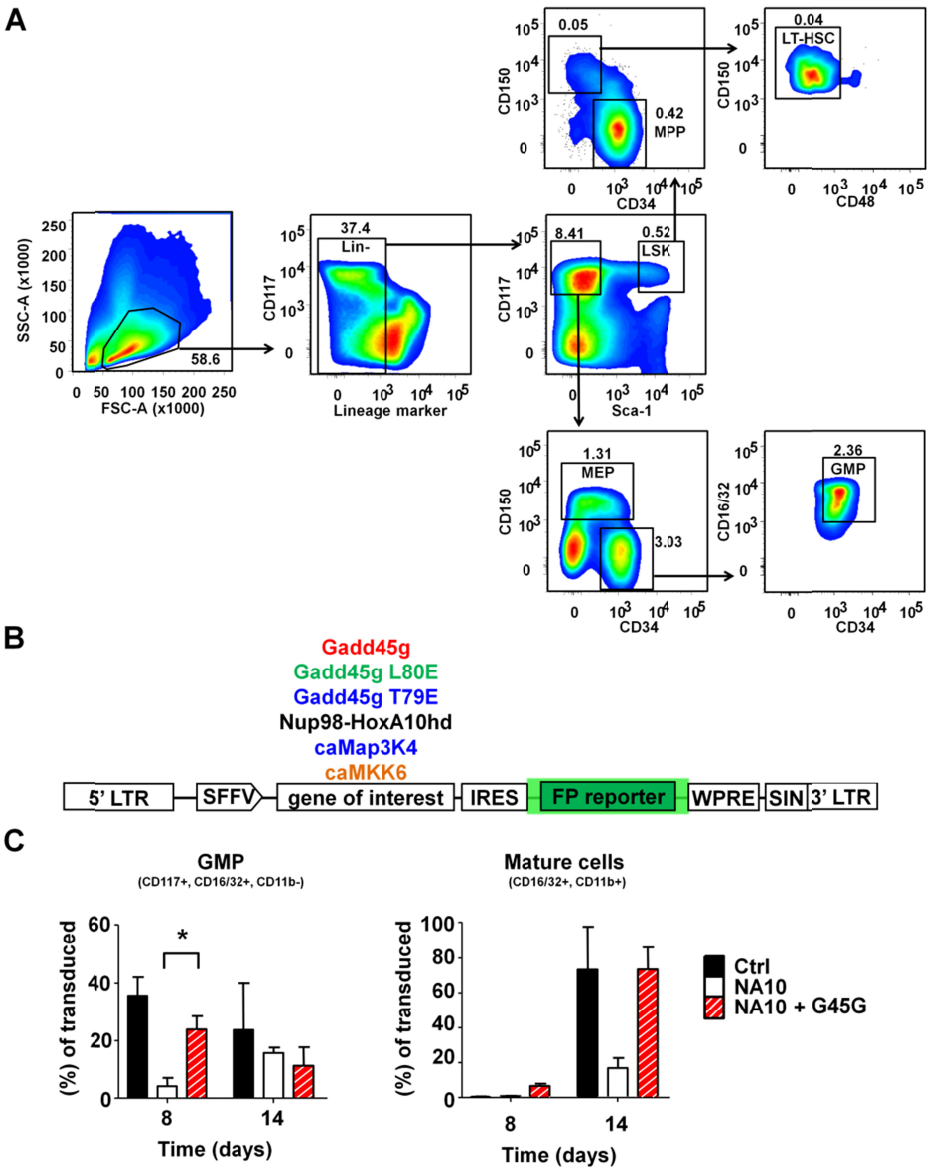

Figure S1. Sorting of HSPC populations and lentiviral transduction of LT-HSCs.

(A) Representative sorting scheme of hematopoietic stem and progenitor populations from adult mouse bone marrow by FACS with indicated percentages, staining and sorting as described in the Experimental procedure section. The sorting purity was always >97% as determined by FACS reanalysis. Cells were prospectively isolated using following surface markers: LT-HSC, long-term repopulating hematopoietic stem cells ($CD150^+$ $CD48^-$ $CD34^{low}$ $Sca1^+$ $CD117^+$ $Lineage^-$); MPP, multipotent progenitors ($CD150^-$ $CD34^+$ $Sca1^+$ $CD117^+$ $Lineage^-$); LSK; $lineage^-$ $Sca1^+$ $ckit^+$ cells ($Sca1^+$ $CD117^+$ $Lineage^-$); MEP, megakaryocyte-erythrocyte progenitors ($CD150^+$ $Sca1^-$ $CD117^+$ $Lineage^-$); GMP, granulocyte-macrophage progenitors ($CD150^-$ $CD34^+$ $CD16/32^+$ $Sca1^-$ $CD117^+$ $Lineage^-$). (B) Scheme of lentiviral transfer vector. The gene of interest was cloned into the multiple cloning site and driven by a SFFV promoter. A fluorescent reporter was coexpressed by an internal ribosomal entry site (IRES). (C) Differentiation kinetics of double transduced LT-HSCs (NA10, NUP98-HOXA10 homeodomain and GADD45G) by FACS gated on GMP-like cells ($CD117^+$, $CD16/32^+$, $CD11b^-$) and mature granulocytes and macrophages ($CD16/32^+$, $CD11b^+$) (N=3 independent experiments). Mean +/- SD is displayed. * $P<0.05$. Figure S1 is related to Figure 1.

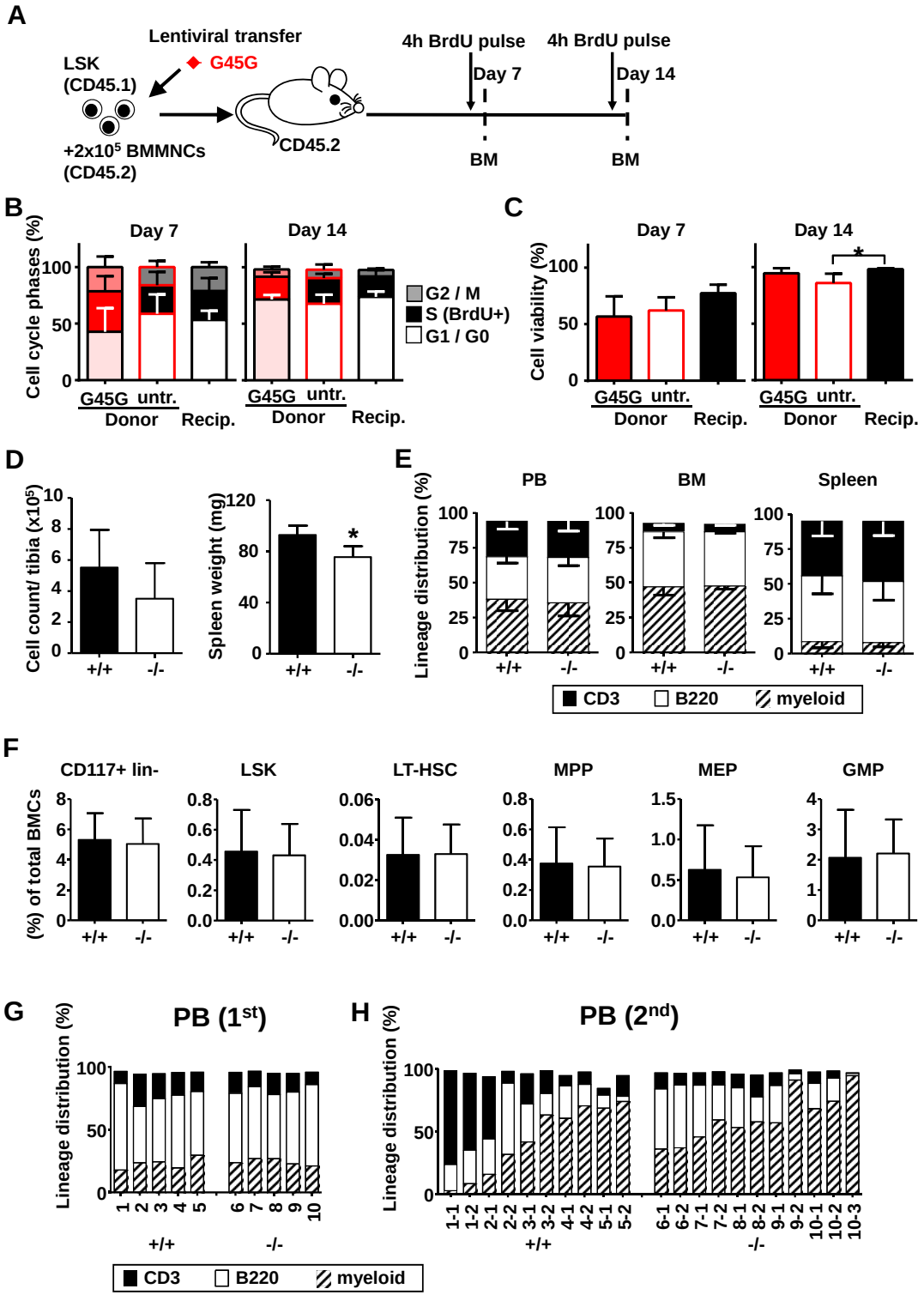

Figure S2. GADD45G does not alter cell proliferation and viability in vivo, and its absence (*Gadd45g*$^{-/-}$) does not change hematopoietic populations in homeostasis and after transplantation.

(A) Experimental scheme (B) Cell cycle distribution and (C) viability of GADD45G expressing cells 7 and 14 days after transplantation of transduced LSK in BM (N=4 mice per time point). (B) BrdU DNA incorporation of a 4h pulse and DNA content were determined by FACS in GADD45G-transduced donor cells (G45G), in untransduced donor cells (untr.) and in transplanted recipient cells. (C) Cell viability was determined by FACS in GADD45G-

transduced donor cells (G45G), in untransduced donor cells (untr.) and in transplanted recipient cells. (D) Cell count of total bone marrow cells of one tibia (N=5) and the spleen weight (N=9) of *Gadd45g*$^{+/+}$ and *Gadd45g*$^{-/-}$ mice. (E) Lineage distribution of mature blood cells in peripheral blood (PB), bone marrow (BM), spleen determined by FACS using CD3, B220 and myeloid markers CD11b and Gr1 (N=9). (F) Stem and progenitor populations in BM of *Gadd45g* $^{+/+}$ and *Gadd45g* $^{-/-}$ mice (N=9). (G) Donor cell distribution in PB by FACS using CD3, B220 and myeloid markers CD11b and Gr1 after 21 weeks of primary transplantation. (H) Donor cell distribution in PB by FACS using CD3, B220 and myeloid markers CD11b and Gr1 after 22 weeks of secondary transplantation. Mean +/- SD is displayed. * $P<0.05$. Figure S2 is related to Figure 2.

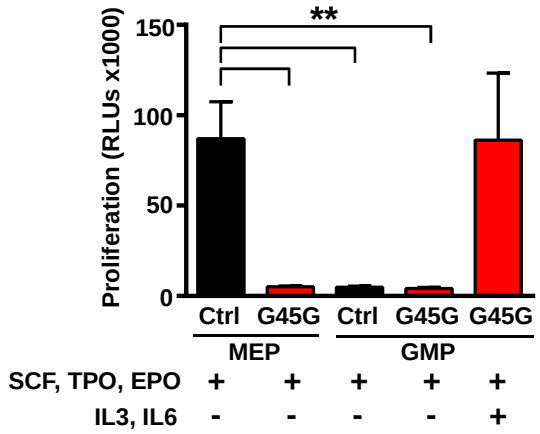

Figure S3. Megakaryocyte/Erythrocyte progenitor (MEP) differentiation is not compatible with the GADD45-mediated differentiation program.

FACS sorted MEPs and GMPs lentivirally transduced with GADD45G or control were cultured for 5 days in indicated cytokine conditions, and viable cell numbers were determined by a proliferation assay kit (RLU, relative light units), N=5 biological replicates. Mean +/- SD is displayed. ** P<0.01. Figure S3 is related to Figure 3.

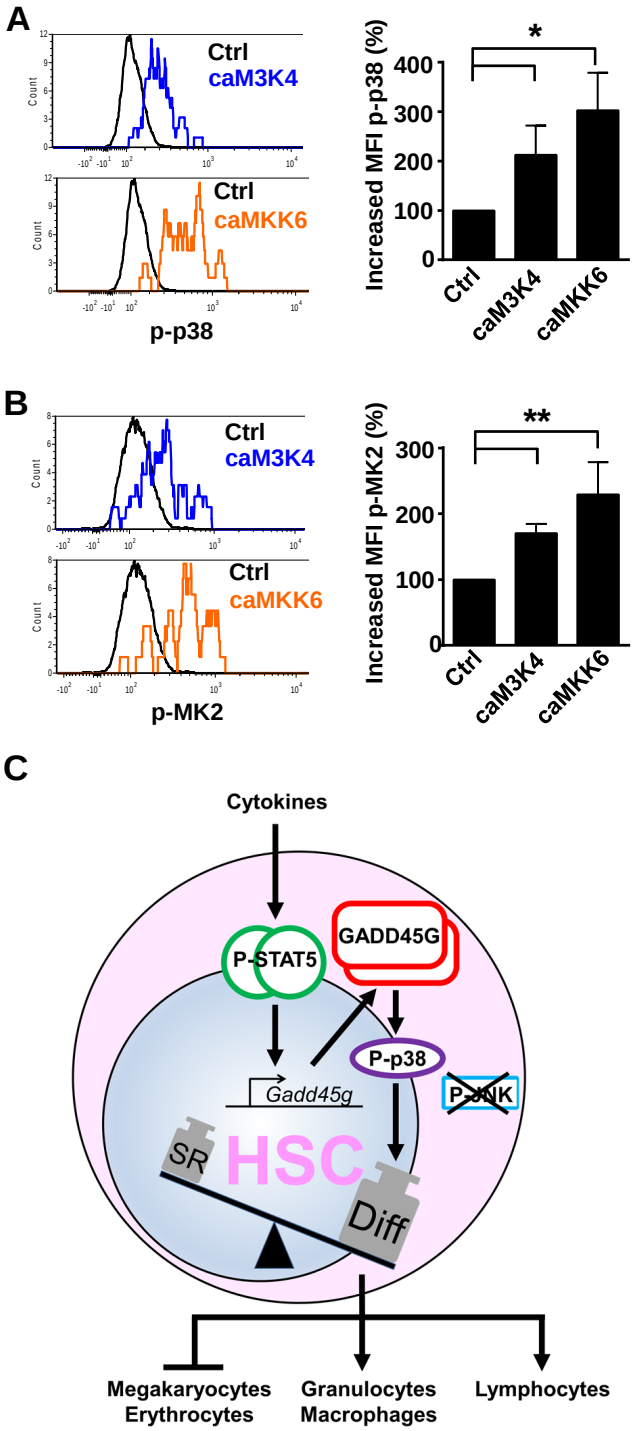

Figure S4. Constitutively active mutants of MAP3K4 and MKK6 lead to phosphorylation of p38 and MK2 in HSPCs.

(A) FACS histogram and quantification of phosphorylated p38 and (B) phosphorylated MK2 in 5 day-cultured MPPs after transduction of constitutively active MAP3K4 (blue line), constitutively active MKK6 (orange line) or control vector (black line) by phosphoflow cytometry. N=3 independent experiments. ca, constitutively active; M3K4, MAP3K4. Mean +/- SD is displayed. * P<0.05; ** P<0.01. (C) Graphical summary. Figure S4 is related to Figure 4.

Movie S1. Long-term observation of a LT-HSC differentiating into GMPs upon GADD45G expression.

LT-HSC and all of its progeny are observed to the fourth generation by time-lapse microscopy and cell tracking (tracks are yellow circles). The observation time span was 12h 17min after sorting of LT-HSCs until day 4 16h 48min of culture in SCF and TPO. Phase contrast images are displayed in the left column, every 4^{th} image frame was used to assemble the video (original temporal resolution 2min per frame). Only the GADD45G expressing daughter cells (fluorescence images of nuclear tdTOMATO in middle column) differentiate into GMPs with CD16/32 surface expression (fluorescence images of anti-CD16/32-A647 antibody, right column). Fluorescence was detected every 60 min, every image is shown. Time bar in d-h:m:s. Movie was assembled using QuickTime 7.7.3 software. Movie S1 is related to Figure 3.

Supplemental Experimental Procedures

Vectors

The open reading frame (ORF) of a fluorescent reporter protein (either VENUS-hImportin subunit α1 (AA2-67) or tdTOMATO-hImportin subunit α1 (AA2-67)) was cloned into the third generation self-inactivating lentiviral vector pRRL.PPT.SFFV.IRES.eGFP.wPRE by replacing the ORF of green fluorescent protein (Schambach et al., 2006). A multiple cloning site (MCS) was inserted after the SFFV promoter. The ORF of murine *Gadd45g* was amplified by RT-PCR (forward 5′-TTAAGGCCGGCCAGCCATGACTCTGGAAGAAGTC CGTGGC-3′, reverse 5′-TTAACGCCGGCGATCGATCAGACCAAGGTCCCTGC-3′) and then cloned into the MCS. *Gadd45g* T79E and L80E were generated by site directed mutagenesis (forward T79E 5′ACATAGCGCTGCAGATCCATTT**CGA**GTTGATTCAGGC GTTC-3′, forward L80E 5′- ACATAGCGCTGCAGATCCATTTCAC**GGA**GATTCAGGC GTTC-3′, common reverse (5′-GTACGCGTCTAGATTTAAATCCGGACTAGTCGCCG-3′). The ORF of NUP98-HOXA10hd (Ohta et al., 2007) was a gift from Keith Humphries, Vancouver, and cloned into the MCS. Constitutively active murine MAP3K4 (caMAP3K4) was cloned by PCR adding a start codon (ATG) 5′ of the murine *Map3k4* ORF coding for 1312asp to the C-terminus (last 286aa) (forward caMAP3K4 5'-CGATACCGTCGACgga tccATGGATACCCCTAAGTCCTATGATAACG-3'; reverse MAP3K4 5'-CGTCTAGATTTAAA TCCGGactagtCTTCACTCTTCATCTGTGC-3') and ligated into the MCS of the lentiviral vector (Mita et al., 2002). CaMAP3K4 lacks the GADD45 binding site, the autoinhibitory domain, and does not require GADD45 binding for auto-activation (Mita, et al., 2002). Constitutively active human MKK6 (caMKK6) was subcloned from Addgene vector #13518 (MKK6glu) into the MCS of the lentiviral vector (Raingeaud et al., 1996).

Virus production

Vesicular Stomatitis Virus-G (VSVG)-pseudotyped lentiviral particles were produced in a split genome approach by calcium-phosphate-mediated transient transfection of human embryonic kidney HEK293T producer cells (Naldini, 1998). After 48 hours, supernatant was collected, filtered (45µm), and enriched by ultracentrifugation (50,000g, 2h). Viral titers were determined by transduction of NIH3T3 cells with different concentrations of virus supernatant and FACS.

FACS of defined HSPC populations

BM cells from femurs, tibias, coxae and sternum were isolated by crushing the bones, stained with biotin-labeled lineage antibodies (CD3, CD11b, CD19, CD41, CD45R, Gr1, Ter119) and subjected to lineage depletion (EasySep^{TM} Biotin Selection Kit, Stemcell Technologies). HSPC populations were sorted with a FACS Aria (BD) after staining with antibodies against CD117, Sca1, CD150, CD48, CD34, CD16/32, and Streptavidin (**Table S1**). HSPC populations were prospectively isolated as shown in **Figure S1A**. Viable sorted cells were counted with trypan blue exclusion. Fcγ receptor block (anti-CD16/32, eBioscience) and isotype controls were routinely used.

Phosphoflow cytometry

FACS-sorted MPPs were transduced with indicated lentiviral particles (MOI 100) and cultured for 5 days. After staining cells with Fixable Viability Dye (eBioscience) for live/dead cell exclusion, the cells were fixed with Fix Buffer I (BD) at 37°C for 20 min and permealized with ice-cold Perm Buffer III (BD) for 30 min on ice. Permealized cells were subsequently stained with antibodies against P-p38-PE and P-MK2-Alexa Fluor 647 (BD) to analyze the phosphorylation status of the cells by FACS. We stimulated 5 day cultured MPPs with 10µg/ml Anisomcyin (Sigma) for 30 min prior to fixation as positive control.

Table S1. FACS antibodies

Antigen	Clone	Conjugate	Company
CD3	145-2C11	Biotin, PE-Cy7	eBioscience
CD3e	500A2	V500	BD
CD11b	M1/70	Biotin	eBioscience
CD11b	M1/70	PE, AlexaFluor (AF)-647	Biolegend
CD16/32	2.4G2	FITC, V450	BD
CD19	1D3	Biotin	eBioscience
CD34	RAM34	eFluor660	eBioscience
CD41	MWReg30	Biotin	eBioscience
CD48	HM48.1	PE, FITC	Biolegend
CD45.1	A20	eFluor450, FITC	eBioscience
CD45.2	104	PerCP-Cy5.5	eBioscience
CD45R (B220)	RA3-6B2	Biotin, PE	eBioscience
CD117 (c-kit)	2B8	PE-Cy7	eBioscience
CD150	TC15-12F12.2	PE, PerCP-Cy5.5	Biolegend
Sca-1	D7	PerCP-Cy5.5, Pacific Blue	eBioscience
Gr-1	RB6-8C5	Biotin, AF-647, AF-700	eBioscience
Ter119	TER-119	Biotin, APC-eFlour780	eBioscience
Streptavidin		APC-Cy7, APC-eFluor780	eBioscience
P- p38 MAPK	36/p38	PE	BD
P-MK2	P24-694	A647	BD
BrdU	BU20A	PE	eBioscience

In vitro cell proliferation assay

10000 FACS-purified MEPs or GMPs per well (96-well format) were lentivirally transduced (MOI 50) and cultured for 5 days in SFEM supplemented with indicated cytokines. Viable cells were determined with the ViaLight Plus Cell Proliferation and Cytotoxicity BioAssay Kit (Lonza) according to the manufacturer´s instructions. Luminescence was measured with a Mithras LB940 luminometer (Berthold Technologies).

Competitive repopulation assay

350 FACS-sorted LT-HSCs from either C57.BL/6 mice ($CD45.2^+$), which were lentivirally transduced 24h prior transplantation, or from *Gadd45g*$^{-/-}$ and *Gadd45g*$^{+/+}$ littermates, were tail vein injected into lethally irradiated B6.SJL ($CD45.1^+$) recipients together with $2x10^5$ BM competitor recipient cells from B6.SJL . Transduction efficiency of the transplanted LT-HSCs was determined with a remaining cell aliquot after three additional days in culture by FACS. Multilineage reconstitution was determined every 4 to 6 weeks post transplantation in PB. Shortly, red blood cells were lysed with PharmLysis Buffer (BD), and cells were stained with antibodies against CD45.1, CD45.2, CD3, B220, Ter119, and CD11b / Gr1 and a dead/live cell exclusion (Fixable Viability Dye, eBioscience). Lentivirally transduced hematopoietic cells were analyzed for VENUS or tdTOMATO expression by FACS (FACS Canto II or FACS Aria, BD). Primary recipients were sacrificed 21 weeks after transplantation, and $5x10^5$ BM cells per mouse were transplanted into a lethally irradiated secondary B6.SJL recipient. For BM reconstitution analyses, ficoll gradient enriched BM cells (Histopaque 1083) were stained with antibodies against CD45.1, CD45.2, CD3, B220, Ter119, and CD11b / Gr1 and a dead/live cell exclusion (Fixable Viability Dye, eBioscience). Used FACS antibodies are listed in **Table S1**.

Short-term transplantation

10000 LSK cells from C57.BL/6 mice ($CD45.2^+$), which were lentivirally transduced 24h prior transplantation, were tail vein injected into lethally irradiated B6.SJL ($CD45.1^+$) recipients together with $2x10^5$ BM competitor cells from B6.SJL**.** Mice were sacrificed 11 days after transplantation and the engraftment and distribution of stem and progenitor cell populations was analyzed by FACS. Transduction efficiency of the transplanted LSKs was determined with a remaining aliquot of the transduced LSKs after three days in culture by FACS.

For the determination of cell viability and proliferation in transplanted cells 7 and 14 days after transplantation, we transplanted 8000 LSK cells from B6.SJL ($CD45.1^+$), which were lentivirally transduced 24h prior transplantation, by tail vein injected into lethally irradiated C57.BL/6 ($CD45.2^+$) recipients together with $2x10^5$ BM competitor cells from C57.BL/6 mice**.** We injected 1.5 mg BrdU (BD) intraperitoneally 4 hours before sacrificing the mice. Dead cells were determined by Fixable Viability Dye staining (eBioscience) and BrdU incorporation and DNA content (7AAD) in living cells were measured according to the manufacturer´s instructions (BD) with a PE-labeled anti-BrdU antibody (eBioscience) by FACS.

Gene expression by quantitative RT-PCR

Freshly sorted LT-HSCs or MPPs were starved for 2h in SFEM at 37°C / 5%CO_2 before adding indicated cytokines (Peprotech) at 100ng/ml for 1h stimulation in the presence of 10µg/ml Cycloheximide (Invitrogen). Cells were washed 2 times and subjected to cDNA synthesis using TaqMan® Gene Expression Cells-to-CT™ Kit (Ambion). Real-time PCR with TaqMan-assays (*Gadd45a* Mm00432802_m1, *Gadd45b* Mm00435121_g1, *Gadd45g* Mm01352550_g1) was performed on a LightCycler™ 430 (Roche). The expression was normalized to B2M and shown relative to the unstimulated control.

Time-lapse imaging

FACS sorted LT-HSCs were seeded in 24-well plates (SFEM medium, 100ng/ml SCF and TPO) equipped with silicon culture inserts (IBIDI) and immediately transduced with lentiviral particles (MOI 100). FITC-conjugated anti-CD16/32 was added to the medium at 50ng/ml. Plates were gas-tight sealed with adhesive tape after 5% CO_2 saturation. Microscopy was performed using a CellObserver (Zeiss) at 37°C. Phase contrast images were acquired every 2-3 min using a 10x phase contrast objective (Zeiss), and an AxioCamHRm camera (at

1388x1040 pixel resolution) with a self-written VBA module remote controlling Zeiss AxioVision 4.8 software. Fluorescence was detected every 1h with HXP illumination (Osram) and the filter sets for EGFP (F45-002) and Cy3 (F4-004, AHF Analysetechnik).

Cell tracking

Cell tracking was performed using a self-written computer program (TTT) as described (Rieger et al., 2009), until the fate of all progeny in the fifth cell generation was determined. The generation time of an individual cell was defined as the time span from cytokinesis of its mother cell division to its own division. Dead cells are easily depicted by their shrunk, non-refracting appearance with immobility. All cell tracking was done by scientists; the current analysis does not rely on data generated by an unsupervised computer algorithm for automated tracking.

Supplemental References

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