The biology of haematopoietic stem cells (HSCs) has predominantly been studied under transplantation conditions. It has been particularly challenging to study dynamic HSC behaviour, given that the visualization of HSCs in the native niche in live animals has not, to our knowledge, been achieved. Here we describe a dual genetic strategy in mice that restricts reporter labelling to a subset of the most quiescent long-term HSCs (LT-HSCs) and that is compatible with current intravital imaging approaches in the calvarial bone marrow. We show that this subset of LT-HSCs resides close to both sinusoidal blood vessels and the endosteal surface. By contrast, multipotent progenitor cells (MPPs) show greater variation in distance from the endosteum and are more likely to be associated with transition zone vessels. LT-HSCs are not found in bone marrow niches with the deepest hypoxia and instead are found in hypoxic environments similar to those of MPPs. In vivo time-lapse imaging revealed that LT-HSCs at steady-state show limited motility. Activated LT-HSCs show heterogeneous responses, with some cells becoming highly motile and a fraction of HSCs expanding clonally within spatially restricted domains. These domains have defined characteristics, as HSC expansion is found almost exclusively in a subset of bone marrow cavities with bone-remodelling activity. By contrast, cavities with low bone-resorbing activity do not harbour expanding HSCs. These findings point to previously unknown heterogeneity within the bone marrow microenvironment, imposed by the stages of bone turnover. Our approach enables the direct visualization of HSC behaviours and dissection of heterogeneity in HSC niches.

Development of an HSC-specific reporter line

The expression of the myelodysplastic syndrome 1 (Mds1) gene is highly enriched in LT-HSCs. Mds1 is transcribed from its own promoter in the Mecom locus, which also produces the well-known EVI1 gene product and the MDS1–EVI1 gene fusion product. We targeted an EGFP expression cassette to the first transcriptional start site of Mds1 (Extended Data Fig. 1a). The resulting allele is predicted to be a hypomorph for MDS1 and MDS1–EVI1 but to have no effect on the expression of EVI1. Mice heterozygous for the GFP-linked allele (Mds1GFP/+) showed normal haematopoietic parameters, frequency of HSCs and cell cycle properties, and response to myelosuppression (Extended Data Fig. 1b–f). Flow cytometric characterization of these
mice confirmed the complete absence of GFP expression in any mature lineage-positive (LIN+) haematopoietic cells (Extended Data Fig. 2a, Supplementary File 2). GFP expression was predominantly restricted to the small fraction of CKit+ SCA-1+ cells (Fig. 1a). Using standard phenotypic parameters, we found that 28.83 ± 11.99% (mean ± s.d.) of bone marrow cells gated solely on GFP could be categorized as LT-HSCs, 26.61 ± 9.86% as short-term HSCs (ST-HSCs) and 30.12 ± 12.8% as MPPs (Fig. 1b). MFG cells constituted only about 12% of the mixed population as haematopoietic stem and progenitor cells (HSPCs). Notably, GFP was not expressed in non-haematopoietic compartments of the bone marrow (Extended Data Fig. 2b, c, Supplementary File 3).

With the aim of eliminating the labelling of MPPs in the Mds1GFP+/+ Flt3Cre model, we reasoned that the additional expression of a gene associated with early differentiation could facilitate exclusive identification of LT-HSCs. Increased brightness of the reporter in phenotypically defined LT-HSCs inversely correlated with the expression of EPCR and little or no CD34 (Extended Data Fig. 3c, Supplementary File 1). MFG cells constituted only about 12% of the phenotypically defined LT-HSC population (Fig. 1b). The specificity of LT-HSC labelling in MFG mice was recapitated in bone marrow from multiple locations (Extended Data Fig. 3d). MFG cells represented a largely quiescent population (Fig. 1c, Extended Data Fig. 3g) that express relatively high levels of SCA1 and EPCR and little or no CD34 (Extended Data Fig. 3e, f), consistent with previously described dormant HSCs.

To further validate our combined Mds1GFP+/+ Flt3Cre model, we performed single-cell RNA sequencing (scRNA-seq) in cells isolated exclusively on the basis of GFP expression. The resulting transcriptomes were then extrapolated to a published single-cell transcriptional map of LT-HSCs, ST-HSCs, and multiple MPP populations (MPP2/3/4)\textsuperscript{16}. Strikingly, virtually all MFG+ transcriptomes mapped to the most unprimed cluster of cells, in which phenotypic LT-HSCs also reside (Fig. 1d, Extended Data Fig. 4a). A small fraction of MFG+
cells showed megakaryocyte lineage priming (Fig. 1d, Extended Data Fig. 3c), which has recently been described in multipotent HSCs. This analysis also highlights the efficiency of our approach in restricting GFP expression to Mds1+ cells (Extended Data Fig. 4b). MFG cells expressed transcripts that were also enriched in dormant HSCs (Extended Data Fig. 4c, d). In addition, single-cell quantitative PCR analysis of a 280-gene haematopoietic gene panel demonstrated clustering of MFG cells with LT-HSCs but no other progenitor signatures (Extended Data Fig. 4e). Finally, we performed long-term reconstitution assays to assess the potency of MFG cells in comparison to cells isolated using traditional flow cytometry markers for HSCs (LIN−SCA-1+C-KIT+CD150+CD48−, here referred to as SLAM cells) parameters. Limiting dilution transplants using 3–25 cells suggested that MFG-HSCs are at least as enriched as SLAM cells in transplantation capacity (Fig. 1e, Extended Data Fig. 4f). MFG cells also repopulated secondary recipients (Extended Data Fig. 4g). In addition, within the LIN+SCA-1+C-KIT+CD150+CD48− compartment, long-term repopulating activity was enriched in cells expressing GFP (Extended Data Fig. 4h). Thus, our MFG animal model allows the isolation of a highly quiescent sub-population of LT-HSCs with potent repopulation potential.

Localization of MFG-HSCs in the calvaria

Using these two reporter models, we performed imaging of GFP+ cells in the calvaria of live mice. As expected, MDS1-GFP HSPCs were more prevalent than MFG-HSCs (Fig. 1a, b, 2a). Both cell types were located peri-vascularily at an average distance of less than 10 μm from the closest vessel (Fig. 2b, Supplementary Videos 1, 2). MFG-HSCs were also found similarly close to the endosteum (Fig. 2c), pointing to a possible dual endosteal-vascular niche, as suggested previously. However, we found that MFG-HSCs were almost exclusively associated with sinusoids rather than arterioles (Fig. 2d). While HSPCs also predominantly localized close to sinusoids, a significant fraction of these were also near transition zone vessels (Fig. 2d) and their distance from the endosteum was more varied (Fig. 2c), suggesting that MFG-HSCs and downstream HSPCs occupy different micro-niches.

MFG-HSCs are not found in deep hypoxic zones

Low oxygen tension (hypoxia) has been historically thought to be a shared niche characteristic that is critical for maintaining stem cell quiescence. However, support for the existence of a hypoxic niche has largely come from indirect evidence and measurements lacking spatial resolution. Using an oxygen sensor and two-photon phosphorescence lifetime microscopy, we measured the local P02 surrounding individual HSPCs and MFG-HSCs in their native microenvironments (Extended Data Fig. 6a–f). First, we confirmed the overall hypoxic status of the calvarial bone marrow, with intravascular P02 in the range of 15–30 mm Hg (mean -23 mm Hg, about 3% O2) and extravascular P02 in the range of 10–25 mm Hg (mean -17 mm Hg, about 2% O2, Fig. 2e). We then measured P02 around individual HSPCs and MFG-HSCs, and found similar oxygen levels (-18 and -19 mm Hg) close to the average.
Heterogeneous HSC response to activation

We next examined the dynamic behaviours of HSPCs and LT-HSCs in their native niches. In vivo time-lapse imaging of the calvarium revealed that MFG-HSCs displayed low baseline motility whereas HSPCs showed enhanced motility (Fig. 3a, b, Supplementary Videos 3–6). To assess whether HSC behaviours would be affected in the context of activation, we used a cyclophosphamide (Cy)/G-CSF protocol that leads to expansion and subsequent mobilization of LT-HSCs28 (Extended Data Fig. 7a). Fluorescence-activated cell sorting (FACS) and imaging analysis demonstrated a tenfold increase in the number of MFG cells after treatment (Fig. 3c–e, Supplementary Video 7, Extended Data Fig. 7b, c). MFG cells are still enriched in the phenotypic LT-HSC fraction in this activated state (Extended Data Fig. 7b, c). MFG cells are still enriched in the phenotypic LT-HSC fraction in this activated state (Extended Data Fig. 7b, c).

Activated MFG-HSCs were found, on average, to be further away from the endosteum than native MFG-HSCs (Extended Data Fig. 7f). Notably, they were even closer to the vasculature, with an average distance of about 1 µm (Extended Data Fig. 7g), and maintained their sinusoidal proximity (Fig. 3f). By assessing the distribution of activated MFG-HSCs in the entire calvarial region, we identified unique patterns of HSC proliferation. First, native MFG-HSCs were found as rare single cells within the bone marrow, whereas activated MFG-HSCs appeared as clusters (Fig. 3c–e), suggesting that that MFG-HSC proliferation occurs within spatially restricted domains. Second, a subset of MFG+ cells remained as single cells while others formed clusters in both the Cy/GCSF and 5-FU models (Fig. 3e, Extended Data Fig. 8c), suggesting that the proliferative response is heterogeneous among HSCs. To assess whether these clusters were clonal, we generated Mds1CreRosa26-loxP-cre mice29 (Extended Data Fig. 8d–f). In untreated mice, labelled cells were usually found as rare single cells of different colours dispersed throughout the bone marrow (Extended Data Fig. 8g). Following treatment with Cy/GCSF, we observed labelled cell clusters made up predominantly of cells of a single colour (Extended Data Fig. 8g, h). Quantitative analysis confirmed that labelled nearby clusters were more likely to have the same colour than a mixture of colours (Extended Data Fig. 8h), providing evidence for clonal HSC proliferation within confined physical domains.

5-fluorouracil (5-FU), the number of MFG+ cells increased (Extended Data Fig. 8a, Supplementary Videos 9, 11) and a subset exhibited higher motility, particularly on day 20 after treatment (Extended Data Fig. 8b, Supplementary Videos 10, 12). These data suggest that enhanced motility is a common feature of the HSC response to injury, although we cannot rule out the possibility that the response is a result of indirect action on the niche by Cy/G-CSF or 5-FU.

Activated MFG-HSCs were found, on average, to be further away from the endosteum than native MFG-HSCs (Extended Data Fig. 7f). Notably, they were even closer to the vasculature, with an average distance of about 1 µm (Extended Data Fig. 7g), and maintained their sinusoidal proximity (Fig. 3f). By assessing the distribution of activated MFG-HSCs in the entire calvarial region, we identified unique patterns of HSC proliferation. First, native MFG-HSCs were found as rare single cells within the bone marrow, whereas activated MFG-HSCs appeared as clusters (Fig. 3c–e), suggesting that MFG-HSC proliferation occurs within spatially restricted domains. Second, a subset of MFG+ cells remained as single cells while others formed clusters in both the Cy/GCSF and 5-FU models (Fig. 3e, Extended Data Fig. 8c), suggesting that the proliferative response is heterogeneous among HSCs. To assess whether these clusters were clonal, we generated Mds1CreRosa26-loxP-cre mice29 (Extended Data Fig. 8d–f). In untreated mice, labelled cells were usually found as rare single cells of different colours dispersed throughout the bone marrow (Extended Data Fig. 8g). Following treatment with Cy/GCSF, we observed labelled cell clusters made up predominantly of cells of a single colour (Extended Data Fig. 8g, h). Quantitative analysis confirmed that labelled nearby cells were more likely to have the same colour than a mixture of colours (Extended Data Fig. 8h), providing evidence for clonal HSC proliferation within confined physical domains.
HSC expansion restricted by bone remodelling

The observation of heterogeneous HSC proliferation in restricted physical domains prompted us to re-examine the characteristics of the microenvironment that either support clonal expansion or maintain cell quiescence. Recognizing that the bone is constantly undergoing remodelling, we hypothesized that the stages of bone turnover impose an additional degree of heterogeneity in the bone marrow microenvironment that is not captured by the prevailing view centred on the endosteal versus perivascular duality. To visualize the stages of bone turnover, we administered two (spectrally distinct) calcium-binding dyes30 48 h apart, and imaged the calvarium immediately after the second dye injection. The two dyes mark the positions of the old and new bone fronts, respectively, and reveal where the old bone front has been eroded (Fig. 4a). We quantified the ratio of the two dyes and classified the cavities as D-type (undergoing predominantly bone deposition), R-type (predominantly bone resorption), and M-type (mixed). We confirmed that osteoblasts are biased towards D-type cavities while osteoclasts are biased towards R-type cavities. A mixture of osteoblasts and osteoclasts were found at intermediate levels in M-type cavities (Extended Data Fig. 9a–g). Using this double-staining scheme, we also found distinct cavity types in the metaphyses of long bones (Extended Data Fig. 11a–f, Supplementary Video 15). The existence of heterogeneity in both the HSC response to injury and the bone marrow microenvironment, coupled to the stages of bone remodelling, that has not been recognized previously to our knowledge3,27,31. Notably, we also found distinct cavity types in the metaphyses of long bones (Extended Data Fig. 11a–f, Supplementary Video 15). The existence of distinct types of bone marrow cavity implies that the traditional way of characterizing the HSC niche as endosteal or perivascular is inadequate, as the microenvironment, including the perivascular niches, contained within these cavities is likely to differ depending on the local calcium

deposition type

P

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and HSPCs. During the steady state, MFG-HSCs were found in baseline varia (Fig. 4f), as well as the spatial distributions of native MFG-HSCs cavities (Extended Data Fig. 9a–g). Using this double-staining scheme, osteoblasts and osteoclasts were found at intermediate levels in M-type cavities while osteoclasts are biased towards R-type cavities. A mixture of (mixed). We confirmed that osteoblasts are biased towards D-type cavities at steady-state and after Cy/GCSF activation. n = 4 mice per group, plotted as different symbols; black line represents mean ± s.d. b. Quantification of HSPCs in D-, M- or R-type cavities at steady-state and after Cy/GCSF activation. n = 4 mice per group, plotted as different symbols. Two-sided Mann–Whitney test used unless otherwise specified, ****P < 0.0001, black line represents mean ± s.d.

Fig. 4 | Heterogeneity of bone remodelling stages governs expansion of MFG-HSCs (Mds1<sup>GFP</sup>/Flt3<sup>Cre+</sup> mice) and HSPCs (Mds1<sup>GFP</sup>/Flt3<sup>Cre−</sup> mice). a. The double calcium staining strategy that identifies D-, M- and R-type cavities. Dye 1, delivered 48 h before imaging, shows the old bone front that has been eroded to varying extents; dye 2, delivered before imaging, shows the new bone front. b–d. Expanded views, showing distinct cavity types defined by the dye 1:dye 2 pixel ratios. e. A sagittal section of bone marrow cavities containing Mds1<sup>GFP</sup>/ Flt3<sup>Cre+</sup> cells. f. Fractions of D-, M- and R-type cavities in the calvaria of non-treated or treated mice (two-tailed t-test, n = 155 cavities from non-treated and 80 or 73 bone marrow cavities from treated animals (n = 3 mice); mean ± s.d.).

deposition, R-type (predominantly bone resorption), and M-type (mixed). We confirmed that osteoblasts are biased towards D-type cavities while osteoclasts are biased towards R-type cavities. A mixture of osteoblasts and osteoclasts were found at intermediate levels in M-type cavities (Extended Data Fig. 9a–g). Using this double-staining scheme (Fig. 4b–e), we quantified the fractions of D-, M-, R- cavities in the calvaria (Fig. 4f), as well as the spatial distributions of native MFG-HSCs and HSPCs. During the steady state, MFG-HSCs were found in baseline numbers in all cavity types, while HSPCs tended to be enriched in M-type cavities (Fig. 4g, h). After activation with Cy/GCSF, however, expanded MFG cells were found almost exclusively in a subset of M-type cavities (Fig. 4g, Supplementary Video 13, Extended Data Fig. 10a). HSPCs were also found to expand preferentially in M-type cavities after activation, although this preference was less pronounced (Fig. 4h, Supplementary Video 14, Extended Data Fig. 10b). This evidence of heterogeneity in types of bone marrow cavity, and of a subset of M-type cavities that favours HSC expansion, supports our earlier observation that HSCs expand clonally in restricted physical domains.

Discussion

Our work here describes the generation and characterization of an animal model in which a single-colour reporter can be used for the identification and live imaging of LT-HSCs in the native niche without transplantation (Extended Data Table 2). We found evidence of heterogeneity in both the HSC response to injury and the bone marrow microenvironment, coupled to the stages of bone remodelling, that has not been recognized previously to our knowledge.3,27,31. Notably, we also found distinct cavity types in the metaphyses of long bones (Extended Data Fig. 11a–f, Supplementary Video 15). The existence of distinct types of bone marrow cavity implies that the traditional way of characterizing the HSC niche as endosteal or perivascular is inadequate, as the microenvironment, including the perivascular niches, contained within these cavities is likely to differ depending on the local calcium deposition (mixed).
gradient\(^3\) and the downstream effects of osteoclast degradation\(^3\). To fully characterize the regulatory factors that govern HSC quiescence versus proliferation, it will be necessary to develop molecular profiling technology\(^4\) that can spatially map distinct bone cavities.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41586-020-1971-z.

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**Methods**

**Mice and genotyping**

The generation of Mds1<sup>Gfp/+</sup> mice was generated by cloning and homologous recombination of the linearized targeting vector via electroporation into v6.5 embryonic stem (ES) cells. After selection with neomycin and clonal screening by PCR, correctly targeted ES cell clones were injected into C57Bl/6 blastocysts. Derived chimaeras were initially bred to C57Bl/6 to obtain germline transmission, followed by crossing with FLP reporter mice<sup>36</sup> to remove the Frt-Neo-Frt cassette that was part of the original targeting vector. Derived mice were backcrossed onto a C57Bl/6 background for more than six generations and mice were analysed via PCR to identify their genotype (′5-AGTGTTAAAGGCTGCTCAACT-3′, ′5-GTACAGGGTAGGCTGCTCAACT-3′, ′5-CTCCTCCACGCTTTTGCT-3′). Some of the displayed data comes from mice that still carried the Frt-Neo-Frt cassette, which showed slightly lower mean fluorescence intensity in bone marrow cells. A similar strategy was used to generate the Mds1<sup>Cre<sup>ere</sup></sup> allele. For all experiments, 2–12-month-old adult mice of both sexes were used and wild-type littermates were used as controls. Flt3<sup>Cre</sup> mice<sup>44,45</sup>, Rosa26-CAG-loxp-stop-loxp-tdtomato reporter mice<sup>20</sup> and Rosa26-CAG-loxp-stop-loxp-Confetti reporter mice<sup>20</sup> have been described. For identification of Flt3<sup>Cre</sup> and Mds1<sup>Cre<sup>ere</sup></sup>, Cre primers were used (′5-TTACTGACCGTACACCAAAATTTGCC-3′, ′5-C TCGGCAGCATGCATCTTTTGCGT-3′). Mice were bred and housed according to NIH guidelines in our AAALAC-accredited, specific-pathogen-free animal care facilities at Boston Children's Hospital or Massachusetts General Hospital. All animal protocols were approved by the Animal Resources at Children's Hospital Boston, Boston Children's Hospital Institutional Animal Care and Use Committee, and Massachusetts General Hospital Institutional Animal Care and Use Committee. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All results involved in the study were acquired according to ethical standards.

**HSC isolation, flow cytometry and cell sorting**

Bone marrow cells were isolated by crushing of the bones using a mortar and pestle in Ca<sup>2+</sup>/Mg<sup>2+</sup>-free phosphate-buffered saline (D-PBS) supplemented with 2% fetal bovine serum (FBS) and 1× penicillin/streptomycin (Pen/Strp) (Invitrogen). Viable cell number was calculated by manually counting with a haemocytometer or using a TC20 Automated Cell Counter (Bio-Rad). The cell suspension was filtered through a 70-µm strainer. For HSC identification via flow cytometry, the cells were stained for C-KIT (eBioscience), SCA-1 (eBioscience, BioLegend), CD48 (BD Pharmingen) and CD150 (BioLegend) as well as a lineage marker cocktail consisting of B220, TER-119, GR-1, CD4 and CD8a (eBioscience). For experiments requiring lineage depletion, antibody staining for B220, TER119, GR-1, CD4 and CD8a biotin-conjugated antibodies was first performed followed by application of anti-biotin beads (Miltenyi Biotec) and depletion using magnetic separation columns (Miltenyi Biotec). For megakaryocyte progenitor staining, the cells were stained for lineage marker cocktail, C-KIT, SCA-1, CD150, CD41 (BioLegend) and FcγR (eBioscience). For identification of common myeloid progenitors (CMPs), granulocyte-monocyte progenitors (GMPs) and megakaryocyte-erythroid progenitors (MEPs), cells were stained for lineage marker cocktail, C-KIT, SCA-1, CD34 (eBioscience) and FcγR. For mesenchymal stem cells, cell suspension was stained for the lineage cocktail, CD45, PDGFRα and integrin-αv (eBioscience). For endothelial cells, lineage cocktail, CD45, CD31 and VE-cadherin (eBioscience) were used. For identification of pre and pro B cells, immature B cells and mature B cells, B220 and IgM (eBioscience) were used. For erythroid cells, the primary marker was Ter-119 (eBioscience); for monocytes and neutrophils, Mac-1 (eBioscience) and Ly6-G (BD Pharmingen) were used; and for T cells CD4 and CD8 were used. Antibody staining of cell suspensions was always performed on ice for 45 min. 4′,6-diamidino-2-phenylindole (DAPI, 10 µg/ml in PBS; Invitrogen) was used for exclusion of dead cells during flow cytometry. Relevant flow cytometry gating strategies for the identification of different mature cell populations, LT-HSCs, STHSCs, MPP2s, MPP3/4s and endothelial cells are available in the Supplementary Information. Transplanted cells were double-sorted to increase purity. For transplantation of low cell numbers, all secondary sorts were performed in a plate using the automated plate reader sorting function. For FACS analysis, a BD LSRII Flow Cytometer was used, while cell sorting was performed using a BD FACSAria II sorter (BD Biosciences). Flow cytometry data were analysed using FlowJo (Tree Star).

**Cell cycle analysis**

As each animal contained an average of only 600–700 MFG<sup>+</sup> sorted cells, the cell cycle analysis demonstrated in Fig. 1c and Extended Data Fig. 3g represent GFP cells isolated from seven Mds1<sup>Gfp/+</sup>Flt3<sup>Cre</sup> mice; thus, the data represent an average from seven mice that were pooled together. Upon identification and sorting purification of corresponding cellular populations as described above, cells were fixed in ice cold 70% ethanol. Cells were then washed and stained with Ki67 (BioLegend) for 30 min on ice to distinguish G0/G1 phase. DAPI was finally used for staining and analysis of G1/G2 versus M/S phase. Cell cycle analysis was performed using a BD FACSAria II sorter.

**Competitive reconstitution assays in irradiated mice and peripheral blood analysis**

Bone marrow transplant recipients were 8–12-week-old B6.SJL-Ptprca<sup>Pec3</sup>/BoyJ (CD45.1) mice. Before transplantation, mice were lethally irradiated using a gamma irradiator with a split dose of 11 Gy with a 3-h interval between the two doses. Cells were transplanted via retro-vascular injection into anaesthetized mice. One hundred thousand whole bone marrow CD45.1 cells were used as competitors unless stated otherwise. For limiting dilution studies, HSC frequency was calculated using Extreme Limiting Dilution Analysis software (http://bioinf.wehi.edu.au/software/elda/) with data taken 4 months after transplantation. The lower stem cell frequency reported here might be due to the incomplete backcrossing of MFG mice (six generations), the presence of constitutive CRE in haematopoietic cells and/or technical reasons. For secondary transplants, two million whole bone marrow cells from primary recipients were transplanted into lethally irradiated secondary recipients. For blood analysis of transplanted recipients, blood was collected at 4-week intervals for at least 16 weeks after transplantation. Peripheral blood was first treated with red blood cell lysis buffer to remove red blood cells followed by antibody staining for B cells (CD19, eBioscience), T cells (CD4, CD8α) and granulocytes (Ly6-G). The percentage chimaerism was estimated using CD45.1 (BioLegend) and CD45.2 (eBioscience) antibody staining.

**Blood cell counts and treatment with 5-FU, Cy/G-CSF and tamoxifen**

Blood samples were collected via the retro-vascular vein in EDTA-coated tubes. Blood cell counts were performed using a HEMAVET950 (Drew Scientific) cell blood counter. For blood cell kinetic analysis upon 5-FU treatment, cell counts were performed on day 0, 3, 7, 10, 13 and 17. 5-FU was delivered via retro-vascular injection as single dose of 150 mg/kg immediately after day 0 and a bleeding sample was collected while control mice were injected with PBS. In addition, bone marrow from treated mice was analysed using flow cytometry or imaging was performed at the indicated time points. For Cy/G-CSF experiments, cyclophosphamide was delivered via intraperitoneal injection as a single dose of 200 mg/kg on day 1, followed by subcutaneous injection of G-CSF on days 2, 3 and 4 at 250 μg/kg per day followed by bone marrow flow cytometry analysis or live animal imaging of the calvarial bone marrow. For Mds1<sup>Gfp/+</sup>Rosa26<sup>Confetti</sup> mouse experiments, cyclophosphamide...
or PBS was administered on day 1 followed by GCSF and tamoxifen injection on day 2 and further GCSF or PBS administration on days 3 and 4 according to each experiment. Tamoxifen was administered at a 2 mg dose via intraperitoneal injection to target labelling to the LT-HSC compartment. Bone marrow analysis of femurs and calvaria was performed 24 h after the final GCSF dose according to each experiment.

**Single cell inDrops RNA sequencing**

GFP cells were sorted from *Mds1<sup>GFP</sup>* Flt3<sup>lo</sup>* mice and single cells were encapsulated using droplet microfluidic technology as previously described<sup>39</sup>. Although 1,200 GFP<sup>+</sup> cells were sorted, only about 400 cells were encapsulated; the loss of more than half of the population is standard for low cell number populations in the inDrop platform. Upon library preparation of barcoded single cells, RNA sequencing was performed. To process the data, we used a previously published workflow and code available at https://github.com/AllonKleinLab/SPRING. Ensemble release 81 mouse mm10 cDNA plus the sequence of loxP-ires-GFP-polyA-loxP was used as reference. SPRING plots were generated using the next four-step process. Initially, cells with few mRNA counts (< 1,000 unique molecular identifiers) and stressed cells (mitochondrial gene set Z-score >1) were filtered out. The remaining high-quality cells were total-counts normalized. We next filtered genes, keeping those that were well detected (mean expression > 0.05) and highly variable (CV > 2). Finally, the data were normalized by Z-scoring each gene and applying principal components analysis (PCA), retaining the top 50 PCs. Following filtration and bioinformatics analysis, only 50 GFP<sup>+</sup> cells passed quality control and were used for plotting. The data acquired from the GFP<sup>+</sup> cells were then plotted with previously published data for LSK cells<sup>28</sup> upon transformation in the PCA space of the previously published data. In brief, the two datasets were integrated using the library sklearndecomposition.PCA (python 2.7). The fit function was used to calculate the first 50 PCs for the single cell LSK dataset<sup>28</sup>. Then, the normalized and filtered count matrices of the GFP and LSK cells were vertically combined. Before combining the two matrices, the GFP matrix was scaled in order to have a comparable amount of normalized counts in correlation to the LSK matrix. The resulting Z-score combined matrix was used as input for the transform function to project the combined dataset onto the original LSK dataset. The output generated by the transform function and the corresponding distance matrix, which was obtained using the SPRING function get_distance_matrix, were used to generate the final SPRING plot. All data were visualized as previously described<sup>28</sup>. This reduced any batch effects between the two experiments. The coordinates generated by the SPRING plots were plotted using R.

**Single cell fluidigm analysis**

GFP cells were primarily sorted from *Mds1<sup>GFP</sup>* Flt3<sup>lo</sup>* mice followed by secondary single cell sorting directly in 96-well plates containing PCR buffer. Sorted plates were frozen on dry ice followed by reverse transcription, pre-amplification and high-throughput microfluidic real-time PCR for 180 transcription factors as previously described<sup>20</sup>. Data analysis and hierarchical clustering were performed using MultiExperiment Viewer (MeV) program. Previously published data for CMPs, GMPs, MEPs, common lymphoid progenitors (CLPs), MPPs and LT-HSCs<sup>28</sup> using the same 180 real-time PCR platform were overlaid for comparison to the GFP cells.

**Synthesis and characterization of phosphorescent Oxyphor PtG4 probe**

The structure of Oxyphor PtG4 is almost identical to that of the previously published Oxyphor PdG4 probe<sup>37</sup>. The synthesis of the core porphyrin and the synthesis of a dendritic probe similar to Oxyphor PdG4 have been published previously<sup>39</sup>. All synthesis steps were identical to those developed for the synthesis of PdG4<sup>40</sup>. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry was used to confirm the identity of the intermediate products and of the target probe molecule. Calibration, assessment of phosphorescence oxygen quenching and absorption spectra measurements for the Oxyphor PtG4 probe were performed as previously described<sup>37,39</sup>.

**In vivo and ex vivo imaging**

All in vivo imaging experiments were performed according to procedures approved by the Institutional Animal Care and Use Committee at Massachusetts General Hospital. In brief, mice were either anaesthetized with an induction dose of 3–4% isoflurane (96% O<sub>2</sub>) and a maintenance dose of 1.25–2% isoflurane or given an intraperitoneal bolus injection of ketamine (100 mg/kg) and xylazine (15 mg/kg). Animals were deemed anaesthetized by the toe pinch method. To minimize pain, mice were treated with buprenorphine (0.05–0.1 mg/kg). The hair on the calvarium was removed with scissors or a mechanical trimmer and then the skin was wiped with alcohol. Next, a calvarial skin flap was created with a U-shaped incision to reveal the underlying calvarial bone as previously described<sup>28</sup>. Mice were injected with imaging agents (for example, vascular labels) retro-orbitally, mounted in a custom-designed heated mouse holder, and secured to the stage of a home-built multiphoton/confocal laser-scanning video-rate microscope (for z-stack or time-lapse imaging) or an Olympus FVMP-OI multiphoton imaging platform (for oxygenation measurements)<sup>39</sup>. A drop of 0.9% saline was applied to the skull to act as the immersion fluid, and a Zeiss 63×/1.15 numerical aperture water-dipping objective, an Olympus 60×1.0 numerical aperture water-dipping objective, or an Olympus 25×1.05 numerical aperture water-dipping objective was used for all imaging. For pointend imaging, mice were killed while under anaesthesia using approved procedures. For survival imaging, the skin flap was closed with 6-0 vinyl sutures (Ethicon). Triple antibiotic ointment (bacitracin, neomycin, and polymyxin-B sulfate) was applied to the top of the surgical site to minimize the chance of infection. Mice were put in a heated cage and monitored until fully awake. For 6-h imaging sessions, mice were given an intraperitoneal injection of -100 µl 0.9% saline solution every hour to ensure proper hydration.

GFP was excited at 491 nm (confocal) or 950 nm (two-photon) and collected at ~505–540 nm using a photomultiplier tube. Angiosense 680EX (~100 µl at 2 nmol/100 µl, Perkin-Elmer) for labelling the vasculature was excited at 635 nm (confocal only) and collected at ~665–725 nm using a photomultiplier tube. Autofluorescence generated from the 491 nm or 950 nm excitations was collected at ~570–610 nm using a photomultiplier tube. Second harmonic generation (SHG) from collagen in the bone was excited at 775 nm or 840 nm (two-photon only) and collected at ~340–460 nm with a photo-multiplier tube. Phosphorescence was excited at 1,150 nm by a Ti:Sapphire laser second harmonic generation (Spectra-Physics) and collected above 750 nm with a photomultiplier tube. For calcium staining of osteoblast bone fronts, calcein blue (30 mg/kg, Sigma), and Alizarin red S (40 mg/kg), were excited at 775 nm and collected at 415–435 nm, 500–550 nm, and 580–650 nm, respectively. Rhodamine B dextran 70 kDa (0.5 mg/50 µl, Sigma) was used as a vasculature contrast with Cat K 680 FAST (2 nmol/100 µl injected 6 h before imaging, Perkin-Elmer) for labelling osteoclasts, excited simultaneously at 532 nm and 638 nm and collected at 570–620 nm and 665–745 nm.

For steady-state in vivo imaging, 15–60 frames from the live video mode were averaged to acquire single 500 × 500 pixel images. Z-stacks were acquired with 1–2-µm steps and time-lapse images were acquired at 30-s intervals for 20 min or longer. For Cy/GCSF in vivo imaging, z-stacks were acquired with 2-µm steps every 20 min for ~6 h. Calvarial cell location maps in Fig. 3d, e were created in Matlab using custom code based on the x, y, and z coordinates of each cell. Data from each mouse were aligned and then overlayed using the locations of the coronal and sagittal sutures.

For P0<sub>1</sub> measurements, ~75 µl of 1.7 mM Pt-G4 suspended in 0.9% PBS (1× PBS, Invitrogen) was injected intravenously before imaging.
In each pO₂ measurement location, multiple pulse excitation/emission cycles were used to record the phosphorescence lifetime decay. For each cycle, the probe was excited for ~10–20 µs followed by ~150 µs for time-resolved photon collection. Quantitative pO₂ values were obtained by fitting the phosphorescence decay with a single exponential to get an average lifetime of phosphorescence. This lifetime value was converted to pO₂ using an in vitro calibration curve for the same batch of Pt-G4.

For 5-FU imaging experiments, 5-FU was delivered via retro-orbital injection as a single dose of 150 mg/kg as described above. In vivo bone marrow two-photon imaging was then performed on day 4 or day 20 after the 5-FU injection. The calvarial cell location map in Extended Data Fig. 8c was made in similar way to the Cy/GCSF cell maps described above.

For Cy/GCSF ex vivo imaging, freshly fixed (4% paraformaldehyde) and excised mouse calvaria were affixed to a plastic dish, immersed in 1× PBS, and immediately imaged for 4–5 h. Tiled z-stacks were acquired with 3-µm steps and a 10% overlap between fields of view. Images were stitched together in 3D using Olympus Fluoview software or ImageJ scripts.

For examining bone remodelling activities in calvaria (in vivo) and metaphysis (ex vivo), calcium binding dyes were administered 48 h apart via retro-orbital injection. Calvarial in vivo imaging was performed as described. Mouse tibia was freshly removed, thinned, and imaged from the bone surface. Tiled z-stacks were acquired with 3-µm steps and stitched using ImageJ.

**Image quantification**

For distance measurements, the distance from each cell to blood vessels or to the nearest bone surface (that is, endosteum identified using SHG) was computed by hand as described previously using the Pythagorean theorem. The bone contains abundant collagen, which enabled us to use SHG imaging to identify the inner bone surface (endosteum). This technique has been used in many previous publications of live bone marrow imaging.

The identity of blood vessels (arterioles, sinusoids, or transition vessels) within the calvaria was determined by a combination of morphology, location within the vessel network, location within the bone marrow, and blood flow. In brief, with our blood pool agent the arterioles appear as narrow (~5–10 µm diameter) and generally straight vessels with a smooth surface upstream of sinusoidal vessels, which are larger (~20 µm diameter or greater) with irregular surfaces. This definition was based on previous work, which confirmed that these small diameter vessels are arterioles with a faster flow speed (~2 mm/s or higher), higher pO₂, and increased barrier function in comparison to sinusoidal vessels. They also stain positive for SCAl. At the transition point between arterioles and sinusoids, the vessel diameter increases. It is from this point of increase to the next vessel branching point downstream that we define as transitional vessels.

Distance measurements were performed in ImageJ v.1.51p. For display purposes, the brightness and contrast of images in the figures were adjusted, but all image analysis was performed on raw data. For motility measurements, frame-to-frame drift was corrected in 3D using the Template Matching plugin in ImageJ. Next, the centroid of the cell was determined for the first and last image of a 20-min sequence, and the 2D displacement was calculated using the distance formula.

For cell clustering analysis, the individual tiled z-stack images were reconstructed into a single z-stack for the whole calvaria using ImageJ. Next, each cell was designated as one of three tags (red, green, or blue) based on the colour of the cell during imaging, and the x, y, z coordinates were recorded by hand. Using a custom Matlab script similar to ClusterQuant, we analysed the spatial clustering (cluster size = 3) of like-coloured cells in this model compared to 10,000 randomized samples to determine the statistical likelihood of the colour clustering in our samples. Graphs and statistical analyses were performed using Graph Pad Prism version 6 or higher. The contrast and/or brightness of figure images and videos were adjusted for display purposes only.

**Classification of bone marrow cavities**

A bone marrow cavity is defined as a 3D inclusion inside bone with a single concave endosteum (Fig. 4e, Extended Data Fig. 11d–f), while deeper down all cavities are interconnected. Once a cavity has been defined using the bone SHG signal, we classified types of bone marrow cavity by sequential staining with two calcium-binding reagents. The first calcium-binding dye (dye 1, tetracycline or calcine blue, Sigma; 35 mg/kg and 30 mg/kg, respectively) was administered 48 h before imaging to track bone resorption activities based on erosion of dye 1, and the second calcium-binding dye (dye 2, Alizarin red, 40 mg/kg) was administered 30 min before imaging to label high-calcium regions (bone fronts). The 48-h interval was chosen on the basis of the estimated lifespan of mouse active osteoclasts. Therefore, the double-staining approach delineated approximately one bone erosion cycle in the bone marrow. As the lack of dye 1 indicates the existence of resorption whereas strong double staining with both dyes indicates ongoing bone deposition, the dye 1:dye 2 ratio contained within a single concave endosteum depicts the status of bone remodelling during the 48-h period. For each cavity, the acquired depth covered the dye 1- and dye 2-labelled regions, typically between 80 and 120 µm beneath the endosteum. For quantification of osteoblast or osteoclast coverage (2.3Col1GFP or cathepsin K pixels) along the endosteum, z-stack double staining, COL1, and cathepsin K images of 2-µm z-stacks were rendered in 2D using maximum intensity projection in ImageJ and then analysed (Extended Data Fig. 9). As cathepsin K is also expressed substantially by endothelial cells, a vascular map (rhodamine B dextran) was acquired simultaneously and subtracted from the cathepsin K map before we retrieved the total pixel counts. For quantifying fractions of cavity types, 3D maps of calvaria were acquired and rendered in 2D using maximum intensity projection, then analysed (Fig. 4f). For quantification of cavity types for Fig. 4g, h, total pixels of dye 1 and dye 2 were retrieved directly from the 3D stacks. Segmentation of dye 1 and dye 2 in each stack was obtained using ImageJ macros combining multiple built-in plugins. Specifically, contrast enhancement was applied consistently (0.1% saturation) for each stack. The images were smoothed using 3D image suite plugins (3D mean filter, kernel size = 1) followed by background subtraction using the rolling ball algorithm with radius size of 100 and 250 pixels for dye 1 and dye 2, respectively. This background subtraction step removed diffuse signals from bone autofluorescence that are more prominent in blue and green channels (dye 1) but still distinct from structured patterns of bone front staining. Segmentation was then performed using ImageJ built-in global or local thresholding algorithms to render matching binary results compared to raw stacks. The total numbers of dye 1 and dye 2 pixels were then obtained from the binary images to calculate the dye 1:dye 2 ratio.

For classification purposes, we defined bone cavities as (i) deposition type (D-type; dye 1:dye 2 > 75%); (ii) resorption type (R-type; dye 1:dye 2 < 25%), and (iii) mixed type (M-type; dye 1:dye 2 25–75%). These definitions emphasize functional perspectives of bone remodelling along the endosteum (dominated by bone deposition or resorption), instead of the presence of osteoblasts or osteoclasts at the time of imaging. This is especially important given that osteoclasts went through apoptosis after each resorption cycle and may not be present at the time of imaging. Of note, bone-lining cells have been reported to occupy the bone fronts of inactive regions that lack both mature osteoblasts and calcium staining. In the calvarium, as neither MDS-HSPCs nor MFG-HSCs were found in fully inactive regions, we characterized cell distribution activities based on the location of cavity types in Extended Data Figs. 4–7. To quantify the number of cells per bone marrow cavity (Fig. 4g, h), Mds1GFP/Ft3GFP and Mds1GFP cells were manually counted. A cell was
considered to belong to a cavity if it was underneath a concave dome; there was no restriction on its distance from the endosteal surface.

**Bone clearing and imaging of femurs**

Tissue preparation, multicolour full-bone imaging of thick femoral sections and quantitative analyses were performed as previously described43. In brief, bones were fixed for 18 h in 4% paraformaldehyde before being decalcified using 10% EDTA (EDTA, pH – 8) for two weeks. Longitudinal bone sections (250 μm thick) were blocked, permeabilized (followed by additional blocking of endogenous avidins and biotins) and stained overnight at room temperature with primary antibodies (anti-GFP (chicken, Aves Labs, GFP-1020), anti-CD117 (goat, R&D, AF1356), anti-CD105 (rat, eBioscience, 14-1051-82) and anti-collagen type I (rabbit, Cedarlane, CL50151AP)), secondary antibodies (Alexa Fluor 555, 680, and CF633) and DAPI (Thermo Fischer Scientific, D1306). The GFP signal was amplified using donkey anti-chicken biotin (Jackson Immunoresearch, 703-065-155) followed by streptavidin Alexa 488 (Thermo Fischer Scientific, S32354). Full-bone scans were performed using a Leica TCS SP8 confocal microscope equipped with three photomultiplier tubes and two HyD detectors using type F immersion liquid (RI: 1.518) and a 20× multiple immersion lens (NA 0.75, FWD 0.680 mm). Images were acquired at 8-bit, 400 Hz and 1,024 × 1,024 resolution with 2.49 μm z-spacing. Segmentation and distance quantification analyses were performed with Imaris (version 8.3.1), using the XT and Distance Transformation XTension modules. To avoid data truncation, data were transformed to 16 bit before distance quantification and then reverted back to 8 bit. Random dots were generated via a Matlab-based, self-developed software (XiT) as previously described44. Graphs and statistical analyses were performed using Graph Pad Prism version 6.

**Reporting summary**

Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

**Data availability**

The GEO accession number for GFP cells is GSE115908. The GEO accession number for LSK cells used for overlay has been published previously (GSE90742)45.

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**Author contributions**

C.C. and F.D.C. designed experiments relevant to the animal models. J.A.S. and C.P.L. designed experiments relevant to live animal calvaria bone marrow imaging and fixed calvaria imaging. S.-C.A.Y. and C.P.L. designed experiments relevant to imaging of bone cavity types in the calvaria and tibia. K.D.K. and T.S. performed femur staining and imaging. C.C., A.R., A.S.P., Y.Z. and S.R. generated the mouse models. C.C. supervised and performed the bioinformatics analysis, respectively. J.A.S. and N.S. performed the live animal calvaria imaging experiments and relevant data analysis. R.T. performed part of the live animal calvaria imaging experiments and relevant data analysis. T.V.E. and S.A.V. generated the pO2 probe and performed relevant characterization. A.S.P. performed the imaging experiments and analysis of bone cavity types and cell proliferation. S.H.O. and K.D.K. designed experiments relevant to bone cavity types in the calvaria and tibia. K.D.K. and T.S. designed experiments relevant to bone cavity types in the calvaria and tibia.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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Extended Data Fig. 1 | Characterization of HSPC (Mds1<sup>F<sup>+/+</sup></sup>) mice demonstrates normal haematopoiesis, HSC frequency, cell cycle and stimuli recovery response. a, Targeting strategy for the generation of Mds1<sup>F<sup>+/+</sup></sup> mice. b, Eight-to-twelve-week-old Mds1<sup>F<sup>+/+</sup></sup> mice (n = 9) show similar bone marrow cellularity to control mice (Mds1<sup>+/+</sup>; n = 7); mean ± s.d. c, Eight-to-twelve-week-old Mds1<sup>F<sup>+/+</sup></sup> mice (n = 14) have similar peripheral blood parameters to Mds1<sup>+/+</sup> control mice (n = 11); mean ± s.d. d, Eight-to-twelve-week-old Mds1<sup>F<sup>+/+</sup></sup> mice (n = 7) showed similar frequencies of CD150<sup>+</sup>CD48<sup>−</sup>LSK (LT-HSCs), CD150<sup>−</sup>CD48<sup>−</sup>LSK (ST-HSCs) and CD150<sup>−</sup>CD48<sup>+</sup>LSK (MPPs) to control mice (Mds1<sup>+/+</sup>; n = 4); mean ± s.d. e, Cell cycle analysis of SLAM cells from Mds1<sup>F<sup>+/+</sup></sup> (n = 3) and wild-type (Mds1<sup>+/+</sup>; n = 2) mice in native conditions. Indicated value per gate represents mean ± s.d. f, Dynamics of recovery of white blood cells (WBC), lymphocytes (LY) and red blood cells (RBC) upon 5-FU treatment in Mds1<sup>F<sup>+/+</sup></sup> and control (Mds1<sup>+/+</sup>) mice. Mean ± s.d., n = 4 mice.
Extended Data Fig. 2 | Flow cytometric analysis of Mds1 **GFP** expression.

**a**, GFP⁺ cells are not present in any mature cellular subpopulations. Data shown are from one representative experiment that was repeated three times. **b, c**, Mds1 **GFP**⁺ cells are not present in the CD45⁻ bone marrow compartment or in mesenchymal (integrin-αV and PDGFRα) or endothelial (CD31 and VE-cadherin) bone marrow niche components. The experiment was performed once. **d**, Flow cytometry analysis reveals an inverse correlation between MDS1-GFP expression and FLT3 staining in LIN⁻SCA'CKIT' cells. The experiment was performed twice with similar results.
Extended Data Fig. 3 | Generation of the MFG (Mds1<sup>GFP</sup>; Flt3<sup>Cre</sup>) mice results in restriction of GFP expression to LT-HSCs. 

**a.** Schematic of genetic strategy to restrict GFP expression to LT-HSC compartment. 
**b.** Analysis of bone marrow from Flt3<sup>Cre</sup>R26<sup>Tom</sup>/L-SL-Tom mice shows Flt3<sup>Cre</sup>-driven activity in compartments downstream of LT-HSCs. 

n = 4 mice; mean ± s.d. 

**c.** Further characterization of the CKIT<sup>+</sup>SCA1<sup>-</sup>GFP<sup>+</sup> cells from MFG mice. CD41<sup>+</sup>CD150<sup>+</sup> cells represent megakaryocyte progenitors. The experiment was performed twice with similar results. 

**d.** Flow characterization of MFG cell in marrow isolated from multiple bones. The experiment was performed three times with similar results. 

**e.** MFG-HSCs are predominantly found within the CD34<sup>-</sup>Flt3<sup>-</sup>CD150<sup>+</sup> bone marrow fraction. The experiment was performed twice with similar results. 

**f.** MFG-HSCs are predominantly found within the SCA1<sup>high</sup>EPCR<sup>-</sup> bone marrow fraction. The experiment was performed once. 

**g.** Cell cycle analysis of SLAM cells that are either GFP<sup>+</sup> or GFP<sup>-</sup> in MFG mice. Pooled data from three mice.
**Extended Data Fig. 4** See next page for caption.
Extended Data Fig. 4 | Additional characterization of MFG-HSCs.

a, b, InDrops scRNA-seq analysis of MFG cells in comparison to multiple populations of HSC and MPPs. MFG cells (46 cells) are predominantly found in areas where *Mecom* (purple, *n* = 742 cells), but not *Flt3* (orange, *n* = 1,111 cells) is expressed. Teal, MPP2; purple, MPP3; light green, MPP4; grey, other cells; bright green, *Mds1*^{GFP}/+ *Flt3*^{Cre} cells. Gradient colour demonstrates normalized read counts. Each dot represents an individual cell. MFG-HSCs represent cells from a single mouse, the rest of the cells represent cells from a separate single mouse. c, d, Heatmaps (c) and spring plot map (d) showing expression levels of previously published 'dormant' HSC genes in scRNA-seq data from LTHSC and MFG cell populations. For the spring plot analysis: MFG, *n* = 46 cells; CD34, *n* = 2,380 cells (teal); each dot represents an individual cell. MFG-HSCs represent cells from a single mouse; the rest of the cells represent cells from a separate single mouse. e, Single-cell transcriptional fluidigm profile of MFG-HSCs demonstrates that they cluster together with LT-HSCs. f, Summary of transplants with 3, 7, or 15 MFG or SLAM HSCs together with 100,000 bone marrow cells, analysed 4 months after transplantation. HSC frequencies were calculated using ELDA software (see Methods). g, Engraftment analysis following secondary transplantsations using whole bone marrow from one primary recipient of 25 MFG' HSCs. Experiment shown is representative of three independent experiments. h, Percentage chimaerism at 4, 8, 12, 16 and 20 weeks in primary recipients transplanted with 25 SLAM cells sorted on the basis of GFP expression isolated from *Mds1*^{GFP}/+ *Flt3*^{Cre} mice (*n* = 12 GFP' mice, *n* = 5 GFP' mice). Our data demonstrate that GFP' cells within the SLAM compartment are more functionally enriched. Each line represents an individual mouse.
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | Multicolour quantitative deep-tissue confocal imaging of complete femoral sections from MFG (Mds1<sup>GFP<sup>+/Flt3<sup>Cre</sup></sup>) mice.

a, Identification of C-KIT<sup>GFP</sup> MFG-HSCs using multicolour quantitative deep-tissue confocal imaging of full bone femoral sections. Pictures are 10-µm xy projections of one area of interest. n = 3 mice. The experiment was performed three times with similar results. 
b, Example of one full-bone femoral section with colour-coded visualization of HSCs based on their distance from bone. Yellow squares represent individual HSCs in proximity to cortical or trabecular bone, whereas green dots represent individual HSCs located more than 10 µm away. The picture represents data from an individual mouse. The experiment was performed three times with similar results. 
c, Example of full-bone femoral section (only Col.1 and DAPI staining are shown). The experiment was performed three times with similar results. 
d, Colour-coded visualization of HSCs based on their distance to bone. Yellow squares represent individual HSCs in proximity to cortical or trabecular bone, whereas green dots represent individual HSCs located more than 10 µm away. This picture represents an independent mouse from b. The experiment was performed three times with similar results. 
e, Quantification of absolute number and anatomical location of C-KIT<sup>GFP</sup> MFG-HSCs per individual experiment. (N = 3 mice)
f, Spatial distribution of HSCs (circles) and random dots (triangles) relative to Col.1 marking bone surfaces (left panel) and CD105<sup>5</sup> vasculature (sinusoids, right panel) (n = 3 mice). P-values were calculated using two-tailed Kolmogorov–Smirnov (distance distributions, left panel P = 0.1516, right panel P > 0.9999) and one-tailed Mann–Whitney (first bin of histograms, left, HSCs: 8.56 ± 5.74, RDs: 6.88 ± 1.94, P = 0.50; right, HSCs: 67.52 ± 10.99, random dots: 68.53 ± 3.51, P = 0.35) tests. Data points with mean ± s.d. (red for HSCs, blue for random dots). NS, not significant. Epi: epiphysis, meta: metaphysis, dia: diaphysis.
**Extended Data Fig. 6** | Synthesis, structure and characterization of phosphorescent probe Oxyphor PtG4. The structure of Oxyphor PtG4 is almost identical to that of the previously published probe Oxyphor PdG4, but it contains Pt instead of Pd at the core of the porphyrin (I: Pt tetra-meso-3,5-dicarboxyphenyl-tetrabenzoporphyrin). a, Synthesis of Oxyphor PtG4. First, eight carboxyl groups on the porphyrin I were amended with 4-aminoethylbutyrate linkers. Upon hydrolysis of the terminal esters in the resulting porphyrin 2, eight aryl-glycine dendrons (H₂N-AG₂(OBu)₄) were coupled to the resulting porphyrin-octacarboxylic acid, giving dendrimer 3. The butyl esters on the latter were hydrolysed under mild basic conditions, and the resulting free carboxylic acid groups were amidated with monomethoxypolyethyleneamino (MeO-PEG-NH₂, Av MW 1000), giving the target probe Oxyphor PtG4. MALDI–TOF (m/z) was used to confirm the identity of the intermediate products as well as of the target probe molecule. Structure 2 (C₁₁₆H₁₂₄N₁₂O₂₄Pt, calculated at MW 2263.85) was found 2264.48 [M⁺]; structure 3 (C₄₆₈H₅₄₀N₆₀O₁₂₀Pt, calculated at MW 9114.76) was found at 9115.68 [M+H⁺] and Oxyphor PtG4 (C₁₇₈₀H₃₁₉₆N₉₂O₁₇₂Pt, calculated at MW 40538) was found at 35952. For Oxyphor PtG4 we identified an additional peak at MW 66123.6 which is probably due to the presence of dimeric species formed during the ionization process. b, Linear (one photon) absorption (green) and emission spectra (red) of PtG4 in 50 mM phosphate buffer solution (pH 7.2, λₑₓ = 623 nm; photophysical constants in PBS, 22 °C: ε(623) ~ 90,000 M⁻¹ cm⁻¹ (molar extinction coefficient), ϕₚₚₜₜ(deox) ~ 0.07 (phosphorescence quantum yield in deoxygenated solution), τₑ₉₅₆ = 16 μs (phosphorescence decay time on air), τₑₙ₉₅₆ = 47 ms (phosphorescence decay time in deoxygenated solution). c, Phosphorescence oxygen quenching plot of Oxyphor PtG4. The calibration was performed as previously described. The experimental points were fitted to an arbitrary double-exponential form and the obtained parametric equation was used to convert the phosphorescence lifetimes obtained in vivo experiments to pO₂ values. d, Two-photon absorption spectrum of PtG4 in deoxygenated dimethylacetamide (DMA, 22 °C). e, Arbitrarily scaled one- (green line) and two-photon (blue line) absorption spectra of PtG4. The two-photon absorption (2PA) spectra of PtG4 and of the reference compounds were measured by the relative phosphorescence method, as previously described. The laser source was a Ti:Sapphire oscillator (80 MHz rep. rate) with tunability range of 680–1300 nm (Insight Deep See, Spectra Physics). All optical spectroscopic experiments and oxygen titrations were performed at least three times, giving highly reproducible results. f, Representative intravital images of an HSPC (green, left image), MFG-HSC (green, right image), vasculature (grey, Rhodamine-B-dextran 70 kDa), and autofluorescence (blue) overlaid with localized oxygenation measurements. White arrows, GFP cells. Black arrow, colour representing 10 mm Hg. Coloured squares represent individual localized oxygen measurement areas. Images represent data from two independent experiments for each mouse model. Scale bars, ~50 μm.
Extended Data Fig. 7  | Increased motility and expansion of activated MFG-HSCs.

a, Schematic illustration of protocol for activating bone marrow HSCs using Cy/GCSF.
b, Flow cytometry analysis of Cy/GCSF-treated MFG mice (n = 3 mice). Data show Lineage− cells. Mean ± s.d.
c, Number of GFP+ cells identified per calvaria in untreated and Cy/GCSF-treated Mds1GFP/Flt3Cre mice (n = 5 and 4 mice, respectively). Red bars indicate mean. P was calculated using two-tailed Mann–Whitney test.
d, Cell cycle analysis of MFG+ cells from Cy/GCSF-treated mice. Three mice were pooled together to acquire the displayed data. e, Graph showing in vivo motility measurements of HSPCs (n = 66 cells) and MFG-HSCs (n = 30 cells) at steady-state and of activated MFG-HSCs (n = 142 cells) in the calvaria. Red bars indicate mean. P were calculated using two-tailed Mann–Whitney test. f, g, Distance from MFG+ cells to the endosteum (n = 24 and 12 cells for untreated and Cy/GCSF-treated, respectively) and to the nearest vessel (n = 20 and 17 cells for untreated and Cy/GCSF-treated, respectively), after treatment with Cy/GCSF. Red bars indicate mean. P values calculated using two-tailed unpaired t-test.
Extended Data Fig. 8  |  See next page for caption.
Extended Data Fig. 8 | Characterization of MFG-HSCs upon activation.  

a, Bone marrow analysis of HSPC (Mds1GFp/+) PBS control (n = 1 mouse) and HSPC (Mds1GFp/) 5-FU-treated mice (n = 2 mice, value represents mean), 17 days after treatment. Data show marked expansion of HSPCs even after recovery of blood (Extended Data Fig. 1e). b, Graph showing in vivo motility measurements of MFG-HSCs at days 4 (n = 14 cells) and 20 (n = 13 cells) after 5-FU treatment. Red bar represents mean. Compare to untreated Mds1GFp/+Flt3Cre mice in Fig. 3a and Extended Data Fig. 7e.  

c, Representative map of the locations of MFG-HSCs in the calvaria on day 20 after 5-FU treatment (n = 2 mice). Scale bar = 500 µm.  

d, Generation of Mds1CreER+ Rosa26Confetti+ mice. e, Schematic illustration of Cy/GCSF treatment protocol for multicoloured Mds1CreER+ Rosa26Confetti+ labelling and activation. Low tamoxifen dosage (2 mg) was used to restrict recombination and expression of fluorescence in LT-HSCs that express higher levels of Mds1.  

f, Detailed flow cytometry analysis of MPP3/4 cells, ST-HSCs and LT-HSCs with differential colour labelling upon treatment of Mds1CreER+ Rosa26Confetti+ mice shows labelling enriched in but not fully restricted to LT-HSCs. The experiment was performed once. g, 2D maps of the 3D locations of activated and labelled HSPCs in the fixed calvaria of control (left top, tamoxifen only, n = 2 mice) and induced mice (left bottom, tamoxifen + Cy/GCSF, n = 3 mice) along with maximum intensity projection (MIP) images (right top and bottom) of the Mds1-labelled cells (red, green, and blue). Scale bars for graphical map and MIP images, ~200 µm and 50 µm, respectively.  

h, Colour purity of cell clusters (original colours) compared to randomized colours (10,000 cycles) in three independent experiments (n = 3 mice). P values calculated using two-tailed unpaired t-test. Bar graphs with error bars represent mean and s.d., respectively.
Extended Data Fig. 9 | Validating bone cavity types using 2.3Col1–GFP (mature osteoblasts) and cathepsin K-activated fluorescent agent (osteoclasts). a, A montage of multiple z-stacks, displayed as the maximum intensity projection, showing double staining of bone marrow cavities in the calvarium. b, The same area as in a, showing the locations of 2.3Col1–GFP osteoblasts in areas of the old bone front that has not been eroded (n = 3 mice). c, Quantification of 2.3Col1–GFP pixels in D-type (n = 10 regions), M-type (n = 16 regions) and R-type cavities (n = 15 regions). Mean ± s.d. d, A montage of multiple z-stacks, displayed as the maximum intensity projection, showing the double staining pattern (blue and red), 2.3Col1–GFP cells (green), osteoclasts (white), and bone marrow vasculature (purple). White arrows, osteoclast clusters. n = 3 mice. e, A zoomed-in region from d (box A), showing correlation between 2.3Col1–GFP cells and the remaining dye 1 (blue) in a D-type cavity, and abundant cathepsin K+ osteoclasts in the R-type region where dye 1 was eroded. f, Examples of an M-type region from d (box B). In this region, dye 1 was eroded to some extent in spite of the presence of abundant 2.3Col1–GFP cells in the cavity. The corresponding cathepsin K panel shows the co-existence of several cathepsin K+ osteoclasts. g, Quantification of cathepsin K+ pixels in D-type (n = 11 regions), M-type (n = 33 regions), and R-type (n = 10 regions) cavities based on maximum intensity projection of montaged z-stacks. Compared to c, cathepsin K coverage shows a larger spread because it does not stain the cell body uniformly. Instead it frequently shows a punctate staining pattern, which is likely to represent lysosomes and endosomes. *P < 0.0189; **P < 0.0015; ****P < 0.0001; two-sided Mann–Whitney test. Mean ± s.d.
Extended Data Fig. 10 | Cell distribution in D-, M- and R-type cavities before and after Cy/GCSF treatment. n = 4 mice per group. Graphs show the fractions of MDS or MFG cells distributed in D-, M- and R-type cavities at the steady state and after Cy/GCSF treatment. The fraction is calculated by the total cells found in each cavity type divided by the total cells found in the calvaria of that mouse.

**a**, The fractions of MFG cells increased in M-type cavities but decreased in D-type cavities after Cy/GCSF treatment. Mean ± s.d. Non-treated groups: 24.5 ± 12.8, 54.3 ± 12.6 and 21.3 ± 15.6 in D-, M- and R-type cavities, respectively. Treated groups: 0.5 ± 1.0, 96.0 ± 4.7 and 3.5 ± 4.4 in D-, M- and R-type cavities, respectively. **P = 0.0096; ***P = 0.0008.

**b**, The fractions of cells decreased in D-type cavities but remained the same in M- and R-type cavities. Mean ± s.d. Non-treated groups: 20.5 ± 5.6, 66.5 ± 2.4 and 13.3 ± 3.6 in D-, M- and R-type cavities, respectively. Treated groups: 6.8 ± 2.5, 75.0 ± 9.6 and 18.8 ± 8.9 in D-, M- and R-type cavities, respectively. **P = 0.004; unpaired, two-tailed t-test.
Extended Data Fig. 11 | Heterogeneous bone remodelling in bone marrow cavities of tibia metaphysis. A mechanically thinned metaphysis was imaged from the bone surface, labelled by sequential calcium staining. a–c. En face views of D-, M- and R-type cavities from tibia metaphysis. d–f, x–z cross-section views from annotated white lines in Supplementary Video 15 show bone marrow cavities of varied remodelling stages similar to mouse calvaria.
Activated MFG-HSCs (Mds1GFP+Flt3Cre+ mice) are characterized by increased motility and various cellular interactions between GFP cells.

|     | Total Cells | Paired Cell interactions | Mobilized cells |
|-----|-------------|--------------------------|-----------------|
| N=1 | 16          | 2                        | 0               |
| N=2 | 44          | 8                        | 0               |
| N=3 | 86          | 32                       | 6               |
| N=4 | 46          | 4                        | 1               |
| Total| 192         | 46                       | 7               |
| Percentage | 24.0%      | 3.6%                     |                 |

Table of observed MFG-HSC behaviours in Cy/G-CSF-treated mice; n = 4.
Extended Data Table 2 | Summary table of findings from live imaging of native HSCs versus transplanted HSCs

| Native HSCs (this work)                                | Transplanted HSCs                                                      |
|--------------------------------------------------------|------------------------------------------------------------------------|
| Adjacent to both endosteum and sinusoidal blood vessels| Adjacent to both endosteum and blood vessels (did not identify type) (Lo Celso C et al, Nature 2009) |
| Do not reside in regions with deepest hypoxia           | Do not home in regions with deepest hypoxia (Spencer JA et al, Nature 2014) |
| Sessile; become motile after activation                | Sessile; become motile after activation (Rashidi NM et al, Blood 2014) |
| Proliferate and form clusters after Cy/GCSF or 5-FU    | Proliferate and form clusters after transplantation (Lo Celso C et al, Nature 2009) |
Corresponding author(s): Fernando D. Camargo, Charles P. Lin

Reporting Summary

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Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

- Confirmed
- The exact sample size \((n)\) for each experimental group/condition, given as a discrete number and unit of measurement
- An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
- Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. \(F, t, r\)) with confidence intervals, effect sizes, degrees of freedom and \(P\) value noted
- Give \(P\) values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Clearly defined error bars
- State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection

Data collection methodology for calvaria imaging and full bone imaging is described in detail in the methods section. For full-bone immunofluorescence confocal imaging was performed using the default acquisition software of the Leica TCS SP8 confocal microscope as previously described (Coutu D.L. et al, Nature Methods 2018). Calvaria imaging data collection was performed as described in detail in the methods section and as previously described in Spencer J.A. et al, Nature 2014.
Data analysis methodology for calvaria imaging and full bone imaging is described in detail in the methods section. Custom code required for the analysis of full-bone immunofluorescence confocal imaging data was previously described (Coutu D.L. et al, Nature Methods 2018) and is available for download at https://www.bsse.ethz.ch/csd/software/XiT.html. In addition, Graph Pad Prism (version 7) and Imaris (8.3.1 and 9.1.2) were used for the corresponding data analysis and insertion of random dots for statistical comparison. For calvaria imaging data analysis Olympus FluoView software, Matlab, ImageJ scripts and Graph Pad Prism (version 6 or higher) were used. Several built-in plugins from image J were used, including contrast enhancement, 3D image J suite, background subtraction, global/ local thresholds, and 3D Euclidean distance measurements. For the single cell RNA sequencing analysis the code is available upon request. For single cell fluidigm experiments, data analysis and hierarchical clustering was performed using MultiExperiment Viewer (MeV) program. For the single cell RNA sequencing experiment, the data were processed using a previously published workflow and code available on https://github.com/AllonKleinLab/SPRING. Any additional python scripts used for graph plotting of the RNA sequencing data is described in detail in the methods section. For the single cell RNA sequencing experiment, the data were processed using a previously published workflow and code available on https://github.com/AllonKleinLab/SPRING. Any additional python scripts used for graph plotting of the RNA sequencing data is described in detail in the methods section. For the synthesis and characterization of the phosphorescent oxyphor probe, MALDI-TOF was used to confirm the identity of the intermediate products and of the target probe molecule. To estimate the HSC frequency (MFG cells) in bone marrow we used the extreme limiting dilution analysis software available on http://bioinf.wehi.edu.au/software/elda/. GraphPad Prism and Excel were used to analyze and display all mouse characterization related data.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

All raw data from all in vivo experiments have been made available with the manuscript as source data in excel format or as supplementary information. The GEO accession code GSE115908 for the RNA seq data is publicly available.

Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences
- Behavioural & social sciences
- Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

No sample-size calculations were performed. The sample size was determined based on previous similar studies (Zhang Y et al, Blood 2011) and was adequate based on consistency of measured results in each group.

Data exclusions

Some data were excluded based on high variability and deviation from the mean in the % GFP positive cells present in Mds1-GFP/+ Fr3-Cre mice. The 5 highest and 5 lowest values were excluded to ensure proper representation of the calculated % GFP positive. Exclusion criteria were not pre-established.

Replication

Experimental findings were reliably reproduced. In rare cases in which large variability was observed it is indicated with corresponding SD. To verify reproducibility of the findings the vast majority of experiments were repeated at least three independent times.

Randomization

Weaned mice from Mds1-GFP/+ x Fr3-Cre crosses, Mds1-CreER/+ x Rosa26-lox-stop-lox-Confetti crosses and Mds1-GFP/+ x C57/BL6 crosses were separated in male and female cages. Adult animals (2-6 months) of both sexes of corresponding genotypes were randomly selected and chosen for all experiments and used for BM isolation.

Blinding

No blinding was performed during data collection and analysis. For the study as we compare specific genotypes with or without treatment we have to pre-determine the genotype of each mouse followed by type of treatment performed and subsequent analysis. Thus, blinding during experiments and data acquisition is not possible in this study.

Reporting for specific materials, systems and methods
### Materials & experimental systems

**Involved in the study**
- [x] Unique biological materials
- [x] Antibodies
- [x] Eukaryotic cell lines
- [x] Palaeontology
- [x] Animals and other organisms
- [x] Human research participants

### Methods

**Involved in the study**
- [x] ChIP-seq
- [x] Flow cytometry
- [x] MRI-based neuroimaging

### Unique biological materials

Policy information about availability of materials

**Obtaining unique materials**

All unique materials used are readily available from the authors.

### Antibodies

**Antibodies used**

All antibodies were purchased from Biolegend, BD Biosciences, eBiosciences, Invitrogen, Cedarlane, Aves and R&D. The corresponding manufacturers per antibody are indicated in methods. All antibodies were used at 1:100 concentration unless otherwise stated.

Antibody Company Catalog number Concentration

- Flt3 PE-Cy5 eBioscience 15-1351-82 1:50
- c-kit APC eBioscience 17-1171-83 1:100
- c-kit AF700 eBioscience 56-1172-82 1:100
- Sca-1 PE-Cy7 Invitrogen 25-5981-82 1:100
- CD34 Biotin eBioscience 13-0341-85 1:33
- Fc R BV421 BioLegend 101331 1:50
- CD150 PE-Cy5 BioLegend 115912 1:100
- CD41 BV605 BioLegend 133921 1:100
- CD48 APC-Cy7 BD Pharmingen 561242 1:100
- CD45.2 V450 BD Horizon 560697 1:100
- B220 APC eBioscience 17-0452-83 1:100
- B220 Biotin eBioscience 13-0452-85 1:100
- CD19 APC-Cy7 eBioscience 47-0193-82 1:100
- CD19 Biotin Invitrogen 13-0193-85 1:100
- Mac1 APC-Cy7 eBioscience 47-0112-82 1:100
- CD4 APC Invitrogen 17-0041-83 1:100
- CD4 Biotin eBioscience 13-0041-85 1:100
- CD8a APC eBioscience 17-0081-83 1:100
- CD8a Biotin Invitrogen 13-0081-85 1:100
- Ly-6G AF700 BD Pharmingen 561236 1:100
- IgM eFluor 450 eBioscience 48-5890-82 1:100
- Gr-1 APC eBioscience 17-5931-82 1:100
- Gr-1 Biotin eBioscience 13-5931-85 1:100
- Ter119 PE-Cy5 eBioscience 15-5921-83 1:100
- Ter119 Biotin Invitrogen 13-5921-85 1:100
- Streptavidin APC eBioscience 17-4317-82 1:100
- Streptavidin APC-Cy7 eBioscience 47-4317-82 1:100
- Streptavidin PE-Cy7 eBioscience 25-4317-82 1:100
- Ki67 PE-Cy7 Biolegend 652426 1:100
- anti-GFP Aves Labs GFP-1020 1:50
- anti-CD117 R&D systems AF1356 1:50
- anti-collagen type I Cedarlane CLS0151AP 1:50
- anti-CD105 eBioscience 14-1051-82 1:50
- Alexa Fluor 488 streptavidin conjugate Thermo Fischer Scientific S32354 1:50
- Alexa Fluor 555 Thermo Fischer Scientific A21432 1:50
- Alexa Fluor 633 Biotium 20137 1:50
- Alexa Fluor 680 Thermo Fischer Scientific A10043 1:50
- donkey anti-chicken biotin Jackson Immunoresearch 703-065-155 1:50
- DAPI Thermo Fischer Scientific, D1306 1:2000

**Validation**

All antibodies used are well characterized and validated by providers. For all flow cytometry antibodies validation was performed in the mouse system using isotype control Abs as well as specific cell types that are known to be negative or positive for the corresponding Ab by the manufacturer. For all flow cytometry used Abs, validation of expression and fluorescence was performed using flow cytometry analysis by the manufacturer. All validation information for each Ab as well as previous publications that have used each Ab can be found on the manufacturer's website.
Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

| Laboratory animals | All animals used in this study for BM and imaging analysis are of Mus musculus species, C57/BL6 background strain independent of genotype and 2-6 months of age. Both males and females were used for all experiments. Mds1-GFP/+, Mds1-GFP/+ Flt3-Cre, Mds1-CreER/+ Rosa26-CAG-lox-stop-lox-Confetti/+, Rosa26-CAG-lox-stop-lox-tdTomato/+ were generated as detailed described in methods or purchased from JAX and crossed with our generated strains. A detailed description of the procedure followed to generate Mds1-GFP/+, Mds1-GFP/+ Flt3-Cre and Mds1-CreER mouse lines is included in the methods section. For Tamoxifen induction and Cyclophosphamide/GCSF experiments the procedure as well as doses are detailed described in the methods section. |
| Wild animals | The study did not involve wild animals. |
| Field-collected samples | The study did not involve field-collected samples. |

Flow Cytometry

Plots

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation | Bone marrow isolation was performed using crushing methodology, followed by red blood cell lysis. MACS beads and quadroMACS (LS columns) were used for lineage depletion or c-kit enrichment. 40um filters were used to ensure single cell suspension prior FACS analysis. Antibody staining was performed for 45min, on ice, in PBS-2% FBS to ensure good staining and high viability of the cells. The detailed BM isolation protocol is included in the methods section. |

Instrument | BD LSR II Flow cytometer was used for flow cytometry analysis, BD FACSAria II was used for cell sorting. |
Software | BD FACSDIVA Software was used for data collection, FlowJo software (Tree Star) was used for data analysis. |
Cell population abundance | Cells were sorted with Purity modes at 80-85% efficiency. Post sort fractions analyzed were at least 95% pure. Sorted samples where double sorted to ensure purity of the sorted populations. In experiments in which low cell numbers of sorted cells was used, cells where secondary sorted directly in plates to ensure accuracy of cell number. |
Gating strategy | FSC/SSC preliminary gating was used to exclude debris (lower FSC) and to restrict the main bone marrow population including larger cells such as granulocytes that are found in higher SSC levels. Back-gating was used to ensure that all excluded populations were debris or dead cells (positive for DAPI) and that all included populations were part of the various positive antibody fractions. FSC-H vs. FSC-A was used to exclude doublet cells. SSC-H vs, SSC-A was used as an additional secondary doublet exclusion step. Dead cells were identified using DAPI staining in all experiments. Gating for negative/positive populations for all antibodies was performed using a negative control (no stain) followed by single color positive control for each antibody. |

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.