Deep imaging of bone marrow shows non-dividing stem cells are mainly perisinusoidal

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Haematopoietic stem cells (HSCs) reside in a perivascular niche but the specific location of this niche remains controversial1. HSCs are rare and few can be found in thin tissue sections2,3 or upon live imaging4, making it difficult to comprehensively localize dividing and non-dividing HSCs. Here, using a green fluorescent protein (GFP) knock-in for the gene Ctnnål in mice (hereafter denoted as α-catulinGFP), we discover that α-catulinGFP is expressed by only 0.02% of bone marrow haematopoietic cells, including almost all HSCs. We find that approximately 30% of α-catulinGFP c-kit+ cells give long-term multilineage reconstitution of irradiated mice, indicating that α-catulinGFP c-kit+ cells are comparable in HSC purity to cells obtained using the best markers currently available. We optically cleared the bone marrow to perform deep confocal imaging, allowing us to image thousands of α-catulinGFP c-kit+ cells and to digitally reconstruct large segments of bone marrow. The distribution of α-catulin–GFP c-kit+ cells indicated that HSCs were more common in central marrow than near bone surfaces, and in the diaphysis relative to the metaphysis. Nearly all HSCs contacted leptin receptor positive (Lepr+) and Cxcl12high niche cells, and approximately 85% of HSCs were within 10 μm of a sinusoidal blood vessel. Most HSCs, both dividing (Ki-67+) and non-dividing (Ki-67−), were distant from arterioles, transition zone vessels, and bone surfaces. Dividing and non-dividing HSCs thus reside mainly in perisinusoidal niches with Lepr+ Cxcl12high cells throughout the bone marrow.

Adult HSCs reside in a perivascular niche in the bone marrow in which Lepr+ perivascular stromal cells and endothelial cells secrete factors that promote their maintenance4–9. Nearly all of the cells that express high levels of Scf (also known as Kitl) or Cxcl12 in the bone marrow are Lepr− (ref. 10). Conditional deletion of Scf from Lepr+ cells and endothelial cells eliminates all quiescent and serially transplanted HSCS from adult bone marrow11. The perivascular niche cells we identified based on Lepr expression have also been identified by others based on their expression of high levels of Cxcl12 (refs 5, 12, and 13) and low levels of the Nes–GFP transgene14,15, PDGFR16,17, and Prol1,Cre18.

Established elements of the HSC niche localize primarily around sinusoids in bone marrow including HSCs19,20, Lepr− stromal cells19, angioptoenin-1-expressing stromal cells19,20, Scf-expressing stromal cells19,20, Cxcl12high stromal cells13,20, and mesenchymal stem/stromal cells10,13,20. Moreover, sinusoidal endothelial cells are functionally important for haematopoiesis after myeloablation11. HSCs have also been suggested to reside in a hypoxic niche19 and the most hypoxic region of the bone marrow is around sinusoids21. Nonetheless, HSC niches have also been suggested to localize near bone surfaces or around arterioles24.

It has been suggested that dividing HSCs reside in a niche that is spatially distinct from quiescent HSCs24. However, dividing HSCs are rarer than non-dividing HSCs, making it difficult to find substantial numbers of those cells within tissue sections. HSC imaging throughout the bone marrow is limited by the inability of even multiphoton microscopy to penetrate more than 150 μm into bone marrow14. Optical clearing techniques have enabled deep imaging of various tissues25,26, including haematopoietic progenitors in embryos27, but have not been used to image rare stem cells or to digitally reconstruct bone marrow.

Gene expression profiling showed that α-catulin is highly restricted in its expression to HSCs5. α-catulin encodes a protein with homology to α-catenin that has been suggested to function as a cytoskeletal linker28. By quantitative reverse transcription PCR (qRT–PCR), we found that α-catulin was expressed at 19 ± 9.3 (mean ± s.d.) fold higher levels in CD150−CD48−Lineage−Sca-1−c-kit− (CD150−CD48−LSK) HSCs as compared to unfractionated bone marrow cells.

To assess α-catulin expression in detail, we knocked GFP into the first exon of α-catulin in frame with the start codon (Extended Data Fig. 1a). Although this was predicted to be a loss-of-function allele, both α-catulinGFP+ and α-catulinGFP− mice were born and survived into adulthood with expected Mendelian frequencies (Extended Data Fig. 1e). Young adult α-catulinGFP+ mice were normal in size and body mass (Extended Data Fig. 1d), as well as bone density and bone volume (Extended Data Fig. 1f) relative to littermate controls. α-catulinGFP+ and α-catulinGFP− mice exhibited normal haematopoiesis as well as normal HSC frequency, HSC cell cycle kinetics, and normal HSC function upon primary and secondary transplantation into irradiated mice (Extended Data Fig. 2).

Only 0.021 ± 0.006% (mean ± s.d.) of whole bone marrow (WBM) cells in α-catulinGFP+ mice were α-catulinGFP− (Fig. 1a). Most of the α-catulinGFP− cells were also c-kit− (Extended Data Fig. 3a), and 49 ± 8.3% of CD150−CD48−LSK HSCs were α-catulinGFP+ (Extended Data Fig. 3c). We did not detect α-catulinGFP− expression among multipotent progenitors, common lymphoid progenitors, common myeloid progenitors, granulocyte macrophage progenitors, or megakaryocyte erythrocyte progenitors (Extended Data Fig. 3c, d). α-catulinGFP− c-kit+ cells seemed to be highly purified HSCs as they represented only 0.007 ± 0.003% of WBM cells and were uniformly CD150− and CD48− (Fig. 1b and Extended Data Fig. 3b).

To test the function of α-catulinGFP+ cells, we performed long-term competitive reconstitution assays in irradiated mice. We found that 1 in 37,000 WBM cells gave long-term multilineage reconstitution; and 1 in 6.7 α-catulinGFP+ cells gave long-term multilineage reconstitution (Table 1). In contrast, only 1 in 2,847,000 α-catulinGFP− bone marrow cells gave long-term multilineage reconstitution (a 77-fold depletion over WBM). The α-catulinGFP+ fraction of CD150−CD48−LSK cells gave long-term multilineage reconstitution (1 in 3.1 cells) but the α-catulinGFP− fraction had little HSC activity (1 in 110 cells; Table 1). Therefore, nearly all HSC activity in adult bone marrow is contained within the α-catulinGFP+ fraction of cells.

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staining of half bones or bone marrow plugs from \(x\text{-catulin}^{\text{GFP}+}\) mice, we cleared the specimens (see Fig. 1c versus d) and then used confocal microscopes to acquire tilted Z-stacked optical sections throughout the bone marrow to a depth of up to 600 μm.

We identified all \(x\text{-catulin}^{\text{GFP}^-}\) c-kit\(^+\) cells within large segments of bone marrow (Fig. 1e–l). Isotype controls showed low levels of background fluorescence that could readily be distinguished from positive signals (Extended Data Fig. 8a, b). We also prepared videos that animate three-dimensional (3D) images of the bone marrow to show HSCs, niche cells, vasculature, and bone surfaces (Supplementary Videos 1–3). When thick specimens are collapsed into a single 2D image, \(x\text{-catulin}^{\text{GFP}^-}\) c-kit\(^+\) cells can appear more frequent than they actually are because all of the cells from the thick specimens are collapsed into a single optical plane. f. Same as e, but digitally masked to reveal only HSCs and bone. \(x\text{-catulin}^{\text{GFP}^-}\) c-kit\(^+\) HSCs are represented by yellow spheres to make them visible at this magnification. g–i, Higher magnification views of the boxed region in f. h, i. All haematopoietic cells other than HSCs (yellow spheres) are digitally masked. i, Blood vessels and haematopoietic cells other than HSCs are digitally masked. j–l, Higher magnification views of the boxed area from i (arrows indicate \(x\text{-catulin}^{\text{GFP}^-}\) c-kit\(^+\) cells). Images are representative of three independent experiments. Supplementary Video 1 shows a 3D digital reconstruction of bone and bone marrow. The positions of HSCs and other structures can appear to change in thick specimens when magnification is changed due to the rendering perspective for 3D display of volume data.

Furthermore, in 3.5 \(x\text{-catulin}^{\text{GFP}^-}\) c-kit\(^+\) cells gave long-term multilineage reconstitution of irradiated primary and secondary recipient mice (Table 1; Extended Data Fig. 4). If HSCs home with near perfect efficiency to engraft haematopoietic tissues, then approximately 30% of \(x\text{-catulin}^{\text{GFP}^-}\) c-kit\(^+\) cells are HSCs. If most intravenously transplanted HSCs fail to home to haematopoietic tissues and therefore do not have an opportunity to exhibit reconstituting potential in transplantation assays, then most \(x\text{-catulin}^{\text{GFP}^-}\) c-kit\(^+\) cells may be HSCs. In either case, \(x\text{-catulin}^{\text{GFP}^-}\) c-kit\(^+\) cells are comparable in purity to cells isolated using the best HSC markers currently available.

\(x\text{-catulin}^{\text{GFP}^-}\) c-kit\(^+\) cells are quiescent, comparable to CD150\(^-\)CD48\(^-\) LSK HSCs, with only 1.2 ± 0.5% of cells in S/G2/M phases of the cell cycle (Extended Data Fig. 4c).

Table 1 | \(\alpha\text{-catulin}^{\text{-GFP}^+}\) cells were highly enriched for long-term multilineage reconstituting (LTMR) HSCs.

| Donor cell population | Donor cell dose | LTMR mice/transplanted mice | HSC frequency |
|----------------------|----------------|-----------------------------|---------------|
| Unfractionated bone marrow | 25,000 | 19/27 | 1 in 37,000 |
| 50,000 | 15/25 |
| 100,000 | 21/23 |
| 300,000 | 14/14 |
| \(x\text{-catulin}^{\text{GFP}^-}\) | 1 | 2/13 | 1 in 6.7 |
| 5 | 13/25 |
| \(x\text{-catulin}^{\text{GFP}^-}\) | 300,000 | 2/20 | 1 in 2,847,000 |
| \(c\text{-kit}^+\) | 1 | 9/34 | 1 in 3.5 |
| 5 | 17/23 |
| 30 | 14/14 |
| \(x\text{-catulin}^{\text{GFP}^-}\) | CD150\(^-\)CD48\(^-\) Lin\(^-\)c-kit\(^+\)Sca-1\(^+\) | 5 | 24/30 | 1 in 3.1 |

Donor cells were prepared against 300,000 recipient WBM cells in irradiated mice. The data reflect means from 2 to 4 independent experiments per cell population. LTMR mice indicates the number of mice that were long-term multilineage reconstituting as a fraction of the total number transplanted with each cell dose.

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10 μm of a bone surface in the diaphysis (Fig. 2d), and 86 ± 6.1% were more than 80 μm away (Fig. 2b). In the metaphysis, the localization of HSCs relative to bone surfaces did not significantly differ from the distribution of random spots as 7.0% of HSCs were within 10 μm of a bone surface (Fig. 2e), and 53 ± 11% were more than 80 μm away (Fig. 2c). These data are consistent with our observation that fewer than 10% of CD150+CD48−LSK HSCs were within 10 μm of bone in femur sections.

Gliai fibrillary acidic protein (GFAP)+ Schwann cells and nerve fibres clustered in the centre of the marrow in the diaphysis (Extended Data Fig. 8d, e). HSCs did not significantly differ from random spots in their distance from GFAP+ cells (Fig. 2f).

Figure 2 HSCs localize adjacent to Cxcl12high and Lepr+ niche cells but distant from bone surfaces. **a** The distribution of α-catenin−GFP+c-kit+ HSCs and random spots in concentric cylinders corresponding to equal volumetric deciles from central marrow (centre) to endosteal marrow near the bone surface (outside) from the tibia diaphysis (2,977 HSCs in 6 bone marrow plugs (390–550 μm thick) in 6 independent experiments). See Extended Data Fig. 6 for further explanation. Relative to random spots, HSCs were significantly enriched in central marrow, **b−e** Distance from HSCs or random spots to the nearest bone in the diaphysis (b) or metaphysis (c, d). Percentages of all HSCs and random spots within 10 μm of a bone surface in the diaphysis (d) or metaphysis (e) are shown. **f** Percentage of all HSCs and random spots within 10 μm of bone surfaces (360–400 μm thick). **g** Distance to the nearest Lepr+ Schwann cell (n = 608 HSCs in bone marrow plugs (430–500 μm thick) from the diaphysis of 3 tibias). **h** Percentage of all HSCs and random spots within 10 μm of a Lepr+ Schwann cell. **i** The distance to the nearest Cxcl12high stromal cell (n = 596 HSCs in bone marrow plugs (235–450 μm thick) from the diaphysis of 4 tibias). **j** Distance to the nearest α-catenin−GFP+c-kit+ cell in apparent contact with a Cxcl12-expressing (j) or a Lepr-expressing (k) cell. All data reflect mean ± s.d. from at least three independent experiments. The statistical significance of overall differences in cell distribution in **a−c, f, h**, and **i** was assessed by Kolmogorov–Smirnov analysis. The statistical significance in **d, e, g, and differences between HSCs and random spots at 5μm in **h**, and **i** was assessed using Student’s t-tests (**P < 0.05**); NS, not significant.

6.2 ± 3.0% of α-catenin−GFP+c-kit+ HSCs were within 10 μm of GFAP+ cells in the bone marrow but 28 ± 3.8% were within 30 μm (Fig. 2f, g). Thus, HSCs and niche cells rarely have contact with Schwann cells or nerve fibres but a subset of HSCs may be regulated by diffusible factors secreted by Schwann cells.

HSCs were significantly more likely than random spots to be close to Cxcl12−DsRedhigh stromal cells, and 97 ± 1.2% of HSCs were within 5 μm of Cxcl12−DsRedhigh stromal cells (Fig. 2h). Although Cxcl12high stromal cells represent only 0.3% of WBM cells, they have long processes that extend throughout the marrow (Supplementary Video 2). Consequently, 89 ± 4.9% of random spots were also within 5 μm of a Cxcl12−DsRedhigh stromal cell (Fig. 2h), and 94 ± 2.5% of HSCs appeared to have cell–cell contact with a Cxcl12−DsRedhigh stromal cell (Fig. 2i).

We also found that 93 ± 3.7% of α-catenin−GFP+c-kit+ HSCs were within 5 μm of a Lepr− cell (Fig. 2i). Lepr− cells were visualized using Lepr-cre;tdTomato mice in these experiments, but 99% of tdTomato+ bone marrow cells in 8–12 week old Lepr-cre;tdTomato mice also stain with an antibody against Lepr. HSCs were significantly more likely than random spots to be close to (Fig. 2i) and almost always contacted (Fig. 2k) a Lepr− cell.

We next imaged the localization of HSCs relative to three kinds of blood vessels in the bone marrow: arterioles, sinusoids, and transition zone capillaries. We distinguished blood vessels based on anatomical position, size, morphology, and continuity of the basal lamina, visualized using anti-laminin antibody staining (Extended Data Fig. 9a−c). α-catenin−GFP+c-kit+ HSCs significantly differed from random spots in their distance to arterioles; they were slightly less likely than random spots to be within 25 μm of an arteriole but slightly more likely than random spots to be 30–50 μm away (Fig. 3a). Only 15 ± 2.3% of HSCs were within 10 μm of an arteriole (Fig. 3d). In contrast, 84 ± 6.2% of HSCs were within 10 μm of a sinusoid (Fig. 3e). HSCs did not significantly differ from random spots in their localization.
Figure 4  | Dividing and non-dividing HSCs are most closely associated with sinusoids. a, Representative images of a Ki-67 \(\alpha\)-catulin–GFP \(c-kit^+\) non-dividing HSCs (a) and a Ki-67 \(\alpha\)-catulin–GFP \(c-kit^+\) dividing HSCs (b) (arrows). c, 15 ± 2.0% of HSCs were Ki-67 \(\alpha\)-catulin–GFP \(c-kit^+\). All data reflect mean ± s.d. from bone marrow plugs (410–440 μm thick) from the diaphysis of 5 tibias. A total of 2,132 HSCs were analysed in 5 independent experiments. d–f. Distance to the nearest arteriole (d), sinusoid (e), or transition zone (TZ) vessel (f). g–i. The percentages of all Ki-67 \(\alpha\)-catulin–GFP \(c-kit^+\) non-dividing HSCs, Ki-67 \(\alpha\)-catulin–GFP \(c-kit^+\) dividing HSCs, or random spots within 10 μm of an arteriole (g), a sinusoid (h), or a transition zone vessel (i). j. Most Ki-67 \(\alpha\)-catulin–GFP \(c-kit^+\) non-dividing HSCs and Ki-67 \(\alpha\)-catulin–GFP \(c-kit^+\) dividing HSCs were most closely associated with sinusoids. k. The distributions of Ki-67 \(\alpha\)-catulin–GFP \(c-kit^+\) non-dividing HSCs, Ki-67 \(\alpha\)-catulin–GFP \(c-kit^+\) dividing HSCs, and random spots in concentric cylinders corresponding to equal volumetric deciles from central marrow (centre) to the marrow near the endosteal bone surface (outside). Non-dividing HSCs were significantly enriched in central marrow, whereas dividing HSCs were significantly enriched towards the endosteum (d–k reflect mean ± s.d. from bone marrow plugs from the diaphysis of 5 tibias. A total of 1,840 Ki-67 \(\alpha\)-catulin–GFP \(c-kit^+\) HSCs were analysed in 5 independent experiments). In d–f and k, the statistical analysis was by Kolmogorov–Smirnov analysis, and in g–j, by Student’s \(t\)-tests (\(P<0.05\); NS, not significant.)
likely than Ki-67- \( \alpha \)-catulin-GFP-c-kit\(^+ \) non-dividing HSCs to localize close to the bone surface (Fig. 4k).

Based on reconstitution assays (Table 1), at least 30% of \( \alpha \)-catulin-GFP-c-kit\(^+ \) cells are HSCs. Therefore, some of the \( \alpha \)-catulin-GFP-c-kit\(^+ \) cells that we imaged are probably not HSCs. If HSCs home to, and engraft, in haematopoietic tissues in competitive transplantation assays with less than perfect efficiency, the HSC purity in this population would be higher than 30%. Even if purity is only 30% and all of the contaminating non-HSCs we imaged were associated with sinusoids, our data would still demonstrate that there are more HSCs associated with sinusoids than arterioles or bone surfaces (for example, subtract 70% from the sinusoid bar in Fig. 3g).

Our results are not consistent with the idea that most quiescent HSCs reside in arterial niches associated with NG2-CreER-expressing stromal cells. While a previous study\(^{24} \) concluded that the Nestin\(^{\text{high}} \) cells, not Lepr\(^{\text{high}} \) cells, expressed the highest levels of Scaf and Cxcl12, the RNA-seq data on which this conclusion was based showed that the Nestin\(^{\text{high}} \) cells analysed in this study were negative for Nes and positive for Lepr expression (see GSE48764 in the Gene Expression Omnibus)\(^{24} \). Thus, these data are consistent with our data in showing that the cells that express Scaf and Cxcl12 are Lepr\(^+ \) (ref. 10).

To address this issue directly, we generated NG2-CreER; Rosaf\(^{\text{Dtmato/Cat}} \); ScfGFP\(^1 \) and NG2-CreER; Rosaf\(^{\text{YFP/Cat}} \); Cxcl12DsRed\(^{\text{hi}} \) mice (YFP, yellow fluorescent protein). While 97% of Scaf-GFP\(^1 \) stromal cells and 96% of Cxcl12–DsRed\(^{\text{hi}} \) stromal cells were Lepr\(^+ \), we did not detect any recombination by NG2-CreER in these cells (Extended Data Fig. 10a, b, g, h). We also conditionally deleted Scf or Cxcl12 with NG2-CreER but did not detect any effect on bone marrow cellularity, HSC frequency, haematopoietic progenitor frequency, or bone marrow reconstituting capacity upon transplantation into irradiated mice (Extended Data Fig. 10c–i, i–l). NG2-CreER-expressing cells are therefore not a source of SCF or Cxcl12 for HSC maintenance in the bone marrow.

Our data provide little support for the idea that dividing and non-dividing HSCs reside in spatially distinct niches, with the exception that dividing HSCs were more likely than non-dividing HSCs to localize near the endosteme. Nonetheless, it remains possible that there are distinct perisinusoidal domains for dividing and non-dividing HSCs.

**Online Content** Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online version.

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METHODS

Mice. The targeting construct for α-catulinFPV-mice was generated by recombineering. Linearized targeting vector was electroporated into Bruce4 ES cells. Correctly targeted ES cell clones were identified by Southern blotting and injected into C57Bl/6-TyrRmice to obtain germine transmission. Then the Frt-Neo-Frt cassette introduced by the targeting vector was removed by mating with FLPe44. These mice were backcrossed onto a C57Bl/6 background and germine transmission was checked by PCR. C57Bl/6-TyrRmice (1CD45.2) and C57Bl/6-TyrR-1.2(CD45.1) mice were used in transplant experiments. Male and female mice from 18–28 weeks old were used for all studies. ScfGFP−/− and Smc3−/− mice, Cxcl12Ddx4tcr and Cxcl12flox mice, Leprcre−/− mice, Rosa26-CAG-loxP-stop-loxP-TdtTomato conditional reporter mice48, Rosa26-loxP-stop-loxP-EYFP conditional reporter mice49, and Ng2–CreER mice50 were all previously described. These mice were housed in AAALAC-accredited, specific-pathogen-free animal care facilities at the University of Texas Southwestern Medical Center. All procedures were approved by the University of Texas Southwestern Institutional Animal Care and Use Committee.

HSC isolation and flow cytometry. Bone marrow cells were isolated by either flushing the long bones (tibias and femurs), or by crushing the bones using a mortar and pestle in Ca2+- and Mg2+-free Hank’s balanced salt solution (HBSS, Gibco) supplemented with 2% heat inactivated bovine serum (Gibco). Spleen cells were prepared by crushing the spleen between two glass slides. The cells were gently passed through a 25-gauge needle then filtered using a 100 μm mesh to generate a single cell suspension. Viable cell number was calculated using a Vi-Cell cell counter (Beckman Coulter) or by counting manually with a haemocytometer. For HSC identification by flow cytometry, the cells were stained with antibodies against CD150 (TC15-12F12.2), CD48 (HM48-1), Scal (E13-167-1), and c-kit (2B8), as well as the following lineage markers: CD42d (IC2), CD2 (RM2-5), CD3 (17A2), CD5 (53-7,3), CD8 (53-6.7), B220 (62B2), Ter119 (TERR119), and Gr1 (8C5). Antibody staining of cell suspensions was always performed with antibodies against CD34 (RAM34), CD127 (IL7Rα), CD41 (MWReg30), anti-CD3 (17A2), anti-CD4 (9C3), anti-CD8 (53-6.7), Sca1 (E13-161-7), c-kit (2B8) and the lineage markers listed above. Stains that achieved a final PFA concentration around 4%. Cells were fixed for 10 min at room temperature. Slides were incubated for 45 min at 4°C to let the sorted cells attach to the slide surface. A 16% paraformaldehyde (PFA) stock solution was then added gently into the drop of staining medium to achieve a final PFA concentration around 4%. Cells were fixed for 10 min at room temperature and washed multiple times with PBS. The cells were then stained with DAPI (2 μg ml−1 in PBS), with 0.1% Igepal603 (Sigma) for 30 min, followed by multiple washes in PBS. ProLong Gold antifade (Life Technologies) was used to mount the cells. An LSM780 confocal microscope (Zeiss) was used to image the cells and Imaris software was used to measure cell diameter.

MicroCT analysis of bones. Dissected intact femurs from 10–12 week old littermate mice were fixed in 4% PFA for 12 h and then washed multiple times with and stored in 70% ethanol until they were scanned using a Scanco Medical microCT 35 machine at the Texas A&M University Baylor College of Dentistry. The scan was performed with a 3.5 μm voxel size resolution, 55 kV, 145 μA, and an integration time of 800 ms. Scanco software was used for analysis. A common reference point was determined for all bones scanned based on the growth plate, and trabecular and cortical regions were analysed for each bone.

PCR genotyping. The following primers were used to genotype α-catulinFPV allele: Cin-G1, 5′-GAATGTGCGAGAACTGAC-3′; Cin-G2, 5′-GGCCGGGTTTGCGAGAAAC-3′; Cin-G3, 5′-GTGCGGCGTCCGTTGCGTTG-3′. Genotyping primers for Cxcl12Ddx4tcr mice45 were previously published.

Bone marrow preparation from metaphysis and diaphysis for FACS analysis. To compare bone marrow from the epiphysis/metaphysis to the diaphysis, the metaphysis was separated from the diaphysis using scissors at the point where the central sinus branches (see Extended Data Fig 7). Then each segment of bone was crushed using a mortar and pestle and small bone fragments were suspended in staining medium (Ca2+− and Mg2+−free Hank’s balanced salt solution (Gibco) supplemented with 2% heat inactivated bovine serum (Gibco) and gently triturated until no marrow was visibly attached to the bone. The cell suspension was filtered through a 100 μm mesh to obtain a single-cell suspension. The cell suspensions were then analysed to determine cellularity and HSC frequency.

Competitive reconstitution assays in irradiated mice. Adult recipient mice were administered a minimum lethal dose of radiation using an X Rad 320 X-ray irradiator (Precision X-Ray) to deliver two doses of 540 rad at least 3 h apart. Cells were transplanted by injection into the retro-oral venous sinus of anaesthetized recipient mice. 300,000 recipient whole bone marrow (WBM) cells were transplanted along with the donor cells. In Table 1, HSC frequency was calculated using Extreme Limiting Dilution Analysis software (http://bioinf.wehi.edu.au/software/elda/) (2–4 independent experiments per cell population). For secondary transplants, 3 million WBM cells from primary recipient mice were transplanted into irradiated secondary recipient mice. Blood was collected from the tail vein of recipient mice at 4-week intervals after transplantation for at least 16 weeks after transplantation. For analysis of the levels of donor cells in peripheral blood, red blood cells were lysed with ammonium potassium buffer, and the remaining cells were stained with antibodies against CD45.1 (A20), CD45.2 (104), CD11b (Mac1, M1-70), Gr-1 (8C5), B220 (62B2), and CD3 (17A2).

Cell cycle analysis. For analysis of DNA content in HSCs and other haematopoietic cells, the cells were isolated by flow cytometry as described above and sorted directly into 70% ethanol then stored at −20°C for at least 24 h. The cells were washed multiple times with staining medium (see above) then incubated in staining medium containing 50 μg ml−1 propidium iodide (Molecular Probes) for 30 min at room temperature and analysed using a FACS Aria or FACS Canto II flow cytometer (BD Biosciences). Data were analysed using FACS Diva (BD Biosciences) or FlowJo (Tree Star) software. To assess 5-bromo-2′-deoxyuridine (BrDU) incorporation in vivo, mice were intraperitoneally injected with a single dose of BrDU (1 mg BrDU per 10 g body mass) then maintained on 0.5 mg BrDU per ml of drinking water for 3 days. For analysis of BrDU incorporation into HSCs, bone marrow cells were stained with the following antibodies that were selected to survive fixation: anti-CD150-BV421, anti-CD48-AF700, anti-CD2-PE, anti-CD3-PE, anti-CD5-PE, anti-CD8-PE, anti-Ter119-PE, anti-Gr1-PE, anti-Sca1-PerCpCy5.5 and c-kit-APCCH7 (BD Biosciences); antibody clones are described above for HSC isolation and flow cytometry. For isolation of α-catulin−/− GFP− c−kit− cells, bone marrow cells were stained with anti-c-kit−/− antibody. After antibody staining, the target cell populations were double sorted to ensure purity, then fixed and stained with an anti-BrDU antibody using the Brdu APC Flow Kit (BD Biosciences) according to the manufacturer’s instructions.

Sorting of α-catulin−/− GFP− c−kit− cells to determine cell diameter. Bone marrow cells from α-catulinFPV−/− mice were prepared for flow cytometric analysis as described above. Biotinylated anti-c-kit antibody (28B clone, Ebioscience) was followed by streptavidin-AF647 (Life Technologies) to stain bone marrow cells. α-catulin−/−GFP− c−kit− cells were sorted into a drop of staining medium on a slide coated with poly-lysine (0.5 mg ml−1 poly-lysine in water was used to coat the slides over night at room temperature). Slides were incubated for 45 min at 4°C to let the sorted cells attach to the slide surface. A 16% paraformaldehyde (PFA) stock solution was then added gently into the drop of staining medium to achieve a final PFA concentration around 4%. Cells were fixed for 10 min at room temperature and washed multiple times with PBS. The cells were then stained with DAPI (2 μg ml−1 in PBS), with 0.1% Igepal603 (Sigma) for 30 min, followed by multiple washes in PBS. ProLong Gold antifade (Life Technologies) was used to mount the cells. An LSM780 confocal microscope (Zeiss) was used to image the cells and Imaris software was used to measure cell diameter.

MicroCT analysis of bones. Dissected intact femurs from 10–12 week old littermate mice were fixed in 4% PFA overnight at 4°C. Freshly dissected tibias were fixed in 4% PFA at 4°C for 8 h at 4°C while shaking. The bones were washed with PBS to remove PFA and cryoprotected in 30% sucrose PBS solution overnight at 4°C with shaking. The bones were embedded in OCT (Fisher) and flash frozen in liquid nitrogen. A Leica cryostat was used to longitudinally bisect the bones. Intact half bone was washed in PBS to remove OCT then processed for staining, clearing and imaging as below. Bone marrow whole-mount plugging preparation for imaging. Intact bone marrow plugs from freshly dissected tibias of 8–12 week old mice were extruded from the bone using a PFA-filled syringe with a 25-gauge needle and placed directly into 4% PFA solution for 3 h at room temperature. Fixed plugs were then washed in PBS before being stained, cleared and imaged as below.

Whole-mount immunostaining. All staining procedures were performed in eppendorf tubes on a rotator at room temperature. The staining solution
contained 10% dimethyl sulfoxide, 0.5% IgG/PBS (Sigma), and 5% donkey serum (Jackson Immuno) in PBS. Half bones and plugs were blocked in staining solution containing anti-CD16/32 mouse Fc-blocking antibody (BD Biosciences) and 1% BSA in PBS overnight at room temperature. After blocking, half bones were stained for three days at room temperature with primary antibodies in staining solution. Then the tissues were washed multiple times in PBS at room temperature for one day and put into staining solution containing secondary antibodies for three days following by a one-day wash to remove any unbound secondary antibodies. Anti-GFP antibodies used for whole mount staining included chicken anti-GFP (GFP-1020, Aves Labs), goat anti-c-kit (BAF1356, R&D Systems), rabbit anti-laminin (ab7463, abcam), rat anti-Ki-67 (SoDa15, ebioscience), Alexa Fluor 647-AffiniPure F(ab')2 fragment donkey anti-chicken IgY, Alexa Fluor 488-AffiniPure F(ab')2 fragment donkey anti-rabbit IgG, AMCA-AffiniPure F(ab')2 fragment donkey anti-rabbit IgA, Alexa Fluor 488-AffiniPure F(ab')2 fragment donkey anti-rat IgG (all from Jackson ImmunoResearch), and 555 or 488 conjugated donkey anti-goat (A-10053 and A-21432 from Life Technologies). For isotype control staining in Extended Data Fig. 4b, goat IgG control (BAF108, R&D Systems), rabbit IgG control (ab27478-100, Abcam), rat IgG control (012-000-003, Jackson Immuno) and non-immune mouse IgG (control (N-1010, Aves Labs) were used along with the secondary antibodies described above. The fixation time of the tissue, using 0.5% IgG/PBS and 10% DMSO in the staining solution, and incubation of the tissue for 3 days in both primary and secondary antibodies were critical factors for efficient deep penetration of antibodies throughout the whole-mount tissue. For anti-Ki-67 antibody penetration, before the blocking step, treatment of the tissues with 0.05% SDS overnight in PBS was necessary.

Comparison of clearing protocols. Scattering and spherical aberration due to refractive index mismatch limit the maximum depth of penetration of visible light into aqueous tissue to about 100 µm. Optical clearing agents can decrease the amount of scattered light and therefore increases the depth of penetration. Most optical clearing agents work by replacing the low refractive index aqueous components of the tissue with agents of higher refractive index to match that of the tissue, such as collagen and cell components. Because each tissue has unique properties, such as density of cells and extracellular matrix, the optimum tissue clearing method must be determined empirically. Bone marrow does not have high lipid content and therefore, unlike brain, is not limited by the opacity of lipids. Therefore clearing methods that remove the lipid with SDS by either electrophoresis or passive flow were ineffective and had the additional disadvantage of SDS destruction of cell surface epitopes. Bone marrow is very densely packed with cells, which probably explains why optical clearing agents and methods with lower refractive indices such as Sca/eA2 (ref. 41), CUBIC42, and Focus Clear39 did not work in our system. We found all of the vessels involved in the transition from arteriole to sinusoid: the SHG signal was used to create bone surfaces. Three-dimensional distances were taken at 512 × 512 pixel resolution with 2 µm Z-steps. SHG signal was used to create bone surfaces. Three-dimensional distances were taken at 512 × 512 pixel resolution with 2 µm Z-steps.

Tissue clearing using modified Murray’s clear. All clearing of half bones and bone marrow plugs was performed in eppendorf tubes on a rotator at room temperature. Immunostained tissues were washed in PBS and dehydrated in either a methanol or ethanol dehydration series then incubated for 3 h in methanol or overnight in ethanol with several changes of 100% alcohol. The alcohol was then exchanged with BABB. The tissues were incubated in BABB for 3 h to overnight with several exchanges of fresh BABB. Half bones or bone marrow plugs were mounted in BABB between two coverslips and sealed with silicone (premium water-based vacuum generalizing Pratex, Permea Flexible). Then we found it necessary to clean the BABB of peroxides (which can accumulate as a result of exposure to air and light) by adding 10 g of activated aluminium oxide (Sigma) to 40 ml of BABB and rotating for at least 1 h, then centrifuging at 2000 g for 10 min to remove the suspended aluminium oxide particles.

Confocal imaging of thick tissue. Three-dimensional (3D) confocal microscopy of the bone marrow at submicron resolution required specialized equipment. We used both a Zeiss LSM780 and a Leica SP8 resonant scanning confocal. Specifications for the Zeiss LSM780: Axio Examiner upright stand; 405, 488, 561, 594 and 633 nm visible laser lines; internal 32-channel GaAsP detector; Prior OptiScan motorized stage; Coherent Chameleon Vision II pulsed NIR laser for two photon excitation; Zeiss BIG two channel nondescanned detector. Specifications for the Leica: Acouto Optical Beam Splitter, Spectral detection, 8 kHz Resonant tandem scanner, HyD hybrid detectors, and 405, 488, 561, 633 nm laser lines. The optimum clearing agent (BABB) for bone marrow has a refractive index of 1.56, similar but not identical to standard immersion oil. Deep imaging also requires a long working distance objective. For the Zeiss LSM780 we found the best available objective was a Zeiss LD LCI Plan-Apo 25×/0.8 multi-immersion objective lens, which has a 570 µm working distance. We used Immersol 518F immersion oil for Zeiss LSM780 imaging. For the Leica SP8, we used an HCX APO 1.20×/0.95 BABB immersion objective with a 1.95 mm working distance. High resolution imaging of large volumes of thick tissue by acquisition of tiled Z-stacks is very time consuming, thus it was important to optimize the acquisition settings for each microscope to minimize acquisition time while preserving adequate resolution and signal to noise ratio. On the Zeiss LSM780, images were taken at 512 × 512 pixel resolution with 2 µm Z-steps, pinhole for the internal detector at 47.7 µm. Bone was imaged by second harmonic generation (SHG) with 850 nm pulsed near infrared (NIR) excitation using the non-descanned detector. On the Leica SP8, images were taken using the resonance scanner using 8× line averaging with the minimum zoom of 1.25× at 812 × 812 pixel resolution, pinhole at 44.7 µm, and 2 µm Z-steps.

Image annotation and analysis. Confocal tiled Z-stack images were rendered in 3D and analysed using Bioplantum Imaris v.7.1.4 software installed on a Dell Precision T7610 64-bit workstation with Dual Intel Xeon Processor E5-2687W v2 (Eight Core HT, 3.4GHz Turbo, 25 MB), 128 GB RAM, and 16 GB AMD FirePro W9100 graphics card. Individual z-catalin–GFP c-kit+ HSCs were identified using the ortho slicer function in Imaris software to visualize digital serial sections of the large 3D image. We identified HSCs as having a round morphology, with GFP throughout the cell, and c-kit expression surrounding the cell surface. These criteria prevented false positive identification of cellular debris or z-catalin–GFP c-kit+ endothelial cells with elongated cell body morphology. HSC coordinates and size were interactively annotated using the Imaris spots function in manual mode. Bone and non-myleneinating Schwann cells were segmented based on thresholding of the SHG (which detects collagen fibres in bone) or GFAP (which labels astrocytes and the CNS, respectively, using the Imaris surface function). Cortical and trabecular bone were then divided into separate surfaces interactively based on SHG signal and morphology. We used anti-laminin antibodies to immunofluorescently label all of the vasculature within the bone marrow. Arteries, arterioles, and capillaries have continuous basement membranes, which are observable as uniform laminin staining. In contrast, bone marrow sinusoids have a discontinuous fenestrated basement membrane43. Laminin staining of sinusoids clearly demonstrates discontinuous basement membranes, thereby allowing unambiguous identification of sinusoidal vessels in the absence of other markers45. Because we were able to image the entire marrow cavity, we were able to trace and digitally label each artery and all of its subsequent branching into smaller arteries and arterioles as they approached the endostal surface. This is an advantage of the deep imaging approach that allowed us to unambiguously identify vessels in a way that is not possible in thin sections where the connectivity of vessels cannot be traced.

Near the endosteam, arterioles connect to the smallest diameter vessels of the capillary network that line the endosteam. These capillaries then connect to larger diameter sinusoidal vessels. By following the blood vessel paths in six samples, we determined that in the diaphysis, the outer 20% of the marrow by volume contained all of the vessels involved in the transition from arteriole to sinusoid: the most obvious identification of the connecting arterioles and veins was the gradual thinning of the vessel wall and the initial portion of sinusoids. We identified this region as the transition zone in keeping with published criteria46. Therefore, we used the published morphological characteristics of orientation, location, and basement membrane continuity to subdivide blood vessels within the bone marrow. The Imaris surface function was used to create three distinct digital surfaces corresponding to each type of blood vessel. SHG signal was used to create bone surfaces. Three-dimensional distances between HSCs and digital vessel or bone surfaces were calculated using the Imaris Distance Transform Matlab Xension and volumetric decile calculations were performed using a Matlab-based Imaris XTension. The annotated programs, which were paired with the raw data in a 3D Tissue (Matlab Extensions for Imaris) are available for download from the Morrison lab protocols webpage at the CRI website under ‘More Information’ (http://cri.utsw.edu/sean-morrison-laboratory-more-information/).

Random spot generation and insertion. The original 3D images of the GFP channel in the Imaris format were used to generate random spots for each sample. In a 16 bit Imaris file of the original bone marrow image, 3D voxels were represented by signal intensity values that ranged between 0 and 65,535. Those signal intensity values were imported into MATLAB using the imreadBF package (http://www.mathworks.com/matlabcentral/fileexchange/32920-imread-for-multiple-life-science-image-file-formats) and the Bio-Formats software (http://www.openmicroscopy.org/site/products/bio-formats). The images were filtered to exclude low-intensity regions that included blood vessel lumens and fat bodies where HSCs were not found and random spots were not generated. The intensity-filtered images, of which the excluded portions were given zero intensity values,
were processed with MATLAB’s median filter to remove ‘salt-and-pepper noise’. Then the intensity images were converted to binary signals by turning any non-zero intensity value into ‘one’. Those ‘one’ signals were used to determine the voxel locations that were used to generate random spots. The locations were randomly permuted, and enough random spot coordinates were designated to approximate the random spot distribution (more than 50,000 per bone). The random spot coordinates were transferred to Imaris to generate the random spots, and distances to cell types or landmarks in the bone marrow were calculated based on the distance transformation files generated using landmark surfaces such as arterioles, sinusoids, transition zone vessels, and bone. We confirmed the randomness of the distribution of the random spots by measuring the frequency of random spots in each percentile of bone marrow volume. Random spots were given a diameter of 6 μm, similar to the observed average HSC diameter.

**Statistical methods.** To assess whether the distribution of HSCs significantly differed from random spots with respect to particular bone marrow landmarks, we used a normalized two-sample Kolmogorov–Smirnov test. The two-sample Kolmogorov–Smirnov test calculates and evaluates the maximum difference between the empirical cumulative distribution functions (ECDFs) of two test groups where each group is a vector of continuous values, which in our case were the distances from HSCs or random spots to particular bone marrow cell types or structures. As we had multiple biological samples, we normalized them by the following approaches: (1) HSCs and random spot distances from the same sample were pooled to determine a range of distances, which was then used to generate 100 equal-length bins for each sample, so that each bin represented 1% of the distance range for that sample; (2) for each sample, the number of HSCs or random spots in each bin was determined and normalized to percentages; (3) the average percentage of HSCs or random spots in each bin was calculated across all samples; (4) the averaged binned percentages of HSCs or random spots were used to approximate the probability density functions (PDF), and the ECDFs were calculated based on those approximate PDFs; (5) the two-sample Kolmogorov–Smirnov test in MATLAB was used and slightly modified so that it accepted the two normalized ECDFs as inputs, and the Kolmogorov–Smirnov P values were adjusted using the Bonferroni method to account for multiple comparisons.

The data presented in the figures reflect multiple independent experiments performed on different days using tissues from different mice. No statistical methods were used to pre-determine sample size. The experiments were not randomized, and the investigators were not blinded during allocation or outcome assessment. Variation is always indicated using standard deviation. For analysis of the statistical significance of differences between two groups, we first determined that variance in the two groups was similar using an F-test, and then two-tailed Student’s t-tests. Single-factor ANOVA and two-way ANOVA with multiple comparisons tests were used for comparisons among three groups in Extended Data Fig. 2.

Not all samples were suitable for image analysis and those that did not meet the criteria were not analysed. Occasionally, the antibody staining was not strong enough for us to detect HSCs or other landmarks in the deepest part of the bone marrow, or the samples were damaged during processing and in these cases the samples were not analysed. All mice used in our studies were between 8 and 12 weeks old, including both male and female mice. We did not observe any differences in HSC localization between male and female mice (Extended Data Fig. 9d–k), so the data were combined for purposes of analysis. All conclusions were based on data obtained from at least three independent experiments involving samples obtained from different mice and processed on different days.

**Code availability.** Code was written to separate bone marrow into volumetric deciles and to identify the transition zone in the outer 20% of bone marrow. This code is available on the Morrison lab protocols webpage at the CRI website under ‘More Information’ (http://cri.utsw.edu/sean-morrison-laboratory/more-information/).

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a-catenin

**Summary**

The research letter describes the genetic manipulation of a-catenin using targeting vectors and presents the results of a genetic cross between two strains of mice. The study compares the body mass and bone density of the offspring generated from this cross.

### Results

#### a-catenin Strains

- **a-catenin^+/+**
- **a-catenin^SPP^+**
- **a-catenin^SPP^SPPGFP**

#### Body Mass

| Gender | a-catenin^+/+ | a-catenin^SPP^+ | a-catenin^SPP^SPPGFP |
|--------|--------------|----------------|----------------------|
| Male   | 25           | 20            | 22                   |
| Female | 15           | 10            | 12                   |

#### Bone Density

- **Cortical Bone**
  - **Mean density [mg HA/cm² of total volume]**
  - **Mean density [mg HA/cm³ of total volume]**
  - **Total volume [mm²]**
  - **Bone volume [mm³]**
  - **Bone volume/Total volume**

- **Trabecular Bone**
  - **Mean density [mg HA/cm³ of total volume]**
  - **Mean density [mg HA/cm³ of total volume]**
  - **Total volume [mm³]**
  - **Bone volume [mm³]**
  - **Connectivity density [1/mm²]**
  - **Trabecular number [mm⁻¹]**

### Methods

The targeted gene was engineered using a combined approach involving the FRT-flanked gene targeting strategy and Cre-mediated recombination. This method allowed for the precise integration of the GFP reporter gene at the a-catenin locus.

### Conclusion

The results suggest that a-catenin plays a critical role in bone density and mass, with the a-catenin^SPP^SPPGFP strain showing a reduced body mass and altered bone structure compared to the control strain.
Extended Data Figure 1 | Generation of α-catulinGFP mice. a, The targeting strategy to generate the α-catulinGFP allele is shown. The targeting vector was generated by retrieving a genomic fragment of the α-catulin gene, including exon 1, from bacterial artificial chromosome clone RP24-146F11 by recombineering31. The retrieved genomic region was then modified to replace most of the exon 1 coding region and the exon 1 to intron 1 junction with an EGFP-bGH-pA-FRT-neo-FRT cassette in frame with the first ATG of α-catulin. The final targeting vector was then linearized and electroporated into C57Bl/ derived Bruce4 ES cells. b, New NsiI and SpeI sites introduced with the EGFP-bGH-pA-FRT-neo-FRT cassette were used to screen correctly targeted ES cell clones by Southern blotting for 5′ and 3′ probes. Correctly targeted ES cells were used to generate chimaeric mice. Upon confirmation of germline transmission by PCR, the α-catulinGFP-neo mice were crossed with FLPe mice32, to remove the neomycin resistance cassette. c, PCR genotyping of α-catulin WT and α-catulinGFP alleles from α-catulin+/+, α-catulinGFP/+, and α-catulinGFP/GFP mice. d, α-catulin+/+ and α-catulinGFP/GFP mice did not show any difference in size or body mass (n = 9 α-catulin+/+ and 8 α-catulinGFP/GFP male mice; n = 7 α-catulin+/+ and 7 α-catulinGFP/GFP female mice; all were 8–10 weeks old). e, α-catulinGFP/+ and α-catulinGFP/GFP mice were born at Mendelian frequencies, survived into adulthood in normal numbers, and were apparently developmentally normal. The statistics reflect mice genotypes at 8–10 weeks of age. f, Cortical and trabecular femur bone (CB and TB, respectively) did not show any statistically significant differences among α-catulin+/+ and α-catulinGFP/GFP mice by microCT (microcomputed tomography) analysis (6 α-catulinGFP/GFP and 5 α-catulin+/+ controls at 10–12 weeks of age). HA, hydroxyapatite. All data represent mean ± s.d. The significance of differences between genotypes was assessed using Student’s t-tests; none were statistically significant.
Extended Data Figure 2 | α-catulin<sup>GFP/GFP</sup> mice had normal haematopoiesis, normal HSC frequency, and normal HSC function.

a, Hindlimb bone marrow cellularity (n = 9 mice for α-catulin<sup>+/+</sup>, n = 4 mice for α-catulin<sup>GFP/GFP</sup> genotype), spleen cellularity (n = 6 mice for α-catulin<sup>+/+</sup>, n = 4 mice for α-catulin<sup>GFP/GFP</sup> and n = 6 mice for α-catulin<sup>GFP/GFP</sup> genotype), and spleen mass (n = 7 mice for α-catulin<sup>+/+</sup>, and n = 7 mice for α-catulin<sup>GFP/GFP</sup> genotype). b, White blood cell (WBC), red blood cell (RBC), and platelet (PLT) counts per microlitre of peripheral blood from 8–12 week old α-catulin<sup>+/+</sup>, α-catulin<sup>GFP/+</sup>, and α-catulin<sup>GFP/GFP</sup> mice (n = 9 mice per genotype). c, d, Frequencies of mature haematopoietic cells and progenitors in the bone marrow of 8–12 week old α-catulin<sup>+/+</sup> and α-catulin<sup>GFP/GFP</sup> mice (pre-pro-B cells were B220<sup>sIgM</sup>CD43<sup>−</sup>CD24<sup>+</sup>; pro-B cells were B220<sup>sIgM</sup>CD43<sup>−</sup>CD24<sup>+</sup>CD4<sup>+</sup>CD135<sup>−</sup>; common lymphoid progenitors (CLP) were Lin−c-kit<sup>hi</sup>Sca-1<sup>i</sup>CD127<sup>hi</sup>CD135<sup>−</sup>CD43<sup>−</sup>; common myeloid progenitors (CMP) were Lin<sup>c-kit</sup><sup>−</sup>Sca-1<sup>−</sup>CD34<sup>−</sup>CD16/32<sup>−</sup>CD48<sup>+</sup>CD24<sup>+</sup>CD135<sup>−</sup>; and megakaryocyte erythroid progenitors (MEP) were Lin<sup>c-kit</sup><sup>−</sup>Sca-1<sup>−</sup>CD34<sup>−</sup>CD16/32<sup>−</sup>CD48<sup>+</sup> (n = 3 mice per genotype). e, Bone marrow CD150<sup>+</sup>CD48<sup>+</sup>LSK HSC frequency, bone marrow CD150<sup>−</sup>CD48<sup>−</sup>LSK MPP frequency (n = 12 mice per genotype), and spleen HSC frequency (n = 3 mice per genotype in 3 experiments). f, Percentage of HSCs and whole bone marrow cells that incorporated a 3 day pulse of BrdU <i>in vivo</i> (n = 6 α-catulin<sup>+/+</sup>, 9 α-catulin<sup>GFP/+</sup>, and 7 α-catulin<sup>GFP/GFP</sup> 8–12 week old mice in 3 independent experiments). g, Colony formation by HSCs in methylcellulose cultures (GM, granulocyte macrophage colonies; GEMM, granulocyte erythroid macrophage megakaryocyte colonies; Mk, megakaryocyte colonies; n = 5 mice per genotype in 5 independent experiments). h, Reconstitution of irradiated mice by 300,000 donor bone marrow cells from 8–12 week old α-catulin<sup>+/+</sup>, α-catulin<sup>GFP/+</sup>, or α-catulin<sup>GFP/GFP</sup> mice competed against 300,000 recipient bone marrow cells (n = 4 donor mice and 16 recipient mice for α-catulin<sup>+/+</sup>, n = 3 donor mice and 9 recipient mice for α-catulin<sup>GFP/+</sup>, and n = 4 donor mice and 18 recipients for α-catulin<sup>GFP/GFP</sup> in 3 independent experiments). i, Serial transplantation of 3,000,000 WBM cells from primary recipient mice shown in h into irradiated secondary recipient mice (n = 4 primary α-catulin<sup>+/+</sup> recipients were transplanted into 17 secondary recipients, and n = 6 primary α-catulin<sup>GFP/GFP</sup> recipients were transplanted into 20 secondary recipients). All data represent mean ± s.d. The statistical significance of differences between genotypes was assessed using Student’s <i>t</i>-tests or ANOVAs; none were significant.
Extended Data Figure 3 | α-catulin-GFP expression among haematopoietic cells is highly restricted to HSCs. a, The frequency of α-catulin−GFP− bone marrow cells in negative control α-catulin+/− (WT) mice and α-catulinGFP+/− mice (n = 14 mice per genotype in 11 independent experiments). In all cases in this figure, percentages refer to the frequency of each population as a percentage of WBM cells. b, α-catulin−GFP− c-kit+ cells from Fig. 1b are shown (blue dots) along with all other bone marrow cells in the same sample (red dots). c, CD150−CD48− LSK HSCs express α-catulin−GFP but CD150−CD48− LSK MPPs do not (n = 17 mice in 12 independent experiments). A minority of the α-catulin−GFP− c-kit+ cells had high forward scatter, lacked reconstituting potential, and were gated out when isolating HSCs by flow cytometry and when identifying HSCs during imaging (see Extended Data Fig. 5 for further explanation). d, Lin−c-kit+CD127−CD135+ common lymphoid progenitors (CLPs), Lin−c-kit+Sca-1−CD34+CD16/32− common myeloid progenitors (CMPs), Lin−c-kit+Sca-1−CD34+CD16/32− granulocyte-macrophage progenitors (GMPs), and Lin−c-kit+Sca-1−CD34+CD16/32− megakaryocyte-erythroid progenitors (MEPs) did not express α-catulin−GFP. α-catulinGFP− and control cell populations had similar levels of background GFP signals that accounted for fewer than 1% of the cells in each population (n = 9 mice per genotype in 2 independent experiments).
Extended Data Figure 4  

α-catulin−GFP⁺ c-kit⁺ bone marrow cells are highly enriched for HSC activity and are quiescent. a, Competitive reconstitution assays in which 1 donor α-catulin−GFP⁺ c-kit⁺ bone marrow cell was transplanted along with 300,000 recipient bone marrow cells into irradiated recipient mice. Each line represents 1 of the 9 mice (out of 34 transplanted; see Table 1) that were long-term multilineage reconstituted by donor myeloid, B, and T cells. b, Three million WBM cells from primary recipient mice 1−4 from a (indicated by an asterisk) were transplanted into secondary recipient mice (7 secondary recipients from primary recipient-1; 4 secondary recipients from primary recipient-2; 3 secondary recipients from primary recipient-3; 3 secondary recipients from primary recipient-4 for an overall total of 17 secondary recipients). Each line shows the average (± s.d.) levels of donor cell reconstitution in secondary recipient mice from each primary donor. c, DNA content of WBM cells, α-catulin−GFP⁺ c-kit⁺ HSCs, and CD150⁺ CD48⁻ LSK HSCs. While 11.5% of WBM cells had greater than 2N DNA content (in S/G2/M phases of the cell cycle), only around 1% of α-catulin−GFP⁺ c-kit⁺ HSCs or CD150⁺ CD48⁻ LSK HSCs had greater than 2N DNA content. d, BrdU incorporation into WBM cells, c-kit⁺ cells, α-catulin−GFP⁺ c-kit⁺ CD150⁻ CD48⁻ LSK cells, α-catulin−GFP⁺ c-kit⁺ CD150⁺ CD48⁻ LSK HSCs, and α-catulin−GFP⁺ c-kit⁺ HSCs after 3 days of continuous BrdU administration (BrdU treated). Untreated negative control mice are also shown. e, Percentage of BrdU⁺ cells in each cell population. In each panel, the number of mice used for analysis (without being pooled) is indicated. All data reflect mean ± s.d. from two to five independent experiments. Statistical significance was assessed using Student’s t-tests (*P < 0.05; **P < 0.01).
a) WT mice WBM cells

α-catenin-GFP

α-catenin<sup>GFP</sup> mice WBM cells

α-catenin-GFP

b) FACS sorted cells

| Cell diameter (µm) | FSC&SSC low cells | FSC&SSC high cells |
|-------------------|-------------------|-------------------|
| 5                 | 5,5               | 6                 |
| 6                 | 6,5               | 7,5               |
| 7                 | 7,5               | 8                 |
| 8                 | 8,5               | 9                 |
| 9                 | More              | More              |

n=134 cells

n=115 cells

c) GFP<sup>+</sup> 300,000 cells/recipient

- c-kit<sup>+</sup>GFP<sup>+</sup> FSC&SSC low 30 cells/recipient
- c-kit<sup>+</sup>GFP<sup>+</sup> FSC&SSC high 20 cells/recipient
- c-kit<sup>+</sup>GFP<sup>+</sup> 100 cells/recipient

d) Donor-derived (%)

- Myeloid
- B
- T
- Overall

Weeks after transplantation

Confocal imaging

n=3063 cells

n=1392 cells

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Extended Data Figure 5 | All HSC activity resides among α-catulin−GFP+c-kit+ cells with low forward and side scatter. a, Most α-catulin−GFP+c-kit+ cells (63 ± 7.2%) had low forward and side scatter, but a distinct minority population (36 ± 7.2%) had higher forward and side scatter that was not typical of HSCs. b, We sorted the low scatter and the high scatter α-catulin−GFP+c-kit+ cell populations gated in a and measured their diameters (three independent experiments). c, Competitive reconstitution assays in irradiated mice revealed that all HSC activity resided in the low scatter cell fraction. For each recipient mouse, the indicated donor cells (based on the number of cells from each population contained within 300,000 bone marrow cells) were transplanted into irradiated mice along with 300,000 recipient bone marrow cells (mean ± s.d. from 2 independent experiments with 20 total recipient mice in the GFP- group, 14 total recipient mice in the c-kit+GFP- FSC&SSC low group, 11 total recipient mice in the c-kit+GFP- FSC&SSC high group, and 9 total recipient mice in the c-kit+GFP- group). d, The size distribution of all α-catulin−GFP+c-kit+ cells identified by confocal microscopy in bone marrow plugs from the tibia diaphysis (6 bones analysed in 6 independent experiments). In keeping with the flow cytometry data, the largest 40% of imaged cells were not considered HSCs, excluding all cells with diameter larger than 7 μm.
Extended Data Figure 6 | HSCs are enriched in the central marrow and depleted near the endosteum in the diaphysis. a, b, The distribution of HSCs from the central marrow to the endosteum can be determined by drawing concentric cylinders that correspond to equal volumetric deciles from the centre of the marrow to the endosteum (a) or to equal radial deciles from the centre to the endosteum (b). c, d, Each volumetric decile (as in a) contains 10% of the marrow volume (c). However, cylinders based on radial deciles (as in b), contain successively larger volumes of marrow as they approach the endosteum because the circumference of the cylinders becomes larger (d). e, f, The distribution of random spots among volumetric deciles (a) is nearly equal because each cylinder contains an equal marrow volume (e). However, the number of random spots per cylinder based on radial deciles (b) increases from the centre to the endosteum as cylinder volume increases (f). g, When we plotted our HSC localization data by volumetric deciles (as in Fig. 2a), HSC were enriched towards the central marrow. h, When we plotted our HSC localization data by radial deciles, the number of HSCs per cylinder increased towards the endosteum as cylinder volume increased, similar to random spots. All data represent mean ± s.d.
Extended Data Figure 7 | HSC density is higher in the diaphysis as compared to the metaphysis. a, Schematic of a femur showing the separation of epiphysis/metaphysis from diaphysis. We divided metaphysis from diaphysis at the point where the central sinus branched (see red line in panels a, f, and i). This is also the point at which the density of trabecular bone declines, moving into the diaphysis. b, A bisected femur before and after clearing. c, The frequency of CD150+CD48+LSK cells and α-catulin−GFP+c-kit+ cells by flow cytometry in the epiphysis/metaphysis versus diaphysis of femurs (n=9 mice in 2 independent experiments). Note that bone marrow cells were extracted from crushed bones. d, The distance (μm) from α-catulin−GFP+c-kit+ cells to the nearest bone surface in the femur diaphysis based on deep imaging (n=368 cells in 3 bisected femurs). e, The distance (μm) from α-catulin−GFP+c-kit+ cells to the nearest bone surface in the femur diaphysis based on analysis of thin (7 μm) sections (n=45 cells). f, Schematic of a tibia showing the separation of epiphysis/metaphysis from diaphysis (red line). g, The frequency of CD150−CD48−LSK cells and α-catulin−GFP−c-kit− cells by flow cytometry in the epiphysis/metaphysis versus diaphysis of tibias (n=9 mice in 2 independent experiments). h, The frequency of α-catulin−GFP+c-kit+ cells in the tibia epiphysis/metaphysis versus diaphysis based on deep confocal imaging (n=3 bisected tibias in 3 independent experiments). i, Deep imaging of a bisected tibia showing the separation of metaphysis and diaphysis (red line) where the central sinus branches. Note that these tibias were digitally reconstructed from two different imaging sessions, above and below the diagonal white line. This image shows a 349 μm thick specimen collapsed into 2D. This causes α-catulin−GFP+c-kit+ cells to appear much more frequent than they actually were because all of the cells from the thick specimen were collapsed into a single 2D optical plane for presentation. j, For comparison purposes, a single 2 μm thick optical slice from the tibia in i is shown. k, High magnification images of single α-catulin−GFP+c-kit+ cells from the same tibia. Note that α-catulin−GFP is also expressed by sinusoidal endothelial cells but these cells are easily distinguished from HSCs because the endothelial cells lack c-kit expression and have a different morphology. Statistical significance was assessed using Student’s t-tests (*P<0.05; **P<0.01; ***P<0.001). All data represent mean ± s.d.
Extended Data Figure 8 | c-kit and α-catenin–GFP staining do not reflect autofluorescence or background staining; and GFAP⁺ non-myelinating Schwann cells tend to localize in the centre of the marrow. a, Four-colour confocal analysis of a bone marrow plug from a tibia diaphysis stained with primary and secondary antibodies against Ki-67, α-catenin–GFP, c-kit, and laminin. A 2 μm optical section is shown from a thick specimen to illustrate typical staining. b, Negative control in which a bone marrow plug from a tibia diaphysis was stained with isotype control and secondary antibodies then imaged under the same conditions as shown in a. c, Ki-67 staining was largely or exclusively nuclear, co-localizing with DAPI. d–g, Low magnification images of bone marrow plugs from tibia diaphysis stained with antibodies against α-catenin–GFP, c-kit, and GFAP. GFAP⁺ non-myelinating Schwann cells are associated with nerve fibres that run longitudinally along the central bone marrow, where innervated arterioles are located24. α-catenin–GFP⁺ c-kit⁺ cells were identified and annotated with blue spheres using the Imaris spot function in e and g. Note, the blue spheres are larger than the actual HSCs because at their actual size, HSCs would be extremely difficult to see at this magnification. As the HSCs are represented as large blue spheres, they appear denser than they actually are. For clarity, other haematopoietic cells and endothelial cells are not shown in e and g. h, A higher magnification image showing two α-catenin–GFP⁺ c-kit⁺ cells (arrows) and their localization relative to GFAP positive glia (white) and α-catenin–GFP⁺ endothelial cells (green). The images in d–g show a 505 μm thick specimen. This causes α-catenin–GFP⁺ cells and c-kit⁺ cells to appear more frequent than they actually were because all of the cells from the thick specimen were collapsed into a single 2D optical plane for presentation. Because these were thick specimens, there were cases in which an α-catenin–GFP⁺ cell and a c-kit⁺ cell were present in different optical planes such that they appeared to be a single α-catenin–GFP⁺ c-kit⁺ cell when collapsed into a single 2D image. For this reason, α-catenin–GFP⁺ c-kit⁺ cells cannot be reliably identified in low magnification 2D projected images. In all cases, cells that we identified as α-catenin–GFP⁺ c-kit⁺ were manually examined at high magnification in 3D to confirm double labelling of single cells, as shown in h. Few HSCs were closely associated with nerve fibres in these images when they were examined at high magnification and in 3D.
Extended Data Figure 9 | Bone marrow blood vessel types can be distinguished based on vessel diameter, continuity of basal lamina, morphology, and position; and no difference in the distribution of HSCs in the bone marrow of male and female mice was detected.  

a, b, Schematic (a) and properties (b) of blood vessels in the bone marrow. Blood enters the marrow through arterioles that branch as they become smaller in diameter and approach the endosteum, where they connect to smaller diameter transition zone capillaries near the bone surface. These transition zone capillaries connect to the large diameter sinusoids that feed blood into the central sinuous through which it leaves the bone marrow in venous circulation. c, Each type of blood vessel was distinguished based on vessel diameter, continuity of basal lamina, morphology, and position, and then colour-coded using published criteria. To create distinct digital surfaces associated with each type of blood vessel, we first designated all laminin-stained blood vessels in the outer 20% of the marrow volume (adjacent to the endosteum) as transition zone (TZ) vessels (blue). Arterioles were identified and manually traced in the remaining 80% of marrow volume based on high intensity laminin staining, continuous basal lamina, and morphology. Remaining blood vessels with low intensity laminin staining, fenestrated basal lamina, large diameter, and sinusoidal morphology were designated sinusoids. The longitudinal images (top) show bone marrow plugs that were 550 μm thick and the cross-sectional images (bottom) were 49 μm thick. d, The distribution of x-catenin–GFP”c-kit” cells in concentric cylinders corresponding to equal volumetric deciles from central marrow to endosteal marrow (near the bone surface) in bone marrow plugs from the tibia diaphysis of male and female mice. e–g, The distance from x-catenin–GFP”c-kit” cells in male or female mice to the nearest arteriole (e), sinusoid (f), or transition zone vessel (g) in tibia based on deep imaging. h–j, The percentage of x-catenin–GFP”c-kit” cells within 10 μm of arterioles (h), sinusoids (i) and transition zone vessels (j) in the tibias of male versus female mice. k, The percentage of x-catenin–GFP”c-kit” cells closest to arterioles, sinusoids, or transition zone vessels in the tibias of male versus female mice. These data show mean ± s.d. for a total of 1,345 x-catenin–GFP”c-kit” cells from 3 female tibias and 1,632 x-catenin–GFP”c-kit” cells from 3 male tibias. The statistical significance of differences was assessed using Kolmogorov–Smirnov tests in d–g and Student’s t-tests in h–k; none of the differences were statistically significant.
Extended Data Figure 10 | Expression of NG2–CreER was not detected in Scf- or Cxcl12-expressing cells; and conditional deletion of Scf or Cxcl12 using NG2–CreER did not affect HSC frequency or haematopoiesis. a, A 20 μm optical section from a 390-μm-thick cleared bone marrow plug from the tibia diaphysis of an NG2–creER;Rosa<sup>TdTomato<sup>1</sup>;Scf<sup>GFP<sup>1</sup> mouse (image is representative of bones from 4 mice). The image shows rare tdTomato<sup>1</sup> periarteriolar smooth muscle cells (arrow) as well as glia associated with nerve fibres (arrowhead); however, we were unable to detect Scf expression by any of these cells. b, Representative flow cytometry plots showing the percentage of Scf–GFP<sup>1</sup> stromal cells that were positive for tdTomato expression (reflecting recombination by NG2–CreER) or Lepr antibody staining (mean ± s.d. from 4 mice in 3 independent experiments). Scf–GFP<sup>1</sup> stromal cells were uniformly positive for Lepr expression but negative for NG2–CreER recombination. c–f, Conditional deletion of Scf in NG2–creER;Scf<sup>GFP<sup>1</sup> mice had no effect on bone marrow cellularity (c), HSC frequency (d), CMP, GMP, or MEP frequency (e), or bone marrow reconstituting capacity upon transplantation into irradiated mice (f) (n = 5 mice per genotype in 5 independent experiments with 4/5 recipient mice per donor in each experiment). g, A 20 μm optical section from the diaphysis of a 130-μm-thick cleared half tibia from an NG2–creER;Rosa<sup>YFP<sup>1</sup>;Cxcl12<sup>DsRed<sup>1</sup> mouse. The image shows rare YFP<sup>1</sup> periarteriolar smooth muscle cells; however, we were unable to detect Cxcl12 expression by these cells. h, Representative flow cytometry plots showing the percentage of Cxcl12–DsRed<sup>1</sup> stromal cells that were positive for YFP expression (reflecting recombination by NG2–CreER) or Lepr antibody staining. Cxcl12–DsRed<sup>1</sup> stromal cells were uniformly positive for Lepr expression but negative for NG2–CreER. i–l, Conditional deletion of Cxcl12 in NG2–creER;Cxcl12<sup>2<sup>fl</sup> mice had no effect on bone marrow cellularity (i), HSC frequency (j), CMP, GMP, or MEP frequency (k), or bone marrow reconstituting capacity upon transplantation into irradiated mice (l) (n = 4 mice per genotype in 4 independent experiments with 4/5 recipient mice per donor in each experiment).