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Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist .

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒	A statement on 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 statistical parameters 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
☐	☒	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	Nikon NIS-elements AR 4.3.1, Zeiss AxioVision 4.7, BD FACS Diva 4.1.1, Micro-Manager 1.4, YouScope v.2.1
Data analysis	GraphPad Prism v7-8, FlowJo v10, Microsoft Office (Excel, Powerpoint) 2016, Windows Movie Maker 8.0.3.8, Matlab 2017a-2018b, R 3.41., Fiji 1.47b, Clone Manager v9, Snap Gene 4.1.1. , Adobe Photoshop CC 2017, Adobe Illustrator CC 2017, Anaconda Navigator 1.9.6 (Python 3.7, Spyder 3.2.2). Software used for data acquisition of immunostainings and time-lapse imaging is commercially available (NIS-Elements 4.3.1) or open sourced (YouScope v.2.1; http://langmo.github.io/youscope/). Software used for single cell tracking and fluorescence quantification used in this study is published and open sourced (https://doi.org/10.1038/nbt.3626). Software used for image Segmentation is published and open sourced (https://academic.oup.com/bioinformatics/article/33/13/2020/3045025). Software used for dimensionality reduction using Uniform Manifold Approximation and Projection (UMAP) is published and open sourced (https://github.com/lmcinnes/umap.git). Software used for time series clustering is open sourced (https://github.com/dmattek/shiny-timecourse-inspector)).

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. 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 data is available in main text or figures. Raw data is available upon reasonable request.

Field-specific reporting

Please select the one below that is 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/documents/nr-reporting-summary-flat.pdf

Life sciences study design

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

Sample size	No statistical methods were used to predetermine sample size. Sample size was determined based on previous experience and choosen based on putative effects sizes in pilot experiments. Sample sizes were considered as sufficient when the obtained results could be reproduced using an independent technique (i.e. immunofluorescence of mitotic cells and live cell imaging of mitotic cells).
Data exclusions	Single cell traces of quantified fluorescence intensities over time were visually inspected and manually curated. In case sudden changes in fluorescence intensity in individual time point were observed, raw images were inspected and time points excluded when imaging or segmentation error were apparent. Living or fixed cells in close proximity to fluorescent debris were excluded but represent usually <1% of all data points. Daughter pairs with extreme sister ratio were inspected manually and excluded in case of apparent imaging artefacts (close to image edge, debris, cell clumps, out of focus etc.). Only daughter pairs which could be fully and reliably tracked and quantified in all channel and did not undergo cell death were used for analysis (= pre-established exclusion criteria).
Replication	Reproducibility of results was ensured by repeating replicates of the same experiments independently from each other. This includes conducting individual replicates of the same experiment at different days as well as preparing separate reagents etc. All results are reproducible and have been validated by independent techniques (i.e. immunofluorescence of mitotic cells and live cell imaging of mitotic cells).
Randomization	Multiple mice were pooled randomly per replicate to reduce potential biological variability per replicate of individual experiments.
Blinding	The investigators were not blinded to allocation during experiments and outcome assessment. Blinding was not required since we did not compare different treatments. Instead we analysed the cellular heterogeneity of one HSC culture condition using unsupervised computational approach like clustering.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology
☐	☒ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☐	☒ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used

antibody company clone LOT catalog concentration Validated species Validated application
anti-CD34-eFluor450 eBioscience RAM34 4329944 48-0341-82 20 µg/mL Fish, Goat, Hamster, Mouse IHC, IF, Flow Cyt
anti-Sca1-PerCP-Cy5.5 eBioscience D7 1931039 45-5981-82 5 µg/mL Fish, Mouse Flow Cyt
anti-CD48-APC eBioscience HM48-1 E07165-1633 17-0481-82 5 µg/mL Fish, Human, Mouse Flow Cyt
anti-cKit-PE-Cy7 eBioscience 2B8 E07592-1634 25-1171-82 5 µg/mL Fish, Mouse, Pig Flow Cyt, IF
streptavidin-APC-eFluor780 eBioscience na E10200-1633 47-4317-82 5 µg/mL Species independent Flow Cyt
anti-CD135-PE-CF594 BD A2F10.1 8156535 562537 5 µg/mL Mouse Flow Cyt
anti-CD150-PE Biolegend TC15-12F12.2 B256074 115904 5 µg/mL Mouse Flow Cyt, IP
anti-CD48-PE eBioscience HM48-1 E01275-1634 12-0481-83 20 ng/mL Fish, Human, Mouse Flow Cyt
anti-CD48-APC eBioscience HM48-1 E07165-1633 17-0481-82 20 ng/mL Fish, Human, Mouse Flow Cyt
anti-CD71-PE eBioscience RI7 217.1.4 E01339-1632 12-0711-81 20 ng/mL Fish, Human, Mouse Flow Cyt
anti-CD71-APC eBioscience RI7 217.1.4 E11016-1635 17-0711-82 20 ng/mL Fish, Human, Mouse Flow Cyt

anti-Sca1-Alexa Fluor® 488 Biolegend D7 B225713 108116 50 ng/mL Mouse Flow Cyt, IP, WB, IHC, IF
anti-CD105-Alexa Fluor® 488 BioLegend MJ7/18 B228680 120405 50 ng/mL Mouse Flow Cyt, IP, WB, IHC, IF
anti-CD41-PE BD MWReg20 4337549 558040 20 ng/mL Mouse Flow Cyt
anti-CD41-APC eBioscience MWReg20 E10381-1631 17-0411-82 20 ng/mL Human, Mouse Flow Cyt
anti-NOTCH1-PE eBioscience 22E5 4277731 12-5765-80 20 ng/mL Mouse Flow Cyt
anti-CD16/32-BV480 BD 2.4G2 746324 746324 40 ng/mL Mouse Flow Cyt
mouse anti-NUMB Santa Cruz 48 D2711 sc-136554 4 µg/mL Mouse ICC-IF, IHC-P, IP, and WB
rabbit anti-PARD3b Santa Cruz F-12 J1014 sc-398887 4 µg/mL Mouse IF
"anti-TOMM20-Alexa Fluor488" abcam EPR15581 GR306988-4 ab205486 4 µg/mL Human, predicted Mouse, Rat ICC/IF
anti-TOMM20-Alexa Fluor555 abcam EPR15581-44 GR3177327-1 ab221292 4 µg/mL Mouse, Human ICC/IF
"anti-LC3β-Alexa Fluor488" abcam EPR18709 GR3239743-1 ab225382 4 µg/mL Human, predicted Mouse, Rat ICC/IF
anti-cMYC-Alexa Fluor488 Santa Cruz 9E10 H2916 sc40 2 µg/mL Mouse, Rat, Human, Cat, Monkey ELISA, FC/FACS, IF, IHC, IHC-P, IP, and WB
mouse anti-mCherry abcam na GR144686-1 ab167453 5 µg/mL Species independent WB, IHC, ICC/IF. Cited in 71 publication(s). Independently reviewed in 12 review(s)
rabbit anti-NUMB abcam na na ab14140 20 µg/mL Mouse, Rat, Human, Zebrafish IHC-P, ICC/IF, IHC-Fr, WB, IP, IHC
goat anti-NUMB abcam na GR257559-4 ab4147 20 µg/mL Mouse, Rat, Chicken, Human IHC-P, WB, IP, ICC/IF
goat anti-NUMB Santa Cruz P-20 I1604 sc-15590 4 µg/mL Mouse IF
rabbit anti-NUMB Santa Cruz H-70 C0111 sc25668 4 µg/mL Mouse IF
Anti-alpha Adaptin antibody [AP6] abcam AP6 na ab2730 2.5 µg/mL Mouse, Rat, Hamster, Cow, Human, Drosophila melanogaster ICC/IF, ICC, Inhibition Assay, Electron Microscopy, ELISA, Blocking, IP, WB, IHC-FoFr, Flow Cyt
rabbit anti-LAMP2 abcam GL2A7 GR143671-2 ab37024 5 µg/mL Mouse, Rabbit. Cited in 72 publication(s). Independently reviewed in 12 review(s). WB, IP, IHC, ICC, Flow Cyt, ICC/IF
anti-α-Tubulin-Alexa Fluor488 Life Technologies B-5-1-2 QA213137 322588 1 µg/mL Human, Mouse, Rat IF, ICC, WB, IHC
rabbit anti-α-Tubulin-Alexa Fluor647 Cell Signaling 11H10 8 5046 100 ng/mL Mouse, Human, Rat, Monkey, Drosophila, Zebrafish, Pig IF
"chicken anti-GFP" Aves Lab na na GFP-1010 10 µg/mL Species independent ELISA, ICC, IHC, WB
donkey anti-rabbit-Alexa Fluor488 Invitrogen na 1674651 A21206 10 µg/mL rabbit IF, ICC, IHN, Flow Cyt
donkey anti-rabbit-Alexa Fluor555 Invitrogen na 1736966 A31572 10 µg/mL rabbit IF, ICC, IHN, Flow Cyt
donkey anti-rabbit-Alexa Fluor647 Invitrogen na 1903516 A31573 10 µg/mL rabbit IF, ICC, IHN, Flow Cyt
donkey anti-mouse-Alexa Fluor488 Invitrogen na 1113537 A21202 10 µg/mL rabbit IF, ICC, IHN, Flow Cyt
donkey anti-mouse-Alexa Fluor555 Invitrogen na 1736967 A31570 10 µg/mL rabbit IF, ICC, IHN, Flow Cyt
donkey anti-mouse-Alexa Fluor647 Invitrogen na 1549801 A31571 10 µg/mL rabbit IF, ICC, IHN, Flow Cyt
donkey anti-goat-Alexa Fluor488 Invitrogen na 1463163 A11055 10 µg/mL rabbit IF, ICC, IHN, Flow Cyt
donkey anti-goat-Alexa Fluor555 Invitrogen na 1620248 A21432 10 µg/mL rabbit IF, ICC, IHN, Flow Cyt
donkey anti-goat-Alexa Fluor647 Invitrogen na 1739289 A21447 10 µg/mL rabbit IF, ICC, IHN, Flow Cyt
anti-CD43-biotin eBioscience na 4298081 13-0431-85 10 µg/mL Mouse Flow Cyt
anti-CD3ε-biotin eBioscience 145-2C11 4308809 13-0031-85 10 µg/mL Human, Mouse Flow Cyt, IHC, IF, IP, WB
anti-CD19-biotin eBioscience eBio1D3 1942197 13-0191-86 10 µg/mL Mouse Flow Cyt
anti-TER-119-biotin eBioscience TER-119 4300555 13-5921-85 10 µg/mL Fish, Human, Mouse Flow Cyt, IHC, IF
anti-B220-biotin eBioscience RA3-6B2 1930460 13-0452-86 10 µg/mL Fish, Human, Mouse Flow Cyt, IHC, IF
anti-Ly-6G-biotin eBioscience RB6-8C5 4340075 13-5931-85 10 µg/mL Dog, Fish, Mouse Flow Cyt, IHC, IF, IP
anti-CD11b-biotin eBioscience M1/70 1937241 13-0112-85 10 µg/mL Fish, Human, Mouse Flow Cyt, IHC, IF, WB
anti-F4/80-PE-eFluor610 eBioscience BM8 1911636 61-4801-80 500 ng/mL Fish, Hamster, Human, Mouse Flow Cyt, IHC
anti-Ly6G-BrilliantViolet570 BioLegend Gr-1 B261199 108431 500 ng/mL Mouse Flow Cyt
anti-CD71-FITC eBioscience RI7 217.1.4 4281163 11-0711-81 1.25 µg/mL Fish, Mouse, Rat Flow Cyt
anti-CD16/32-BrilliantViolet421 BioLegend 93 B238223 101331 500 ng/mL Mouse Flow Cyt
anti-Ter119-PE-Cy7 eBioscience TER-119 E0746-1634 25-5921-82 250 ng/mL Dog, Fish, Mouse Flow Cyt
anti-Gr1- PE-Cy7 eBioscience Ly6G E07647-1635 25-5931-81 500 ng/mL Fish, Human, Mouse Flow Cyt, IHC
anti-CD11b-eFluor450 eBioscience M1/70 E10253-1633 48-0112-82 500 ng/mL Fish, Human, Mouse Flow Cyt, IF, IHC
anti-CD16/32- PerCP-Cy5.5 Biolegend 93 B250025 101324 500 ng/mL Fish, Fruit fly, Mouse Flow Cyt
anti-Ter119-PE eBioscience TER-119 E01922-1631 12-5921-81 250 ng/mL Fish, Human, Mouse Flow Cyt
anti-c-KIT-APC-eFluor780 eBioscience 2B8 E10200-1633 47-1171-82 500 ng/mL Fish, Human, Mouse, Pig Flow Cyt

Validation

All antibodies used were titrated and tested extensively on freshly isolated mouse bone marrow. Isotype and/or secondary only staining controls were used. Staining pattern was compared to existing literature if available and confirmed by using several commercially available antibodies clones from different companies. Details of antibodies including species and validated / reported applications from the manufactures are can be found in material and methods and are listed above and is Supplementary Table 1.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)

OP9 were a gift from Shin-Ichi Nishikawa, 7F2 (ATCC® CRL-12557™) purchased from ATCC, HEK293T and NIH 3T3

Authentication	cell lines were not authenticated
Mycoplasma contamination	All cell lines were tested negative for mycoplasma contamination.
Commonly misidentified lines (See ICLAC register)	HEK and 3T3 lines are listed. These lines were used solely for Virus production and titration. The conclusion drawn here, do not rely on these cell lines.

Animals and other organisms

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

Laboratory animals	12-16 week old, male C57BL/6J and B6;129-Myctm1Slek/J (GFP-c-MYC KI)
Wild animals	No wild animals were used in this study.
Field-collected samples	No samples were collected from the field
Ethics oversight	Experiments were conducted with 12-16 week old, male C57BL/6J from Janvier Labs or B6;129-Myctm1Slek/J (GFP-c-MYC KI) purchased from The Jackson Laboratory when indicated according to Swiss federal law and institutional guidelines of ETH Zurich, approved by local animal ethics committee Basel-Stadt (approval number 2655) and Regierung von Oberbayern (AZ55.1-2-54-2531-59-08)

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:

☒ 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	femurs, tibiae, coxae, humeri, ulnae and vertebrae were isolated and crushed in PBS (2% FCS, 2mM EDTA) and filtered through a 100 µm nylon mesh. Erythrocytes were lysed for 3 min on ice in ACK lysis buffer (Lonza), stained with biotinylated lineage antibodies against CD3e (145-2C11), CD19 (eBio1D3), TER-119 (TER-119), B220 (RA3-6B2), Ly-6G (RB6-8C5) and CD11b (M1/70), labelled with streptavidin conjugated magnetic beads (Roche). After immuno-magnetic depletion cells were stained with anti-CD34-eFluor450 (RAM34), Sca1-PerCP-Cy5.5 (D7), CD48-APC (HM48-1), cKit-PE-Cy7 (2B8), streptavidin-APCeFluor780 (all eBioscience), CD135-PE-CF594 (A2F10.1; BD) and CD150-PE (TC15-12F12.2; Biolegend) for 90 minutes on ice and sorted using BD FACS Aria I or III with 70 um nozzle, single cell purity mode and sorting purities ≥98%.
Instrument	BD FACS Aria I-III and BD Fortessa
Software	BD FACS Aria I-III and BD Fortessa for data acquistion. Software used for analysis BD FACS Diva and FlowJo v10, BD FACS Aria I-III and BD Fortessa for data acquisition. Software used for analysis BD FACS Diva and FlowJo v10
Cell population abundance	50% of phenotypically isolated hematopoietic stem cells are functional HSCs. Optimal settings prior to sort were determined by reanalysis of cKIT+Lineage- population and was usually >98%. Due to low HSC numbers post sort purities were estimated based in parallel sorted more abundant surrogate population if applicable.
Gating strategy	Gating strategy was done according to previous published reports and included: Commonly used FSC/SSC gates for leukocyte isolation, doublet exclusion and common landmarks to isolate indicated hematopoietic stem and progenitor populations. Since common landmark gating depends on relative marker expression levels between populations instead of positive versus negative gates of unstained controls were used to determine gates for HSPC sorts. In all other flow cytometry related end point analysis (i.e. colony assays), unstained controls were included and gates set based on a false positive rate of <1%.

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