SUPPLEMENTARY APPENDIX

Aging of human hematopoietic stem cells is linked to changes in Cdc42 activity

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Supplemental Methods

Primary cells: Low-density mononuclear cell fraction (<1.077 g/cm^3) was harvested by centrifugation on Ficoll gradient (#07801, StemCell Technologies) and cryopreserved in freezing medium (# PB-044-50, PeloBiotech). Additional details of BM donor age used in the study are shown in Table S1.

Table S1: The number of donor BM samples within specified age groups

Cohort	Age	Number of donors
Young	20-25	6
	26-30	16
	31-35	6
	36-40	2
Aged	56-60	5
	61-65	18
	66-70	17
	71-75	8
	76-80	5
	81-85	4

Flow cytometric analysis and cell sorting: MNCs were thawed and stained at a density of 10x10^6 per ml in PBS supplemented with 3% FBS with human specific antibodies. Antibodies used were: Fitc-conjugated anti-human lineage cocktail ((# 22-7778-72: anti-CD2 (RPA2.10), anti-CD3 (OKT3), anti-CD14 (61D3), anti-CD16 (CB16), anti-CD19 (HIB19), anti-CD56 (TULY56), anti-CD235a (HIR2)) from eBioscience, APC-conjugated anti-human CD34 (581 # 555824), PE-conjugated anti-human CD38 (HIT2 # 555460), PE Cy7-conjugated anti-human CD90 (5E10 # 561558), PerCP Cy5.5-conjugated anti-human CD45ra (HI100 # 563429) all from BD Pharmingen.

Single cell division assay: M4 media was prepared using serum-free expansion media (SFEM, #09560) supplemented with 100 ng/ ml stem cell factor (SCF), 100 ng/ ml thrombopoietin (TPO), 50 ng/ ml interleukin-6 (IL-6), 10 ng/ ml interleukin-3 (IL-3), 20 ng/ ml Fms-like tyrosine kinase 3/ fetal liver kinase 2 (Flt3/Flk2) ligand. M5 media consisted of SFEM supplemented with 100 ng/ ml SCF, 50 ng/ ml TPO and 100 ng/ ml Flt3/Flk2 ligand. Media and cytokines were all obtained from StemCell Technologies. Cells were incubated at 37°C under hypoxic (3% oxygen) or normoxic (21% oxygen) conditions and checked every 12 hours under a light microscope for division.

For CASIN treatment, cells were incubated in SFEM, 3% oxygen for 1 hour at 37°C and subsequently in media ± CASIN or DMSO (controls) for 4 hours. Cells were washed with

PBS/3%FBS, incubated with antibodies and single cells collected in M4 media. Plates were incubated under 3% oxygen for 7 days at 37°C and checked every 12 hours for division.

Colony Forming Unit assay: The myeloid and erythroid generative potential of young, aged and CASIN-treated aged cohorts were assessed on methylcellulose medium (#HSC003, R&D Systems). 200 HSCs were sorted and pre-treated or not with CASIN (as described) prior to seeding. Plates were incubated at 37°C, 21% oxygen and colonies scored based on morphology on day 14.

Cell cycle: Staining and analysis was performed on an LSR Fortessa SORP with Hoechst and Ki-67 antibody. Cells were pre-stained with the following antibodies: Fitc-conjugated anti-human lineage cocktail (# 22-7778-72) from eBioscience, CD45RA-conjugated Fitc (L48 # 335039), PE-conjugated mouse anti-human CD38 (HIT2 # 555460) from BD Biosciences, PE-Cy7-conjugated anti-human CD34 (581 # 343516), alexa fluor 700-conjugated anti-human CD90 (5E10 # 328120) from Biolegend. Subsequently, cells were fixed and permeabilised on ice for 30 minutes with BD Cytofix/Cytoperm (# 554722), washed and incubated with alexa fluor 647-conjugated mouse anti-Ki 67 (B56 # 561126) from BD BioSciences. Prior to flow, cells were incubated with 1 μl Hoechst 33342 solution (# 62249) from ThermoFisher Scientific.

Immunofluorescent staining: Cells were seeded in serum-free expansion medium (SFEM), incubated for 2 hours and fixed as previously described to assess polarity in (Florian et al., 2012).[9] Cells were categorized exclusively with respect to the protein of interest such that a cell could be polar for Cdc42 and apolar for tubulin. The frequency of cells was then quantified in direct reference to the specific protein. Primary antibodies used were: mouse-anti-Cdc42 (M152 # ab41429, Abcam) and rat-anti-tubulin (YL1/2 # ab6160, Abcam). Secondary antibodies used were from Jackson ImmunoResearch: donkey anti-mouse IgG conjugated to alexa fluor 594 (# 715-585-150) or alexa fluor 488 (# 715-545-150) and donkey anti-rat IgG conjugated to alexa fluor 647 (# 712-605-153), alexa fluor 594 (# 715-475-151) or alexa fluor 488 (# 712-545-153).
In quantifying fluorescence intensity of Cdc42-GTP, an antibody specific for the active form of Cdc42 was utilized (Althoff et al., 2020).[22] HSCs were seeded and fixed as described and incubated with mouse-anti-Cdc42-GTP (# 26905, NewEast Biosciences) overnight. Cells were subsequently incubated with donkey anti-mouse IgG conjugated to alexa fluor 594 and mounted onto microscope slides. Imaging was done with the confocal laser scanning microscope LSM 710. Mean fluorescence intensity was obtained automatically after manually outlining cells.

Xeno-transplantation: 500 HSCs were injected via the tail vein into 8 to10-week-old non-conditioned or low dose irradiated (1.6 Gy) NBSGW mice. 8- and 12-weeks post-transplant, aspirates were drawn from the BM of mice after administering anesthesia. Human cells were identified using these antibodies: VioBlue-conjugated anti-human CD45 (5B1 # 130-113-684) and APC-Vio 770-conjugated anti-human CD3 (BW264/56 # 130-113-126) from Miltenyi, alexa fluor 647-conjugated anti-human CD19 (HIB19 # 302220) from Biolegend, Fitc-conjugated rat anti-mouse CD45 (30-F11 # 553080) and PE-conjugated anti-human CD33 (WM53 # 555450) from BD Biosciences.

Western blot: Equal numbers of LDBM cells were collected into tubes for lysis to determine relative Cdc42 activity. A rac/cdc42 assay reagent (# 14-325, Millipore) was used in pull down

assays according to the manufacturer's protocol. Samples were boiled at 95°C for 10 minutes and separated on 12% reducing SDS-PAGE at 110 V. Membranes were blocked with 3% BSA/0.1% PBS-T (BSA, Sigma-Aldrich; Tween-20, Sigma-Aldrich) and incubated with primary antibodies overnight at 4°C (Rabbit anti-Cdc42 # 07-1466, Millipore; Mouse anti-GAPDH # TA802519, OriGene Technologies). After washing, membranes were incubated with secondary antibodies conjugated to horse-radish peroxidase (Goat anti-rabbit IgG conjugated to HRP # 4030-05; Goat anti-mouse IgG conjugated to HRP # 1015-05; both purchased from Southern Biotech) and developed using chemilluminescent substrate (SuperSignal West Femto Chemilluminescent substrate # 34096, ThermoFisher) on ChemiDoc™ MP Imaging System (Bio-Rad Laboratories).
To quantify the relative Cdc42 activity, the ratio of GTP-bound Cdc42 to the total Cdc42 (normalized to GAPDH) was determined using the Image Lab Software Version 5.1 (Bio-Rad Laboratories).

Statistical analysis: Statistical analyses were performed within GraphPad Prism 8 (version 8.1.2) and are presented as mean ± SD or mean ± SEM and box plots as minimum and maximum points. To assess the normality of our data, the Shapiro-Wilk test was used. For skewed distribution, the Mann-Whitney test was used to determine statistical significance and the Student's *t*-test with Welch's correction for normally distributed data. Curves were derived using the line of best fit for a non-linear regression curve (cumulative Gaussian-percents). To determine the slopes of the curves, linearized portions were fitted to a straight line (non-linear regression) with the elimination of outliers and *probability values* calculated using the correlation coefficients in a Z-test statistic for a two-tailed test. Regression analyses were tested for significance using Pearson, Spearman or Kendall correlation (with 95% confidence intervals) and in determining the correlation between Cdc42 activity and polarity, activity values below 0.4 were excluded. Outliers were excluded using the ROUT method. Kendall's correlation analysis was performed with R version 4.0.3, RStudio Team (2020) version 1.3.1093. RStudio: Integrated Development for R. RStudio, PBC, Boston, MA URL http://www.rstudio.com/

Supplemental Figures

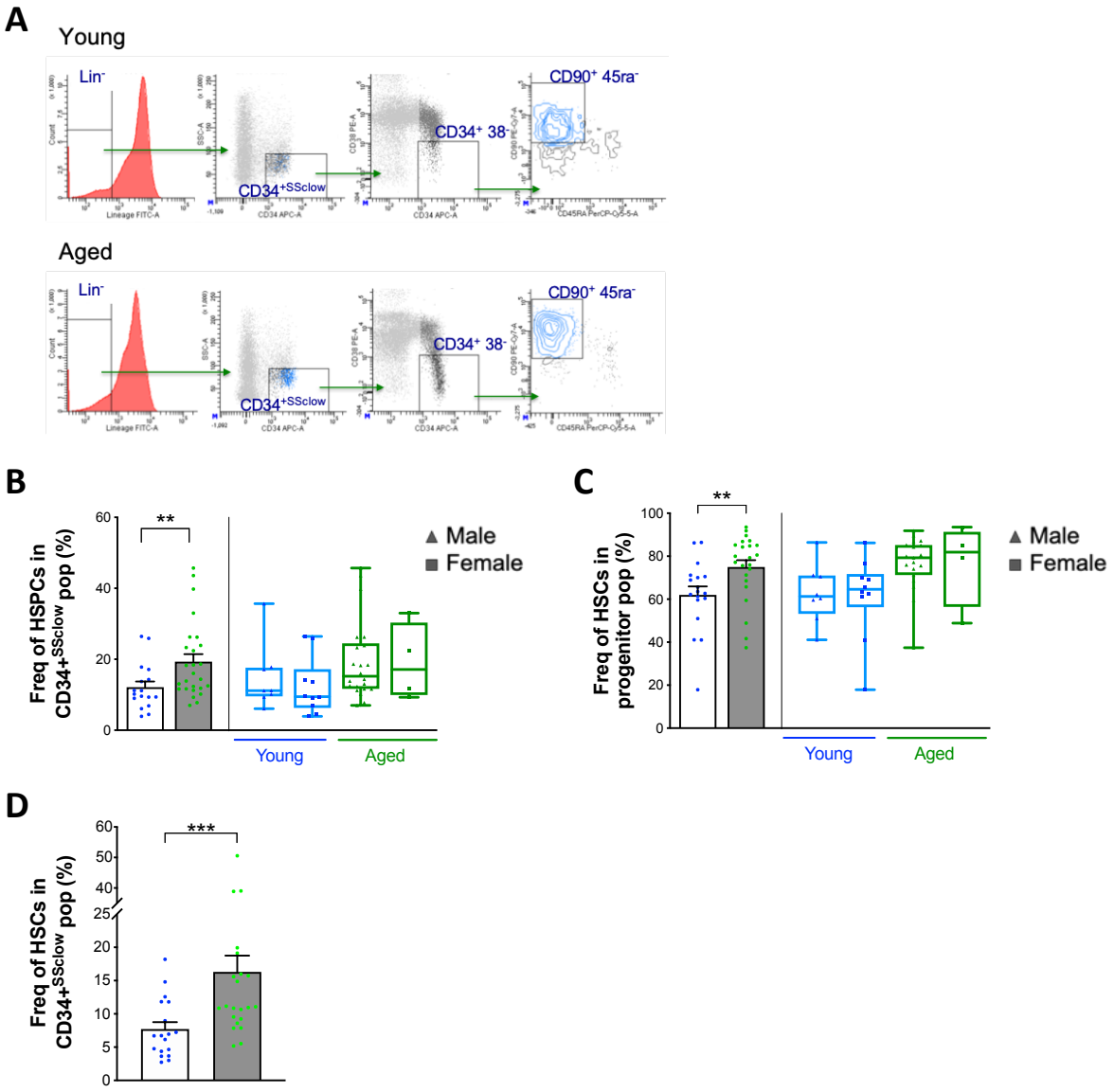

Figure S1: Flow cytometric analysis of HSCs and HSPCs. (A) Representative image shows the gating used in isolating HSCs and HSPCs in low-density mononuclear cell fraction (MNC). Frequencies of (B) HSPCs and (C) HSCs of young (blue) and aged (green) donor BM segregated according to gender. Mann-Whitney test, n_{young} = 8 males, 10 females; n_{aged} = 20-23 males, 4 females. Box plot represent minimum and maximum points. (D) Frequency of HSCs within the CD34+^{SSclow} population of young (blue) and aged (green) donors. ** p = 0.0035, Mann-Whitney test, n_{young} = 18; n_{aged} = 24. Bars = mean ± SEM. Donor age: young = 23-39 yr, median = 27 yr; aged = 58-82 yr, median = 65 yr.

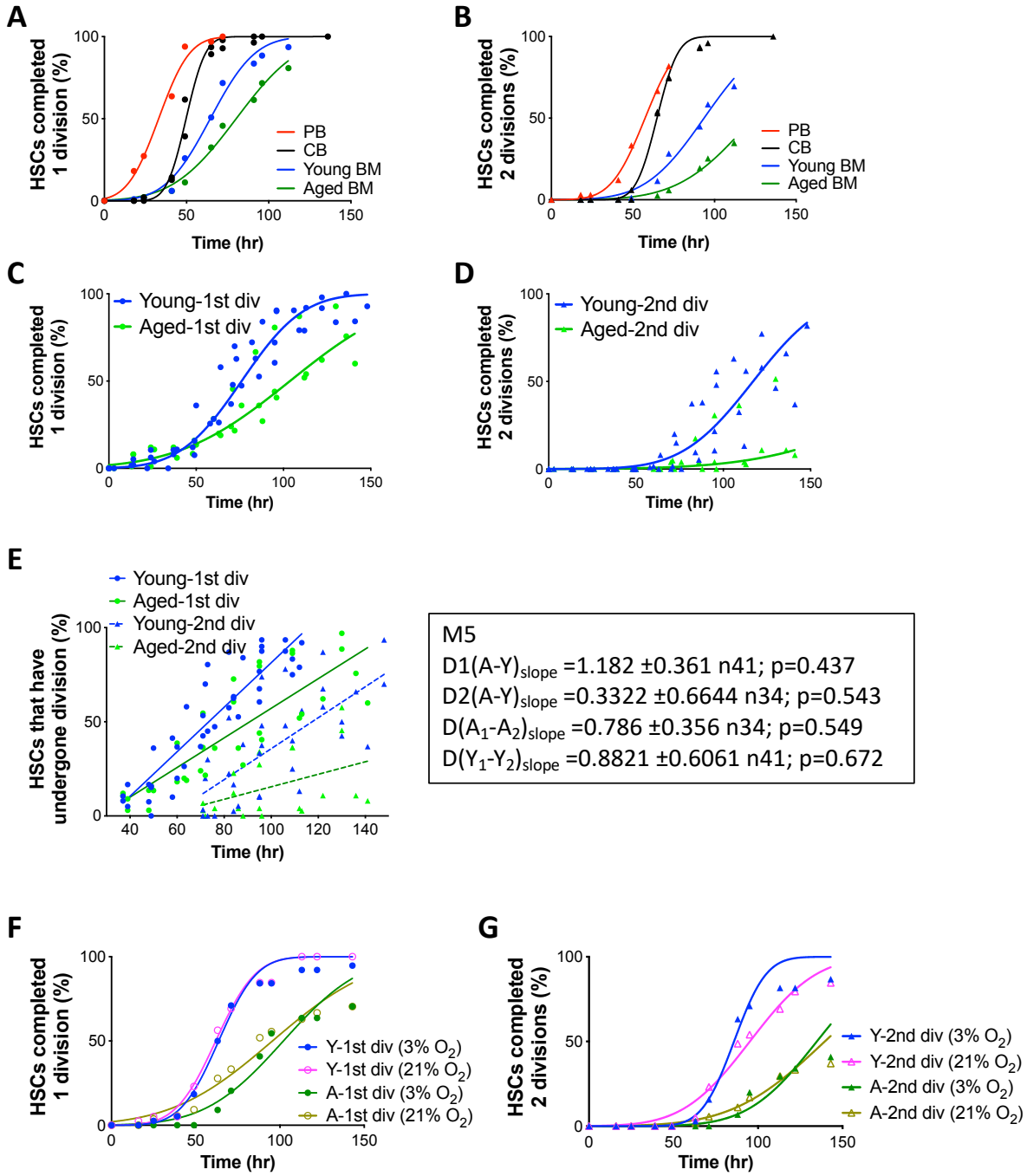

Figure S2: Kinetics of single cell division of HSCs cultured *ex vivo* in different cytokine media under different oxygen conditions. Cumulative (A) first and (B) second division of singly sorted HSCs isolated from peripheral blood (PB; red), cord blood (CB; black) and bone marrow (BM; blue and green) incubated at 37℃, 3% oxygen in M4 media. n_{PB} = 1; n_{CB} = 2; n_{BM} = 6 for blue and 4 for green. Singly sorted cells were seeded into plates containing M5 media and incubated at 37℃, 3% oxygen. Cumulative (C) first division and (D) second division of live young and aged HSCs. (E) Slope was derived as linear regression fits (left) and *probability values* calculated for the correlation coefficients (M5 calculations in the box). n_{young} = 6; n_{aged} = 4. Donor age: young = 27-39 yr, median = 28 yr; aged = 64-75 yr, median = 69 yr. Cumulative curves of cells incubated at 37℃ in M4 media, comparing kinetics under 21% oxygen (open circles/ triangles) with 3% oxygen (closed circles/ triangles). Cumulative (F) first

division and (G) second division of live young and aged HSCs. n_{young} = 1 (39 yr); n_{aged} = 2 (65, 66 yr). Each curve was derived from cumulative gaussian fits with robust regression.

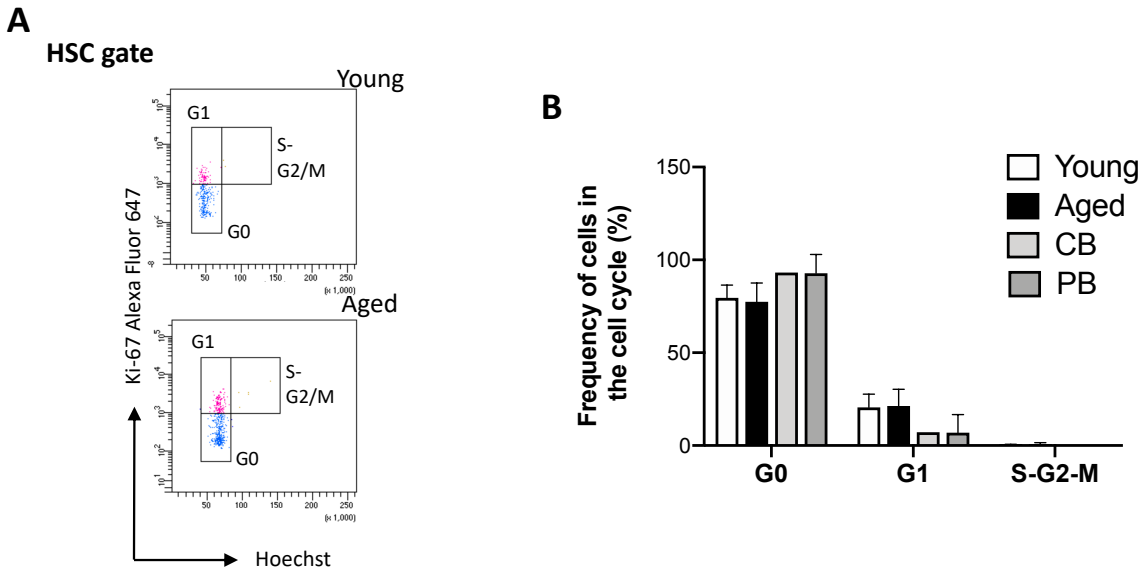

Figure S3: Gating strategy and quantification of cell cycle distribution of HSCs. (A) Representative image gated on fixed HSCs stained for DNA content and the proliferation marker Ki-67 (Hoechst, anti-Ki67 antibody). (B) Proportion of quiescent (G_0) and non-quiescent (G_1 and S-G_2M) HSCs determined in young (white = 3), aged (black = 5), cord blood (light grey = 1) and peripheral blood (dark grey = 2). Bars represent mean ± SD. Donor age: young = 24-31 yr, median = 27 yr; aged = 63-82 yr, median = 72 yr.

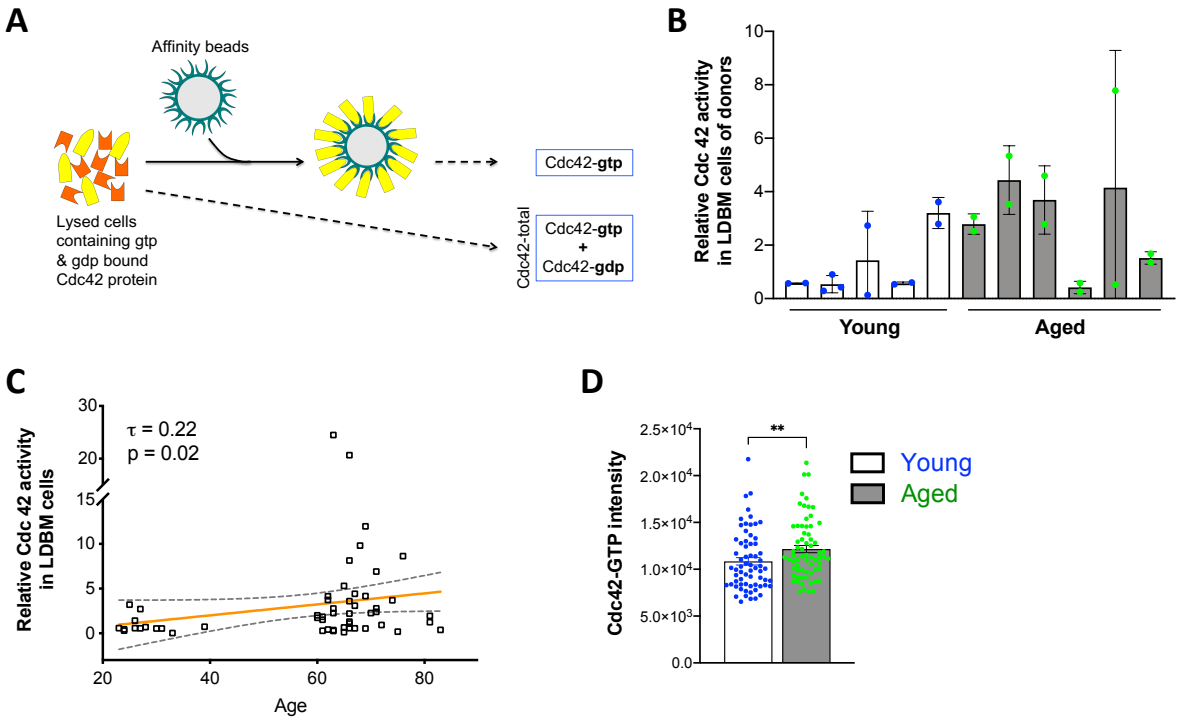

Figure S4: Cdc42 activity. (A) Schematic of the experimental design for western blot. (B) The means and standard deviation of relative Cdc42 activity of individual samples determined from western blot quantification. Young (blue dots) donor age = 23-31 yr, median = 26 yr; aged (green dots) donor age = 61-75 yr, median = 63 yr. Bars represent mean ± SD. (C) Kendall correlation analysis of relative Cdc42 activity and age (τ = 0.2, p =0.023, n = 54. Broken grey lines represent 95% CI). (D) The actual Cdc42-GTP mean fluorescence intensities of young and aged HSCs. ** p =0.0092, Mann-Whitney test. Bars = mean ± SEM. n_{young} = 66 (27-31 yr, median = 27 yr) and n_{aged} = 67 (63-76 yr, median = 76 yr).

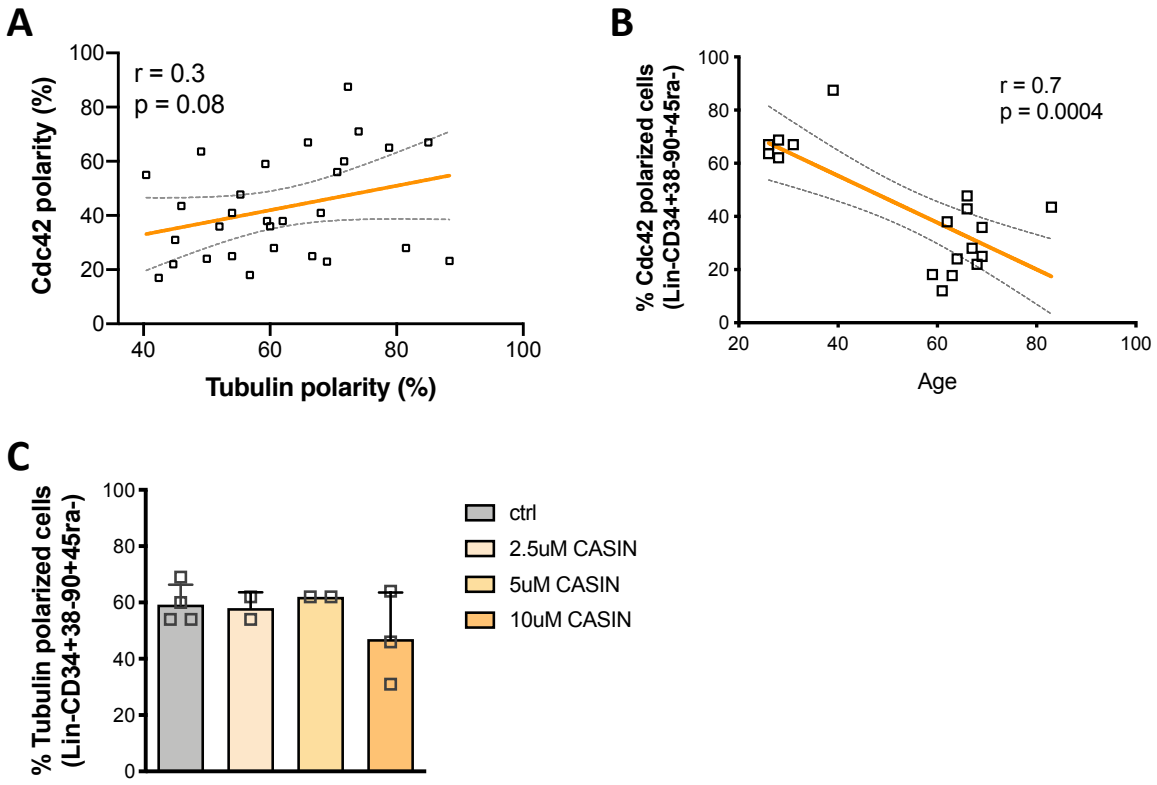

Figure S5: Polarity assessment. (A) Spearman correlation analysis of Cdc42 and tubulin polarity, r = 0.3, *p* = 0.08, n = 29 (broken grey lines represent 95% CI). (B) Correlational assessment of the percentage of HSCs polar for Cdc42 polarity and age (Pearson, r = 0.7, *p* = 0.0004, n = 18). (C) Frequency of cells repolarized with respect to tubulin upon treatment with different doses of CASIN. Bars = mean ± SD, Mann-Whitney test. n_{aged} >2. Donor age: aged = 61-81 yr, median = 62 yr.

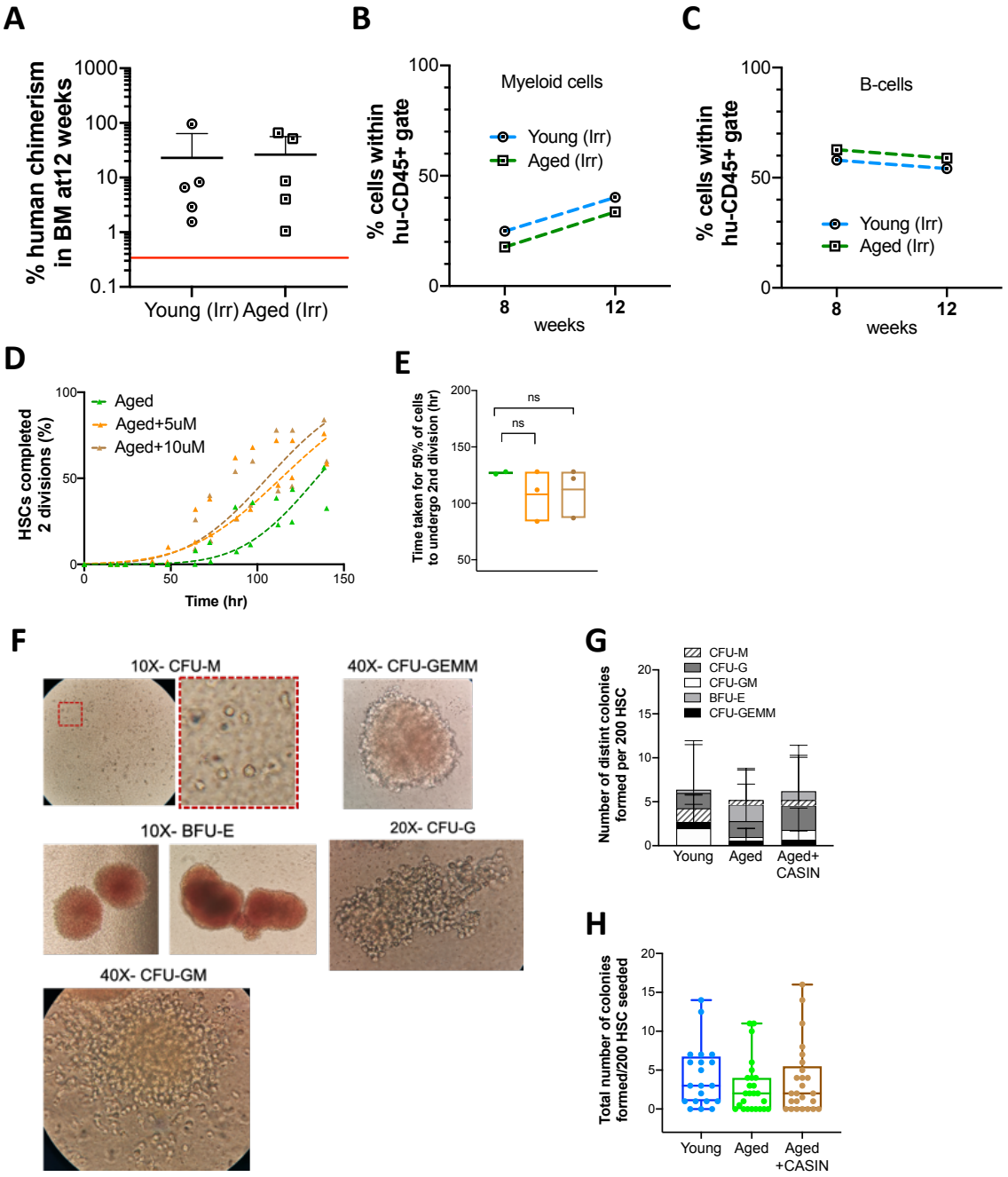

Figure S6: *In vivo* and *in vitro* functional analysis. Assessment of xenotransplantation experiment with low dose irradiation (Irr) (A) Frequency of human chimerism in the BM of NBSGW mice 12 weeks post-transplant. Emergence of (B) human myeloid cells and (C) B-cells post-transplant. Bars = mean ± SD. n_{young} = 2 (25, 27 yr) and 2 aged (63, 66 yr), 10 mice. (D) Cumulative second division of aged HSCs with and without CASIN and (E) time by which 50% of the cells have undergone second division. Curve was derived from cumulative gaussian fits with robust regression. n_{aged} and $n_{aged+CASIN}$ = 4. Donor ages: aged = 63-71 yr, median = 66 yr. *In vitro* experiments evaluating the myeloid and erythroid potential of HSCs. (F) Representative images of colonies taken under a light microscope after 14 days of culture. (G) Quantification of distinct colonies formed from young, aged and CASIN-treated aged (10 μM) HSCs after 14 days of culture under 37℃, 21% oxygen conditions. Bars = mean ±

SD. Two-way ANOVA with Tukey's multiple comparison. (H) Total number of colonies derived. Box plot represent minimum and maximum points. One-way ANOVA with Holm-Sidak's multiple comparison test. n_{young} = 4 (27-32 yr, median = 29 yr) and n_{aged} and $n_{aged+10\ \mu M\ CASIN}$ = 5 (63-77 yr, median = 71 yr).