Kinase-inactivated CDK6 preserves the long-term functionality of adult hematopoietic stem cells

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Supplementary Methods

Animals

Mice (C57BL/6N (*Cdk6*$^{+/+}$), Ly5.1^{+} [B6.SJL-Ptprca]/CD45.1^{+}), *Cdk6*$^{KM/KM}$ (*Cdk6*$^{K43M/K43M}$)[1] and *Cdk6*$^{-/-}$ [2] were bred and maintained under special pathogen-free (SPF) conditions at the Institute of Pharmacology and Toxicology, University of Veterinary Medicine Vienna, Austria. Age-matched (8-12 weeks) male and female mice were used unless indicated otherwise. All procedures and breeding were approved by the Ethics and Animal Welfare Committee of the University of Veterinary Medicine, Vienna in accordance with the University´s guidelines for Good Scientific Practice and authorized by the Austrian Federal Ministry of Education, Science and Research (BMMWF-68.205/0093-WF/V/3b/2015, 2022-0.404.452, BMMWF-68.205/0112-WF/V/3b/2016, BMBWF-68.205/0103-WF/V/3b/2015 (TP), 2023-0.108.862) in accordance with current legislation.

BM isolation and maintenance of BM derived HSC/LSK cells

BM cell suspensions were prepared from *Cdk6*$^{+/+}$, *Cdk6*$^{-/-}$ and *Cdk6*$^{KM/KM}$ mice by crushing femora, tibiae, and iliac crests in PBS. Subsequently, cell suspensions were filtered through 70 µm cell strainers (Falcon), followed by washing and centrifugation for 5 min at 250 x g and re-suspended in ice cold 2% fetal bovine serum (FBS) (Capricorn Scientific)/PBS (Sigma Aldrich). Cell numbers of the isolated BM were calculated from both hind legs throughout the manuscript. Cells expressing lineage markers were depleted using, MagniSort^{TM} mouse hematopoietic lineage depletion magnetic kit (Thermo Fischer Scientific) according to manufactures instructions, followed by staining the remaining lineage-negative cells described below. Single HSCs were sorted and cultured at 37°C and 5% CO_2 in U-bottom plates in 150 µl StemSpan SFEM (Stem Cell Technologies) and 1000 sorted LSK cells in 200 µl StemSpan SFEM medium. Both medias were supplemented with 5% heat-inactivated FBS (Capricorn

Scientific), 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), 20 ng/ml human Interleukin-11 (R&D Sytems), and SCF (generated in-house) used at 2% final concentration.

Cell culture maintenance

HPC^{LSK} cell lines generated from *Cdk6*$^{+/+}$, *Cdk6*$^{-/-}$ and *Cdk6*$^{KM/KM}$ mice were established, cultured and maintained as previously reported[3]. HPC-7 cells were maintained as described[4]. Platinum-E (plat-E) cells were cultured and maintained in DMEM medium supplemented with 10% FBS (Capricorn Scientific) and 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma-Aldrich). All cells were routinely tested for mycoplasma contamination.

Knockout, knockdown and re-expression experiments

3 clonal *Cdk6*$^{-/-}$ HPC-7 lines were generated by CRISPR technique using a guide RNA targeting the first exon of Cdk6, as previously described[5]. Briefly, $1x10^6$ HPC-7 cells were resuspended in 100 µl 1M medium (5mM KCl; 15mM MgCl2; 120mM Na2HPO4/NaH2PO4 pH7.2; 50mM Manitol), mixed with 1µg control plasmid pSpCas9(BB)-2A-GFP(px458) or the plasmid expressing sgRNAs against Cdk6 pSpCas9(BB)-2A-GFP(px458)- hCdk6del, then transferred to 0.2 cm cuvette (VWR). Cells were electroporated using the program X-001 of Lonza® Nucleofector® II electroporation system. Transfected cells were cultured for 24h and GFP^+ cells were sorted using a FACSAria^{TM} II cell sorter (BD Biosciences, San Jose, CA, USA) and plated as single cell suspension into 96-well plates and checked for Cdk6 knockout by immunoblotting.

For the re-expression of Cdk6 in the *Cdk6*$^{-/-}$ HPC-7 cell lines HA-CDK6 and HA-$CDK6^{KM}$ pMSCV-IRES-GFP plasmids were used. Knockdown experiments were performed using shRNAs targeting NFY-A cloned into the pLENC (pMSCV-miRE-PGK-NeoR-IRES-

mCherry) vector, as previously described[6]. Briefly, Plat-E cells were separately transfected with the following retroviral vectors: pLENC-shNFYA, pLENC-shRenilla, pMSCV-CDK6-HA-IRES-GFP, pMSCV-CDK6^{KM}-HA-IRES-GFP using Turbofect (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instruction. The following day DMEM medium was changed to IMDM culture media supplemented with 5% heat-inactivated FBS (Capricorn Scientific), 100 U/ml penicillin, 100 µg/ml streptomycin and 1.5×10^{-4} M 1-thiolglycerol (Sigma-Aldrich) and incubated 24hrs [3]. Viral supernatants were harvested, filtered (0.45 µm) and supplemented with 4 µg/ml polybrene and 2% SCF for HPC-7 cells; 4 µg/ml polybrene, 2% SCF and 12.5 ng/ml IL-6 (Peprotech) for HPC^{LSK} cells. Four to seven days after transduction, mCherry positive HPC^{LSK}s and EGFP positive HPC7 cell pools were sorted with Cytoflex (Beckman Coulter).

To silence Maz, the cell penetrating Accell siRNA technology (Horizon Discovery) was used according to the manufacturer's instruction. Briefly, 2000 sorted LSK cells were targeted with 2µM non-targeting Accell siRNA or Accell SMART pool directed against *Maz* (Horizon Discovery) from 12hrs to 72hrs in IMDM medium supplemented with 2% heat-inactivated FBS (Capricorn Scientific), 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma-Aldrich), 20 ng/ml human Interleukin-11 (R&D Sytems), and 2% SCF.

Human HSPC isolation

Human HSPCs were isolated of umbilical cord blood donations at the general hospital of Vienna. For that purpose, mononuclear cells (MNCs) were extracted using Lymphoprep (StemCell Technologies, Vancouver, Kanada), red blood cells (RBCs) were depleted using ammonium chloride lysis buffer and CD34+ cells were extracted via magnetic beats cell sorting (MACS) using CD34 MicroBead Kit Ultra Pure (Miltenyi Biotec GmbH, Bergisch Gladbach,

Germany). The experimental protocols involving human cord blood samples was approved by the Ethics Committee of the Medical University of Vienna (EK1553/2014).

Flow cytometry staining, acquisition and FACS sorting

For flow cytometry sorts and analyses, BM cells or lineage-depleted BM cells were stained in 2% FBS (Capricorn Scientific)/PBS (Sigma Aldrich) for 45 min with corresponding antibodies followed by PBS washing and centrifugation for 5 min at 250 x g.

Throughout the whole study the following lineage panel was used: TER-119-APC/Cy7, CD45R-APC/Cy7, Gr-1-APC/Cy7, CD3-APC/Cy7, Mac1-APC/Cy7. For characterization of the HSC compartment under homeostatic conditions and single treated pI:pC (InvivoGen), BM-derived from $Cdk6^{+/+}$, $Cdk6^{-/-}$ and $Cdk6^{KM/KM}$ mice were stained with the lineage panel plus antibodies directed against c-Kit-PE-Cy5, Sca1-PE/Cy7, CD150-APC and CD48-PE. Following repetitive pI:pC (InvivoGen) injections, $Cdk6^{+/+}$, $Cdk6^{-/-}$ and $Cdk6^{KM/KM}$ BM cells were stained with the lineage panel antibodies and c-Kit-PE-Cy5, CD86-PE/Cy7, CD150-APC, CD48-PE.

To detect HSC/MPP1 cells after serial BM transplantation and homing assay the lineage panel together with antibodies directed against c-Kit-PE-Cy5, Sca1-PE/Cy7, CD150-APC, CD48-PE, CD34-FITC, CD45.1-FITC, CD45.2-PB were used.

KI67/DAPI cell cycle staining was achieved as previously described[7]. Briefly, for intracellular staining cells were fixed, permeabilized and stained using the BD Fixation/Permeabilization Solution Kit (BD Biosciences) according to manufacturer's instructions. Cells were stained using the lineage panel and antibodies directed against Sca1-PE/Cy7 or alternatively CD86-PE/Cy7, c-Kit-PE-Cy5, CD150-APC and CD48-PE, KI67 in combination with 2 µg/ml DAPI. Intracellular stainings using antibodies against CDK6 (Invitrogen) and MAZ (Abcam) were carried out in $Cdk6^{+/+}$, $Cdk6^{-/-}$ and $Cdk6^{KM/KM}$ BM cells as previously described[8].

Following staining cells were washed with PBS, filtered through a 70 µM filter and acquired by a flow cytometer (Cytoflex or Cytoflex S, Beckman Coulter or FACS Canto II, Becton Dickinson) or cell sorter (FACSAriaIII or Cytoflex machine equipped with 561 nm, 633 nm, 395 and 488nm lasers, Becton Dickinson) for analysis or sort, respectively. For sorting a 100 µm nozzle and a maximum sort rate of 3000 cells/second was used. Data was analysed with FACSDiva, CytExpert or FlowJo software.

Cell proliferation analysis

Single HSCs or 1000 LSK cells were sorted in one well of a U-shaped 96-well plate in several replicates and kept at 37°C, 5% CO_2 up to 10 days in StemSpan SFEM medium described above. HSC and LSK cell numbers were determined either by bright field microscopy (Olympus) for 3 days or by flow cytometry for 10 days, respectively.

Homing Assay

Lineage depleted BM cells isolated from CD45.2$^+$ *Cdk6*$^{+/+}$, *Cdk6*$^{-/-}$ and *Cdk6*$^{KM/KM}$ mice were prepared as described above. 4-6 mice were pooled and stained with the linage panel plus antibodies directed against c-Kit-PE-Cy5, Sca1-PE/Cy7. 200.000 LSK cells/mouse were *i.v.* injected into CD45.1$^+$ recipient mice. 18 hours after transplantation, recipient mice were sacrificed and homing of immune-phenotypically identified CD45.2$^+$ HSC-MPP4 was analysed with flow cytometry.

For the Palbociclib homing assay, LSK cells (isolated and immunophenotyped as above) were treated for 24 hours with either 200 nM Palbociclib or PBS in StemSpan SFEM medium (Stem Cell Technologies), supplemented with 2% SCF. After 24 hours, cells were collected, and cell number was normalized to 200.000 cells/sample and *i.v.* injected into CD45.1$^+$ recipient mice. 18 hours after transplantation, recipient mice were sacrificed and homing of immune-

phenotypically identified CD45.2$^+$ HSC-MPP4 was analysed with flow cytometry. Palbociclib (PD-0332991) was obtained from Pfizer (New York City, USA).

Competitive transplantation experiments of Palbociclib pre-treated cells

For competitive transplantation assay, 1000 CD45.2$^+$ and 1000 CD45.1$^+$ LSK cells were sorted from gender- and age-matched mice. CD45.2$^+$ LSK cells were treated with 200 nM Palbociclib (Palbo) for 72 hours and CD45.1$^+$ LSK cells with PBS (Ctrl) in SFEM medium supplemented with 2% SCF and 25 ng/ml TPO (PeproTech). Palbociclib treatment was refreshed after 48 hours. After 72 hours incubation, cells were counted and equalized to a 1:1 ratio of PBS:Palbo and injected together with 1x10^5 GFP$^+$ carrier BM cells into lethally irradiated CD45.2$^+$ recipient mice by tail vein injection. 16 weeks post transplantation BM was analysed by flow cytometry for the contribution of Ly5.1+ and Ly5.2+ cells to individual HSPC.

Competitive transplantation experiments of Cdk6$^{+/+}$ and Cdk6$^{KM/KM}$ BM cells

For 1:1 competitive transplantation of CD45.1$^+$*Cdk6*$^{+/+}$ and CD45.2$^+$*Cdk6*$^{+/+}$ or CD45.2$^+$*Cdk6*$^{KM/KM}$ BM cells were injected to lethally irradiated recipient mice and analysed, as previously described in Scheicher *et. al.*[9].

In vivo Palbociclib treatment followed by serial plating CFA

Age- and gender-matched C57BL/6N wildtype animals were randomly assigned and orally exposed to either Palbociclib (50 mg/kg once per day) or equivalent volume of PBS vehicle. After 10 days of treatment, mice were sacrificed and BM cell populations were analysed by flow cytometry. As a second analysis, 250 HSC/MPP1 cells were sorted from the PBS- or Palbociclib-treated mice and seeded in methylcellulose for serial plating assay as described below. Additionally, 1000 LSK were treated either with 2 μM non-targeting Accell siRNA or

Accell SMART pool directed against *Maz* (media described above) for 24 hours before embedding in methylcellulose for colony formation assay as described below.

Colony formation assay (CFA) and serial plating assay

Either 250 HSC/MPP1 or 1000 LSK cells were re-suspended in 100 µl IMDM (Sigma-Aldrich) medium supplemented with 20 ng /ml IL-3, IL-7, GM-CSF (R&D Sytems), IL-6 (PeproTech), 0.5% SCF, 200 ng/ml holo-transferrin, 10 µg/ml insulin (Sigma-Aldrich) and 5 U/ml erythropoietin (EPO, Johnson & Johnson). Cell suspensions were subsequently mixed with 2 ml of methylcellulose (MethoCult, Stem Cell Technologies), plated in 35 mm dishes and kept at 37°C, 5% CO2 for 7 days. Colonies were counted and collected and analysed by flow cytometry using CD71-FITC, Ter119-Violet660, CD3/CD19-PE, Gr-1-PB450, CD11b-APCCy7, Sca1-PE/Cy7, c-Kit-PE-Cy5 antibodies. 30.000 cells were used for each re-plating round up to three rounds.

Palbociclib CFA and serial plating assay

1000 sorted LSK cells from BM of wild type mice were treated either with PBS or 200 nM Palbociclib in StemSpan SFEM medium (Stem Cell Technologies) supplemented with 2% SCF. After 72 hours treatment in liquid culture, cells were plated in methylcellulose as described above for 7 days. Colonies were counted and collected and analysed by flow cytometry as described above and 30 000 cells were used for further replating rounds.

Human CFA

1000 $CD34^+$ cord blood cells were seeded either with PBS or Palbociclib [200 nM] in methylcellulose enriched with cytokines (H4435, MethoCult). 10 days after plating, colonies

and cells were counted and analysed with flow cytometry. 25 000 cells were used for further replating rounds.

Proximity ligation assay

Proximity ligation assays were performed using the Duolink® flowPLA Detection Kit – Far Red (Sigma-Aldrich) according to the manufacturer instructions. Briefly, 10.000 HSC/MPP1 cells from *Cdk6*$^{+/+}$, *Cdk6*$^{-/-}$ and *Cdk6*$^{KM/KM}$ mice were FACS-sorted using the lineage panel plus antibodies directed against c-Kit-PE-Cy5, CD86-PE/Cy7, CD150-APC, CD48-PE and fixed with 2% paraformaldehyde (Sigma-Aldrich)/PBS at 37 °C for 10 min. Cells were permeabilized with 90% ice cold methanol in PBS/2% FBS/0.2% Tween-20 (Roth/Lactan) overnight at -20 °C, followed by two washing rounds with PBS/2% FBS/0.2% Tween-20 (Roth/Lactan). Cells were incubated with the Duolink® Blocking Solution for 60 min at 37 °C, labelled with antibodies directed against CDK6 (1:250) and MAZ (1:250) on ice for 90 min and washed twice with Duolink® Wash Buffer. Following PLA probe incubation, ligation, amplification (37 °C, overnight) and detection steps (37 °C, 30 min) cells were analysed by flow cytometry.

Transwell assay

For migration analysis, a two-chamber transwell migration system (Thermo Fischer Scientific) was used. 50.000 LSK cells in 100 µl IMDM medium supplemented with 5% heat-inactivated FBS (Capricorn Scientific), 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma-Aldrich) and 2 mM L-glutamine (Sigma-Aldrich) was seeded to the upper chamber. The lower chamber contained the attractant medium of 450 µl IMDM supplemented with 2% SCF and 100ng/ml SDF-1α (R&D Systems). Cells were incubated for 4h at 37°C and 5% CO_2. Migrated cells were counted and analysed via flow cytometry using the lineage panel plus the following antibodies: c-Kit-PE-Cy5, Sca1-PE/Cy7, CD150-APC and CD48-PE.

Nuclear and cytoplasmic cell fractionation

$12x10^6$ HPC^{LSK} cells were washed with PBS and centrifuged for 5 min, 500 x g at 4°C. Cell pellets were resuspended in 800 µl buffer A (10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) pH 7.9 (Sigma-Aldrich), 0.1 mM EDTA (Carl Roth), 0.1 mM EGTA (Sigma), 2 mM DTT (Sigma), 25 mM Na-fluoride (Merck), 1 mM PMSF (Sigma), 0.4 mM Na-vanadate (Sigma), 10 mM KCl (Carl Roth), 20 µg/ml leupeptin (Sigma), 20 U/ml aprotinin (Sigma)). Samples were incubated for 15 min on ice and 10% NP-40 (0.6% final concentration) was added to the cells for 60 sec followed by a centrifugation step for 60 sec, 13.000 g, 4°C. Cytoplasmic supernatant was collected into a new 1.5 ml tube and stored at -80°C. The nuclear pellet was washed five times with 1 ml ice cold PBS and centrifuged for 1 min with 13.000 x g at 4°C. 80µl buffer B (20 mM HEPES pH 7.9, 25% glycerol, 0.4 mM Na-vanadate, 400 mM NaCl, 1 mM EGTA, 2 mM DTT, 1 mM EDTA, 25 mM Na-fluoride, 1 mM PMSF, 20 µg/ml leupeptin, 20 U/ml aprotinin) was added to the nuclear pellets. Samples were vigorously mixed on the shaker for 45 min at 4°C and centrifuged (5 min, 13.000, 4°C) afterwards. The supernatant (soluble nuclear fraction) was collected and stored at -80°C. The remaining pellet contained the chromatin residue, in which 80-100 µl Laemmli buffer (100 mM Tris/Hcl (pH 6.8) (Carl Roth), 2.5% sodium dodecyl sulfate (SDS) (Carl Roth), 10% glycerol (Carl Roth)) was added, sonicated for 15 min and mixed for 5 min at 95°C at the shaker. Protein concentrations were measured using either BCA Protein Assay Kit (Thermo Fischer Scientific) or Bradford Assay Kit (Thermo Fischer Scientific).

NFY-A immunoprecipitation

$12x10^6$ HPC^{LSK} cells per sample were washed with ice-cold PBS and lysed by snap-freezing in liquid nitrogen. Lysates were thawed on ice were pre-cleared with Protein-A/G-Agarose beads

(Thermo Fischer Scientific) in ELB buffer containing 0.1% NP-40, 50 mM HEPES (Sigma-Aldrich), 250 mM NaCl (Carl Roth), 5mM EDTA (Carl Roth) and proteinase inhibitors (Sigma-Aldrich) at 4°C for 2 hours on the rotation wheel. Protein concentration was determined with BCA Protein Assay Kit (Thermo Fischer Scientific). 300 µg protein lysates were used for further protocol procedures and input was kept as a control. 6 µg NFY-A antibody/sample was added and incubated under rotation at 4°C overnight. Next day, 40 µl Protein-A/G-Agarose beads (Thermo Fischer Scientific) per sample were prepared and pre-washed with ELB buffer. Samples were incubated for 2 hours at 4°C while rotating. Beads and supernatant were separated and 30 µg supernatant per sample was saved as a control. Beads were washed six times in ELB buffer and were eluted with 4x Laemmli buffer (100 mM Tris/Hcl (ph 6.8), 2.5% sodium dodecyl sulfate (SDS), 10% glycerol) for 10 minutes at 95°C. Eluates were loaded for immunoblot analysis as described below.

Immunoblotting

Western blot analysis was done according to standard protocols. Briefly, HPC^{LSK} cells were collected, washed with PBS and whole-cell lysates were made with 1x Laemmli buffer. Samples were incubated at 95°C for 5 min and sonicated for 15 min. Proteins were separated on a 12% SDS polyacrylamide gel and transferred to a PVDF (Millipore) membrane. Following antibodies were used for immunoblotting all at 1:1000 dilution: CDK6, NFY-A, GAPDH, RCC1, Lamin B1, HSC-70 and HSP-90. RCC1, Lamin B1 and HSP-90 served as fractionation controls, while GAPDH and HSC-70 as loading controls. For secondary antibodies, anti-mouse or anti-rabbit HRP conjugated antibodies were used. Chemiluminescent visualization was done with the ChemiDoc™ Touch imaging system (BioRad) after incubation of the membranes with Clarity Western ECL (BioRad) reagents.

Nuclear co-IP, liquid chromatography, and LC-MS/MS mass spectrometry of HPC-7 cell lines

$60x10^6$ *Cdk6*$^{-/-}$ HPC-7[4,9] cells re-expressing either HA-CDK6 or kinase-inactive HA-CDK6-K43M were used (see above) (two cell lines/genotype in duplicates). Nuclear and cytoplasmic fractions were collected as previously described[10]. Chromatin pellet was re-suspended in 200 µl benzonase buffer (20 mM Tris HCl (pH 8.0), 20 mM NaCl, 2 mM MgCl2, 200 U benzonase and proteinase inhibitors (Sigma-Aldrich)). Samples were incubated for 15 min at 4°C on the shaker and centrifuged 5 min 13000g at 4°C. Supernatant was collected which contained the chromatin fraction. Nucleoplasm and chromatin fraction were combined for protein measurement using BCA Protein Assay Kit (Thermo Fischer Scientific). 600 µg protein was used for overnight immunoprecipitation of HA-tagged CDK6[6].

After washing of the beads four times with 200 µl 100 mM TEAB (Sigma), beads were dissolved in 50 µl 100 mM TEAB. For reduction 2.6 µl of 100 mM TCEP (Roth) in 100 mM TEAB (30 min 37°C) were added, followed by alkylation with 2.1 µl of 400 mM IAA (Sigma) in 100 mM TEAB (30 min 25°C). Finally, remaining IAA was quenched with 2.9 µl 100 mM TCEP in 100 mM TEAB (10 min 37°C).

For tryptic digest 2 µl of Trypsin/LysC Mix (Promega, Madison, WI) were added. Digest proceeded for 14 hours at 37°C, followed by cool-down to 4°C. Digested peptides were removed using a magnetic rack and transferred to a fresh tube. Beads were washed twice with 50 µl 100 mM TEAB. The three portions of extracted peptides were combined, and digest was stopped by addition of 0.8 µl conc. TFA (Fisher Scientific). Before LC-MS analysis peptide extracts were desalted and cleaned up using C18 spin tips according to the manufacturers protocol. The digested protein sample was dissolved in 24 µl 0.1% TFA and 6 µl were injected to the LC-MS system. Two technical replicates were analysed using a nanoRSLC-nESI-QExactive-Orbitrap HF MS/MS system (Thermo Fisher Scientific)[11].

Raw data evaluation was performed with Proteome Discoverer software (version 2.4.1.15, Thermo Fisher Scientific). For database search a combination of the UniProt mouse database (taxonomy 10090, download March 2023, www.uniprot.org) and a common contaminant database (https://www.thegpm.org/crap/, accessed on 25 June 2019) was used. The sequence for the K43M mutant of CDK6 was included in a custom-made database. Search parameters were applied as follows: enzyme trypsin (full); maximally 2 missed cleavages; 10 ppm precursor mass tolerance and 0.02 Da fragment mass tolerance; dynamic modifications allowed were oxidation/+15.995 Da (M)/deamidation/+0.984 Da (N, Q)/Gln->pyro-Glu/−17.027 Da (Q) and static modification carbamidomethylation/+57.021 Da (C).

For intensity-based label-free quantification, protein abundance raw values were extracted in Proteome Discoverer software (Thermo Fisher) followed by normalization to total area sums and aggregation of protein abundances of technical replicates by the mean. After filtering and exclusion of proteins with one or two missing values per group, we calculated mean log2 foldchanges of normalised abundance values for CDK6 vs $CDK6^{-/-}$ and for $CDK6^{KM}$ vs $CDK6^{-/-}$ samples.

Chromatin Immunoprecipitation (ChIP) quantitative PCR

$1.5x10^7$ HPC^{LSK} cells were collected, crosslinked and sonicated as described previously[8]. Following steps were performed differently: Sheared chromatin was incubated either with 8 µg anti-MAZ or anti-IgG (as control) antibodies at 4 °C overnight. For Selected pulled down genomic regions were quantified as described in [10]. Primer sequences for ChIP qPCR are listed in supplementary resource table.

Transcriptomic analysis

Low-input RNA-Seq of transplanted HSC/MPP1 cells

We sorted 100 cells into a 96 well plate equipped with 4 µl lysis buffer following the protocol for Smart-seq2 library preparation as described previously[12]. Lysed cells were frozen at -80°C until further processing following the Smart-seq2 workflow. cDNA was amplified in a PCR reaction with 18cycles. 0,1ng of cDNA were subjected to library preparation. cDNA and final libraries were quality checked using D1000 and D5000 screen tapes (Agilent Technologies) on an Agilent 4200 Tapestation instrument. DNA concentration was determined using a Qubit Fluorometer (ThermoFisher/Invitrogen). Pooled libraries were sequenced on an Illumina Hi-seq4000 instrument using the 50bp single end configuration. Samples were sequenced at the Biomedical Sequencing Facility (BSF), CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences in Vienna, Austria.

Read alignment and read counting. Sequencing reads were quality controlled using the FastQC software (version 0.11.6)[13]. Adapter trimming and read filtering was performed using trimmomatic (version 0.36)[14]. The quality-controlled and filtered reads were then aligned against the mouse reference genome sequence (GENCODE version M13, primary assembly)[15] using STAR version 2.5.2b[16] with default parameters. Reads overlapping genes in the GENCODE M13 comprehensive gene annotation on the primary assembly were counted using the FeatureCounts function of the Subread package (version 1.5.1)[17].

Differential gene expression analysis. The R package DESeq2 was used for differential gene expression analysis. Genes with very low expression levels (less than 10 counts across all samples) were excluded from the analysis. For the remaining genes, gene expression was modeled as a function of genotype (factor-encoded as "wt" = $Cdk6^{+/+,}$ "km" = $Cdk6^{KM/KM}$, and "ko" = $Cdk6^{KO/KO}$ with "wt" as the reference level). Results for the three pairwise comparisons between the genotypes were extracted using the "results" function with the "contrast" argument set to the respective contrasts.

*Gene set enrichment analysis***.** A "quiescent stem cell gene set" and an "activated stem cell gene set" were generated from data provided in Cabezas-Wallscheid *et al*, Cell Stem Cell 2014.[18] Table S2 from the publication was downloaded from https://www.cell.com/cms/10.1016/j.stem.2014.07.005/attachment/5c5b3af3-fb97-4d01-805a-1a55217a775d/mmc3.zip (last accessed 8 May 2023). The sheet termed "HSC_MPP1 to MPP4_quant_29,841" contained gene expression data for HSC, MPP1, MPP2, MPP3 and MPP4 cells. The raw read counts (columns 49 to 64) were extracted and the DeSeq2 package's "estimateSizeFactors" function and "count" function with the parameter "normalized" set to TRUE were used to generate normalized data. Table S2 also contained results from differential gene expression analyses. The comparison HSCs vs MPP1, resulted in 479 genes with an adjusted pvalue < 0.1 (column named "HSC:MPP1.padj") which were regarded as differentially expressed. The heatmap in Supplementary Figure 4A of our paper depicts the normalized and z-transformed expression levels of these 479 genes in HSC and MPP1 cells and was drawn using the "pheatmap" function from the pheatmap R package.

The 479 genes were split into genes that were upregulated in HSC vs MPP1 cells ("UP_in_HSC" = "quiescent stem cell gene set"; 277 genes) or downregulated in HSC vs MPP1 cells ("UP_in_MPP1" = "activated stem cell gene set"; 202 genes).

These two sets were used for gene set enrichment analysis (GSEA) using the pre-ranked method. First, ranked gene lists were generated from the results of each of the three differential gene expression analyses. For each ranked list, the ranking score was calculated for individual genes as *-log10(p-value) * sign(log2 fold-change).* An enrichment analysis of the "quiescent stem cell gene set" and the "activated stem cell gene set" against the three ranked gene lists was performed using default "GSEApreranked" parameters.

Single cell transcriptomic analysis – single cell RNA-sequencing (scRNA-seq)

30.000 LSK cells were FACS-purified from isolated BM and subjected to the single cell RNA-sequencing workflow using the Chromium NextGem Single Cell 5' v2 Kit solution (10X Genomics), according to the manufacturer's instructions. cDNA and final libraries were quality checked using D1000 and D5000 screen tapes (Agilent Technologies) on an Agilent 4200 Tapestation instrument. DNA concentration was determined using a Qubit Fluorometer (ThermoFisher/Invitrogen). Pooled libraries were sequenced on an Illumina Nova-seq 6000 instrument using the 75bp paired end configuration. Raw sequencing data were processed with the Cell Ranger v7.0.0 software (10x Genomics) for demultiplexing and alignment to the GRCmm10 mouse reference transcriptome. The Seurat software package[19] was used to analyse single cell RNA sequencing data in R. *Analysis of $Cdk6^{+/+}$, $Cdk6^{KM/KM}$ and $Cdk6^{-/-}$ samples:*

Quality control and filtering. Cells with less than 200 detected features were excluded from further analysis. Next, cells that had less than 2000 or more than 17000 UMI counts in total were excluded, as were cells that had less than 0.7 % or more than 2.5 % of their UMI counts mapping to mitochondrial genes.

Data integration. After filtering, the data were normalized using the NormalizeData function and variable features were identified using the FindVariableFeatures function, both using default parameters. The samples were then integrated using the FindIntegrationAnchors (with 2000 anchors and the $Cdk6^{+/+}$ sample as a reference) and IntegrateData functions.

UMAP construction. The integrated data were scaled (ScaleData function) and a principal component analysis (PCA) was performed on the scaled data. Using the first 30 principal components, a uniform manifold approximation and projection (UMAP) was calculated.

Clustering. A k-nearest-neighbor graph was generated, based on the first 30 principal components with a k-parameter of 7, followed by calculation of a shared nearest neighbour graph (FindNeighbors function). The FindClusters function was used with a resolution parameter of 0.4 to identify clusters.

Subclustering of the HSPC cluster. The entire dataset was then subset for cells within the hematopoietic stem and progenitor cell cluster (HSPC cluster). For this subset of cells a new UMAP was constructed as described above. Clustering was performed as described above, except that the k-parameter (FindNeighbors function) was set to 12 and the resolution parameter (FindClusters function) to 1 in this analysis. Relative cluster sizes and cluster marker genes were calculated as described above.

Relative cluster sizes were calculated as the percentage of cells in a cluster relative to all cells in a sample.

Cluster marker genes were identified using the FindConservedMarkers function. The resulting lists of marker genes were used to manually annotate clusters based on published literature[20,21]. For that, clusters were annotated by finding previously described marker genes, which were amongst the top cluster-enriched genes. The full lists of the LSK and HSPC subcluster marker genes are summarized in Supplementary Table 1-2. For the LSK cells, we identified 11 individual clusters, which were annotated based on common expression of defined stem/lineage markers including: *Mecom* (stem cell), *Il7r, Dntt, Tcf7, Id2* (lymphoid), *Mcm3, Mki67, Ccnb2* (replication/cell cycle) and *Apoe, Ctsg* (myeloid). For the HSPC cluster, 6 clusters were identified and following defined marker genes were used for annotation: *Cd48* (differentiation), *Flt3, Cd69, Dntt* (lymphoid), *Mpo, Apoe* (myeloid) Atpif1, Mki67 (cycle), Irf7, Stat1 (interferon, IFN) and *Cd34, Mllt3, Pdzk1ip1, Mecom* (stem). Among the HSPC subcluster, the HSC cluster showed the nearest native dormant gene signature compared to the other subclusters.

Differential gene expression analysis. Pairwise differential gene expression analyses were performed between the three genotypes, separately for each cluster. We used the FindMarkers function with default parameters and considered only genes with an absolute log2 fold-change of at least 0.3.

"PSig" and "QSig" gene signatures.[22] The "PSig" and "QSig" gene sets were downloaded from https://journals.plos.org/plosbiology/article?id=10.1371/journal.pbio.0020301 (last accessed on 8 May 2023) as "Table S6 Genes in P-Sig" and "Table S7 Genes in Q-Sig", respectively. These gene sets were used to attribute a "PSig score" and a "QSig score" to each cell of the HSPC cluster as follows of the 338 genes in the PSig gene set, 202 were found expressed in the HSPC cluster of our single cell data. The normalized gene expression values for these 202 genes were centered and scaled for each gene across all cells and samples of the HSPC cluster. Of these normalized, centered and scaled expression values the mean expression across the 202 genes was calculated for each cell. This value constituted the PSig score for the cells. A QSig score was calculated likewise using the 156 genes of the QSig gene set (298 in total) that were expressed in our data set.

Analysis of the Palbociclib treated sample and the PBS-treated control sample:

Single cell RNA-Seq analysis for the Palbociclib treated sample and the PBS-treated control sample was performed as described above, with the following exceptions:

Quality control and filtering. Low and high filtering cutoffs for UMI counts were 2500 and 20000 counts, respectively. Low and high filtering cutoffs for the percent UMI counts mapping to mitochondrial genes were 0 % and 2.5 %, respectively.

Data integration. The reference sample for data integration was the PBS-treated control sample.

Subclustering of the HSPC cluster. The k-parameter of the FindNeighbors function was set to 10 and the resolution parameter of the FindClusters function was set to 0.3.

Cluster Marker genes. The resulting lists of marker genes were used to manually annotate clusters based on published literature[20,21]. For that, clusters were annotated by finding previously described marker genes, which were amongst the top cluster-enriched genes. The full lists of the Palbociclib treated LSK and HSPC subcluster marker genes are summarized in Supplementary Table 3-4. For the LSK cells, we identified 13 individual clusters, which were

annotated based on common expression of defined stem/lineage markers including: *Cd34, Ifitm1* (stem cell), *Ube2c*, *Hist1h2ae* (cycle), *Dntt*, *Flt3* (lymphoid), *Pf4* (megakaryocyte), *Car1*, *Gata2* (erythroid), *Elane*, *Ctsg* (neutrophil), *Cd74*, *Siglech* (dendritic), *Cpa3*, *Gzmb* (granulocyte), *Lyz2* (dendritic/macrophage), *Ccl5*, *Tcf7* (innate lymphocyte), *Rps26* (Ribosome).

For the HSPC subcluster, 4 clusters were identified and following defined marker genes were used for annotation: *Elane*, *Mpo*, *Ctsg* (Neutrophil), *Cd74* (Dendritic), *Ctsg*, *Prtn3* (Granulocyte), *Cd63*, *Cd9* (immature, naïve).

Heatmaps. Log2 fold-changes used in heatmaps were calculated using the FoldChange function of the Seurat package. Heatmaps were plotted using the pheatmap function of the pheatmap R package.

Motif enrichment analysis

Genelists (as RefSeq Ids) of significant differential expressed genes (DEG) from previous DE analysis (Split into 4 groups for analysis: Cdk6 ko vs wt downregulated, Cdk6 ko vs wt upregulated, Cdk6 K43M vs wt downregulated, Cdk6 K43M vs wt upregulated) were pasted into the web interface of Pscan (http://159.149.160.88/pscan/, Version: 1.6 (Last update: 23. Jun 2021)) for a motif enrichment analysis. The settings were selected from the web interface as followed: Selected Organism: Mus musculus, Selected Region: -450 / +50 (Distance from TSS) and selected descriptors: Jaspar_2020_NR. The resulting predictions were downloaded as lists with names of enriched motifs (sorted by p-value), z-score tables of enriched motifs vs. gene promoters (of RefSeq IDs) and the motif logo of the top 10 enriched motifs (sorted by p-value).

CDK6 ChIP-seq and MAZ ChIP-seq overlap

Bed files from MAZ ChIP-seq aligned to GRCm38/mm10 were obtained from Encode (https://www.encodeproject.org/files/ENCFF058WWE/@@download/ENCFF058WWE.bed.

478 gg).[23] MAZ peaks were overlapped with CDK6 consensus peaks from CDK6-HA ChIPseq of
479 BCR/ABL^{+} cell lines[6] using Homer (version 4.9.0). CDK6 consensus peaks represent peaks
480 which are present in at least two of three biological replicates and absent in the consensus peak
481 set of CDK6 ChIP-seq from *Cdk6*$^{-/-}$ control cell lines (*Cdk6*$^{-/-}$ consensus peak set are again peaks
482 present in at least two out of three biological replicates). Homer was also used for annotation
483 of peaks.

484 *Quantification and statistical analysis*

485 Unless otherwise indicated, data are presented as mean +/- standard error of the mean (SEM).
486 Statistical analyses were carried out either between groups or in comparison to the control
487 group. For pairwise comparisons of single variables, two-sided Mann-Whitney tests were
488 applied. Comparison of three groups or more were performed by one-way analysis of variance
489 (ANOVA) test. If the ANOVA provided evidence that group means are different, Dunn's
490 multiple comparison tests were done to determine which means amongst the set of means
491 differed from the rest. Variables that showed a skewed data distribution were Log2 transformed.
492 Statistical significance is indicated by one ($p < 0.05$), two ($p < 0.01$) or three ($p < 0.001$)
493 asterisks. Analyses were performed using GraphPad Prism 8.4b software.

494 *Data Sharing Statement*

495 The datasets are available via ArrayExpress (https://www.ebi.ac.uk/biostudies/arrayexpress)
496 with the following accession numbers:
497 Low-input RNA-seq data: E-MTAB-13145, scRNA-seq data of *Cdk6*$^{+/+}$, *Cdk6*$^{KM/KM}$ and *Cdk6*$^{-}$
498 $^{/-}$ LSK cells: E-MTAB-13149, and scRNA-seq data of Palbociclib or PBS pre-treated LSK cells:
499 E-MTAB-13268.

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501 **Resource Table**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		

Antibodies used for Immunoblotting, IP or ChIP		
NFY-A	Santa Cruz	#Sc-17753
CDK6	Invitrogen	#PA5-27978
RCC1	Santa Cruz	#55559
Lamin B1	Cell Signaling	#134355
HSC-70	Santa Cruz	#sc-7298
GAPDH	Cell Signaling	#51745
HSP-90	Santa Cruz	#sc-13119
MAZ	Abcam	#ab85725
IgG	Abcam	#ab171870
Murine antibodies for flow cytometry		
TER119-APC/Cy7	Invitrogen	TER-119
Gr1-APC/Cy7	Biozym	RB6-8C5
CD19-APC/Cy7	eBioscience	eBio1D3
CD3-APC/Cy7	Biozym	17A2
CD11b-APC/Cy7	Invitrogen	M1/70
Sca1-PE/Cy7	eBioscience	D7
c-kit-PE/Cy5	Invitrogen	2B8
CD150-APC	Biozym	TC15-12F12.2
CD48-PE	Biozym	HM48-1
CD34-FITC	eBioscience	RAM34
CD19-PB	eBioscience	eBIO1D3
Gr1-PB	Invitrogen	RB6-8C5
CD11b-PB	eBioscience	M1/70
TER-119	eBioscience	TER-119
CD3-PB	Biozym	17A3
c-kit-FITC	Biozym	2B8
CD150-BV521	Biozym	TC15-12F12.2
Ly5.1(CD45.1)-FITC	Biozym	A20
Ly5.2(CD45.2)-PB	eBioscience	104
CD86-PE/Cy7	Biozym/BioLegend	GL-1
CD71-FITC	eBioscience	R17217
CD3-PE	Biozym/Biolegend	17A2
CD19-PE	BD Biosciences	1D3
FITC Mouse Anti-KI67 Set	BD Biosciences	556026

Human antibodies for flow cytometry		
CD38-PE/Cy7	Biozym	Kappa HB-7
CD34-PE	Biozym	581
CD11b-APC/Cy7	Biozym	ICRF44
Cytokines		
Murine Cytokines		
SCF	VetMed (in-house)	
CXCL12/SDF-1α	R&D Systems	460-SD-050
IL-3	R&D Systems	403-ML
IL-7	R&D Systems	407-ML
GM-CSF	R&D Systems	415-ML-010
TPO	PeproTech	135-14
Human Cytokines		
IL-6	PeproTech	200-06
IL-11	R&D Systems	218-IL-025
Erythropoietin	Janssen, Johnson & Johnson	
Holo-transferrin	Sigma-Aldrich	T0665-50MG
Insulin	Sigma-Aldrich	I2643-25MG
Chemicals, Peptides, and Recombinant Proteins		
Disuccinimidyl Glutarate	Thermo Scientific	20593
PenStrep	Sigma	P4333-100ML
2-Mercaptoethanol	Sigma	M3148-250ML
Fetal Bovine Serum (FBS)	Capricorn Scientific	FBS-12A
IMDM medium	Sigma	I3390-500ML
StemSpan™ SFEM medium	Stem Cell Technologies	09650
RPMI medium	Sigma-Aldrich/Merck	R8758
L-glutamine	Sigma-Aldrich	G7513-100ML
DAPI	Sigma-Aldrich	D9542
Clarity Western ECL Substrate	BioRad	1705061
Immobilon® -P PVDF membrane	Millipore	IPVH00010
Protein-A/G-Agarose	Thermo Fisher Scientific	11826784
cOmplete™ Protease Inhibitor (PI) tablets	Sigma-Aldrich/Roche	4693116001
Critical Commercial Assays		
Duolink™ flowPLA Mouse/Rabbit Starter	Sigma-Aldrich/Merck	DUO94104
Deposited Data		

GEO Single cell RNA-Seq in progress	hscs	
Experimental Models: Cell Lines		
Murine HPC^{LSK}	Generated in house	[3]
Murine BCR/ABL^{p185+}	Generated in house	[5]
Murine HPC7		[4]
Experimental Models: Organisms/Strains		
Cdk6-/-	VetMed	C57BL/6
Cdk6KM/KM	VetMed	C57BL/6
C57BL/6N	VetMed	C57BL/6
$Ly5.1^{+}$ ($CD45.1^{+}$)	Charles River	B6.SJL-Ptprca
Software and Algorithms		
GraphPad Prism (8.4.3.686)	GraphPad Software	
FlowJo (v10.6.1)	DeNovo Software	
Cytexpert (2.4.0.28)	Beckman Coulter	
FACSDiva	BD	
Venny software 2.1.	By Juan Carlos Oliveros, BioinfoGP, CNB-CSIC	
R 4.0.3[24]	R Foundation	https://www.r-project.org/
RStudio 1.3.1093[25]	Rstudio	https://posit.co/download/rstudio-desktop/
Seurat 3.2.3[19]	CRAN	
DESeq2 1.30.0[26]	Bioconductor	https://bioconductor.org/packages/
GSEA 4.1.0[27]	T	http://www.broad.mit.edu/gsea/
Proteome Discoverer software	Thermo Fisher Scientific	www.thermofisher.com
Other		
SureBeads™ Protein Magnetic Beads	BIO-RAD	161-4023
Polyinosinic:polycytidylic acid (pI:pC,)	InvivoGen	tlrl-pic-5
Transwell™ permeable Supports 6,5 mm; 5	Thermo Fisher Scientific	10107341
MethoCult M3231	StemCell Technologies	03231
BD Cytofix/Cytoperm Plus Fixation Permeabilization Kit	BD Biosciences	555028

MagniSort™ Mouse hematopoietic Lineage depletion kit	Thermo Fisher Scientific	8804-6829-74
BCA Protein Assay Kit	Thermo Fisher Scientific	23225
Bradford Assay Kit	Thermo Fisher Scientific	23246
Murine Primer (qPCR)	Forward	Reverse
Mlec	GGACAGTAGACGATGTACCA	GAACATGAGGCTGCTGTT
Rplp0	AGATTCGGGATATGCTGTTGG	AAAGCCTGGAAGAAGGAGGTC
Fosb	CCGAGAAGAGACACTTACCCCAG	GCGATCTCCGACTCCAGC
Murine Primer (ChIP qPCR)	*Forward*	*Reverse*
Mlec	GAGCAACCTACCCCGACG	CTTCAACCGAACTCCCTCC
Fosb	CCGACTGAGCTCCTTGTGG	CCAAACAAACACTGGGCCG
Hmgb2 pos. region	CGCCATTTTTCAAACGCTTTTC	AGGCTAAGCCTAGCAGGTTC
Hmgb2 neg. region	TCCAGAACACCAGAGCATAG	AGGAACCAGAAACCAAATGAG

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Supplementary Figure Legends

Figure S1: CDK6 shapes the HSC transcriptomic landscape in a kinase-inactivated, kinase -dependent and -independent manner

CDK6 levels in **(A)** LSK and **(B)** HSC/MPP1 cells isolated from *Cdk6*$^{+/+}$, *Cdk6*$^{-/-}$ and *Cdk6*$^{KM/KM}$ mice analysed by a flow cytometry based intracellular CDK6 staining (n = 4 per genotype, mean ± SEM, mean fluorescence intensity (MFI)). **(C)** (left) Western blot analysis and (right) protein quantification of CDK6 levels in lineage depleted BM of *Cdk6*$^{+/+}$, *Cdk6*$^{-/-}$ and *Cdk6*$^{KM/KM}$ mice. HSC70 served as loading control (n=3 per genotype). **(D)** Flow cytometry analysis of *Cdk6*$^{+/+}$, *Cdk6*$^{-/-}$ and *Cdk6*$^{KM/KM}$ BM cellularity. **(E-F)** Relative LSK, HSC, MPP1-MPP5 cell numbers (n = 10 per genotype, mean ± SEM). **(G)** Expression levels of key marker genes on UMAPs of *Cdk6*$^{+/+}$, *Cdk6*$^{-/-}$ and *Cdk6*$^{KM/KM}$ LSK cells **(H)** Bar graph showing the relative abundance of each cell cluster *Cdk6*$^{-/-}$ or *Cdk6*$^{KM/KM}$ compared to *Cdk6*$^{+/+}$ control (Log_2FC relative to *Cdk6*$^{+/+}$). HSPC: Hematopoietic stem and progenitor cells, Rep: Replication, Lym1-5: Lymphoid cells, Myel 1-2: Myeloid cells, Cycle 1-2: Cell cycle. **(I)** (top) Representative flow cytometry plots of *Cdk6*$^{+/+}$, *Cdk6*$^{-/-}$ and *Cdk6*$^{KM/KM}$ HSC/MPP1 cells depicting KI67/DAPI gating and percentages of cell cycle phases. (bottom) Percentages of cell cycle phase distributions of HSC/MPP1 cells (n ≥ 6; mean ± SEM). **(J)** Proliferation curves of sorted *Cdk6*$^{+/+}$, *Cdk6*$^{-/-}$ and *Cdk6*$^{KM/KM}$ (left) LSK or (right) HSC cells (LSK n = 3, HSC n = 10, mean ± SEM). **(K)** Expression levels of key marker genes in *Cdk6*$^{+/+}$, *Cdk6*$^{-/-}$ and *Cdk6*$^{KM/KM}$ cells on the UMAPs of the HSPC subcluster.

Figure S2: Kinase-inactivated CDK6 maintains HSPC potential upon long-term challenge

(A) Experimental overview of single pI:pC treatment. **(B-C)** Cell numbers of $L^-K^+CD86^+$ and $L^-K^+CD86^+$ HSC-MPP4 cells in *Cdk6*$^{+/+}$, *Cdk6*$^{-/-}$ and *Cdk6*$^{KM/KM}$ mice upon single pI:pC injection (n=4 per genotype, mean ± SEM). **(D)** (top) Representative flow cytometry plots of

Cdk6$^{+/+}$, *Cdk6*$^{-/-}$ and *Cdk6*$^{KM/KM}$ L$^-$K$^+$CD86$^+$ HSC/MPP1 cells depicting KI67/DAPI gating and percentages of cell cycle phases. (bottom) Percentages of cell cycle phase distributions of L$^-$K$^+$CD86$^+$ HSC/MPP1 upon single pI:pC treatment (n=3, mean ± SEM). **(E)** Total BM cellularity in *Cdk6*$^{+/+}$, *Cdk6*$^{-/-}$ and *Cdk6*$^{KM/KM}$ mice upon serial pI:pC treatment (n ≥ 4 per genotype, mean ± SEM). **(F)** Flow cytometry analysis of *Cdk6*$^{+/+}$, *Cdk6*$^{-/-}$ and *Cdk6*$^{KM/KM}$ (left) MPP2 (L$^-$K$^+$CD86$^+$) and (right) MPP3/4 (L$^-$K$^+$CD86$^+$) cells upon serial pI:pC injection (n ≥ 3 per genotype, mean ± SEM). **(G)** Cell counts of serially plated *Cdk6*$^{+/+}$, *Cdk6*$^{-/-}$ and *Cdk6*$^{KM/KM}$ BM cells upon serial pI:pC treatment (n ≥ 3 per genotype, mean ± SEM). **(H)** (left) Myeloid and (right) lymphoid lineage distributions of *Cdk6*$^{+/+}$, *Cdk6*$^{-/-}$ and *Cdk6*$^{KM/KM}$ BM cells during 3 rounds of serial plating (n = ≥ 3 per genotype, mean ± SEM).

Figure S3: Kinase-inactivated CDK6 enhances HSC homing and self-renewal

(A) (left) Experimental overview of an *in vitro* transwell assay. Percentage of migrated *Cdk6*$^{+/+}$, *Cdk6*$^{-/-}$ and *Cdk6*$^{KM/KM}$ (middle) LSK or (right) HSC/MPP1 cells upon 4 hours of incubation (mean ± SEM, n = 3 per genotype). **(B)** Flow cytometry analysis of *in vivo* homed *Cdk6*$^{+/+}$, *Cdk6*$^{-/-}$ and *Cdk6*$^{KM/KM}$ (left) MPP2 and (right) MPP3/4 (CD45.2$^+$) cells in CD45.1$^+$ recipients 18 hours post-injection (n ≥ 11/genotype, mean ± SEM). **(C)** Percentage of engrafted CD45.2$^+$ *Cdk6*$^{+/+}$, *Cdk6*$^{-/-}$ and *Cdk6*$^{KM/KM}$ (left) MPP2 and (right) MPP3/4 cells during 4 rounds of serial transplantation (n = 3-6/genotype, mean ± SEM). **(D)** (left) Myeloid and (right) lymphoid lineage distribution of engrafted *Cdk6*$^{+/+}$, *Cdk6*$^{-/-}$ and *Cdk6*$^{KM/KM}$ cells in the peripheral blood (n ≥ 3 / genotype, mean ± SEM). **(F-G)** Endpoint analysis of the competition transplantation assay depicting the competitor *Cdk6*$^{+/+}$ and *Cdk6*$^{KM/KM}$ **(F)** LSK, **(G)** (left) MPP2 and (right) MPP3/4 cells (n = 6/group, mean ± SEM).

Figure S4: Kinase-inactivated CDK6 balances quiescent and activated transcriptional programs of long-term HSCs

(A) Heatmap presenting genes differentially expressed between HSC and MPP1 cells in Cabezas-Wallscheid, *et al*[18]. These genes formed the basis of the "quiescent" (277 genes) and "activated" (202 genes) gene sets which were used for gene set enrichment analysis. **(B)** Fold change (FC) of the dead cells of *Cdk6*$^{+/+}$, *Cdk6*$^{-/-}$ and *Cdk6*$^{KM/KM}$ HPC^{LSK} cells with shRenilla or shNFY-A analyzed by flow cytometry (n=3/genotype, mean ± SEM). **(C)** (left) Western blot analysis of *Cdk6*$^{+/+}$and *Cdk6*$^{-/-}$ HPC^{LSK} cells expressing either shRenilla or shNFY-A constructs and (right) NFY-A level quantification. CDK6 served as genotype, while HSC70 as loading control.

Figure S5: CDK6-MAZ interaction maintains HSC stemness

(A) Venn diagram showing *Cdk6*$^{KM/KM}$ and *Cdk6*$^{-/-}$ specific motifs over *Cdk6*$^{+/+}$ found in the quiescent stem cell gene set ($p < 0.01$). **(B)** Pathway analysis of common CDK6 and MAZ ChIP-seq peaks. **(C)** MAZ or IgG control ChIP-quantitative polymerase chain reaction (qPCR) analysis of *Cdk6*$^{+/+}$ HPC^{LSK} cell lines treated either with PBS (Ctrl) or Palbociclib (200nM) for 24 hours: qPCR was used to quantify MAZ enrichment on *Fosb*, *Mlec* and *Hmgb2* promoter or negative region for *Hmgb2* (n = 3/group, mean ± SEM). **(D)** qPCR analysis of *Fosb* and *Mlec* mRNA levels in sorted LSK cells treated with siRNA MAZ (24 hours) +/- Palbociclib (200nM, 4 hours), depicted as FC relative to non-treated scramble control (n = 3/group, mean ± SEM)**.** **(E)** Analysis of MAZ knockdown in sorted *Cdk6*$^{+/+}$ LSK cells. (left) Overlay of representative histograms of MAZ MFI levels in scramble Ctrl and siMAZ knockdown LSKs. (right) Flow cytometry analysis of MAZ levels in LSK cells relative to scramble Ctrl (n = 3/group, mean ± SEM). **(F)** Flow cytometry analysis of LSK cells upon MAZ knock down and/or Palbociclib treatment depicted as Log_2FC relative to corresponding scramble control (n = 4 per genotype, mean ± SEM).

Figure S6: CDK4/6 kinase inhibition protects HSC fitness

(A-B) Expression levels of key marker genes on UMAPs of Palbociclib or Ctrl treated **(A)** LSK cells or **(B)** in the HSPC subcluster. **(C)** Heatmap of 282 upregulated common genes identified in Fig.5G of either in the comparison *Cdk6*$^{KM/KM}$ vs *Cdk6*$^{+/+}$ or *Palbo* vs *Ctrl* of the dormant or immature HSPC subcluster [Log_2FC]. **(D)** Schematic representation of the *in vivo* homing assay upon Palbociclib treatment. **(E)** Flow cytometry analysis of homed CD45.2$^+$ LSK and HSC/MPP1-MPP4 cells pretreated either with PBS (Ctrl) or Palbociclib into CD45.1$^+$ recipients 18h post-injection ($n \geq 10$ recipients and donors, mean ± SEM). **(F)** (left) Experimental workflow of sorted LSK cells treated with either PBS (Ctrl) or Palbociclib followed by a serial plating assay. (right) Colony numbers and percentage of LSK and lineage$^+$ cells treated with either Palbociclib or Ctrl are depicted as Log_2FC relative to PBS control during 3 rounds of serial plating (n = 4/treatment, mean ± SEM). **(G-I)** Flow cytometry analysis of BM cells following *in vivo* Palbociclib treatment (1st analysis described in Fig.6F): **(G)** percentages of LSK, MPP2 and MPP3/4 cells and **(H)** cell numbers of LSK, HSC/MPP1, MPP2 and MPP3/4 cells ($n \geq 4$/group, mean ± SEM), **(I)** Flow cytometry analysis of BM myeloid cells. **(J, K)** Flow cytometry analysis of serially plated BM cells following *in vivo* Palbociclib treatment (2nd analysis described in Fig.6F): **(J)** Colony and cell numbers, **(K)** percentage of LSK cells upon *in vivo* Palbociclib treatment followed by 2 rounds of serial plating (n = 4/group, mean ± SEM). **(L)** Colony and **(M)** LSK cell numbers of siMAZ knockdown following the *in vivo* Palbociclib treatment (described in Fig.6F) of sorted and embedded LSK cells depicted as Log_2FC relative to the corresponding scramble control (n = 2/group, mean ± SEM). **(N-O)** Endpoint analysis of competition transplantation assay described in Fig.6I: **(N)** Percentage of engraftment of *Cdk6*$^{+/+}$ and *Cdk6*$^{KM/KM}$ LSK, HSC/MPP1, MPP2 and MPP3/4 cells and **(O)** cell numbers of engrafted

MPP2 and MPP3/4 subfractions (n = 7/group, mean ± SEM). **(P)** Percentage of $CD11b^+$ human cells after two rounds of serial plating ($n \geq 3$, mean ± SEM).

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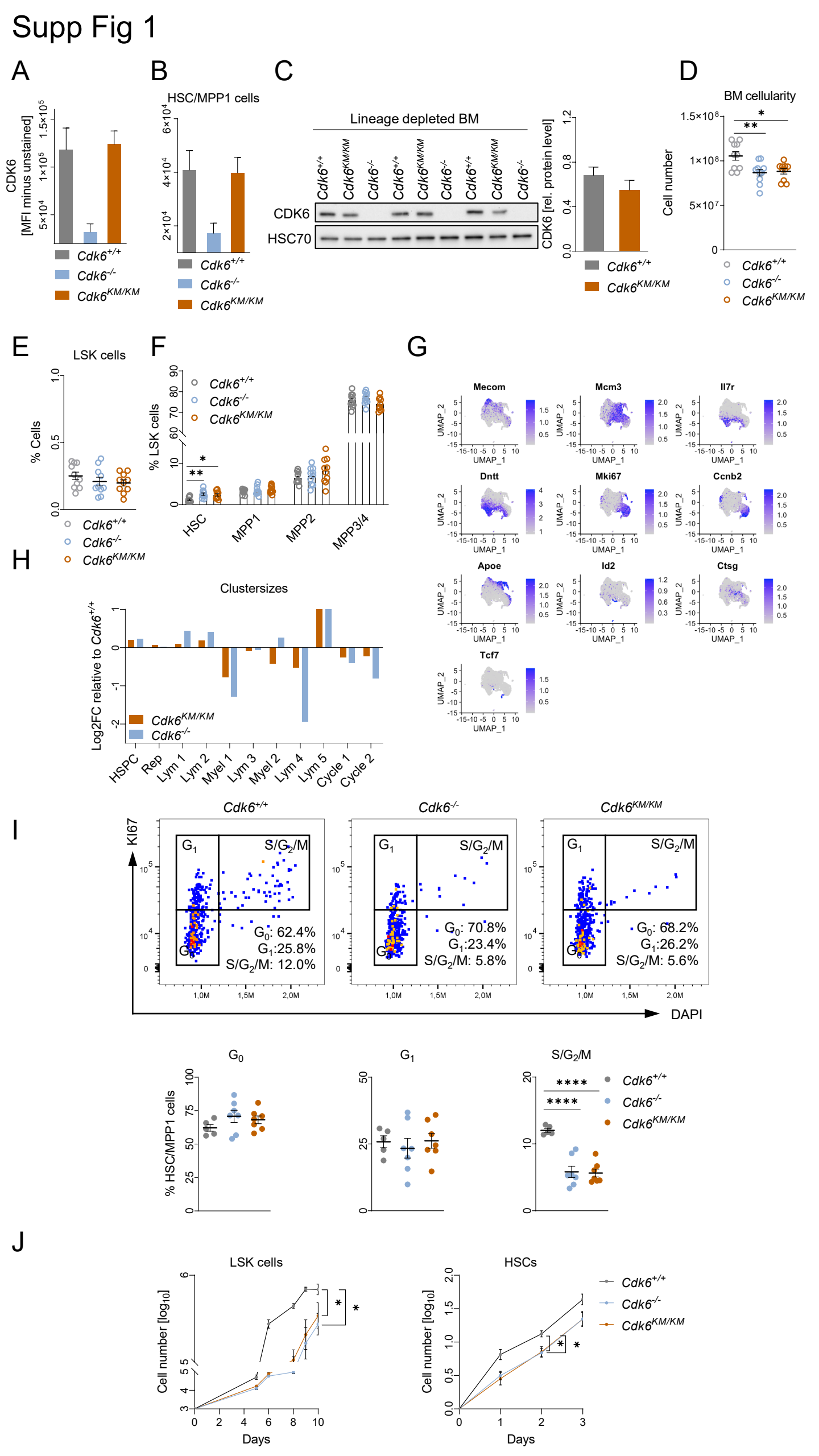
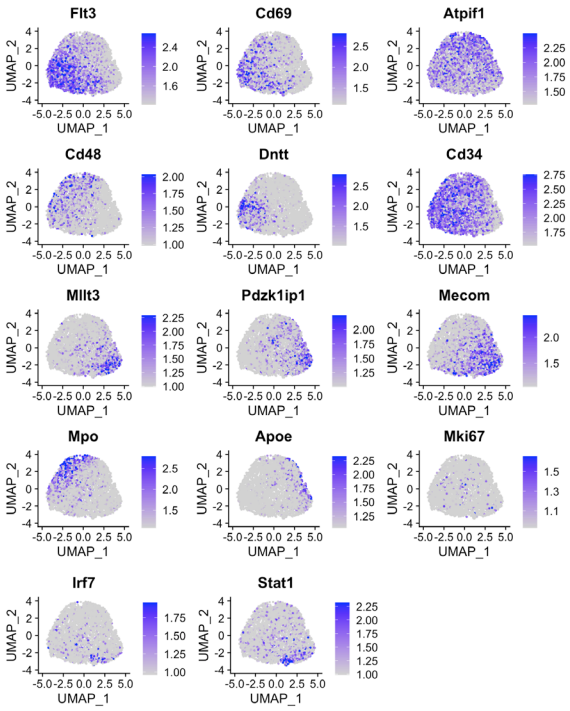
Supp Fig 1

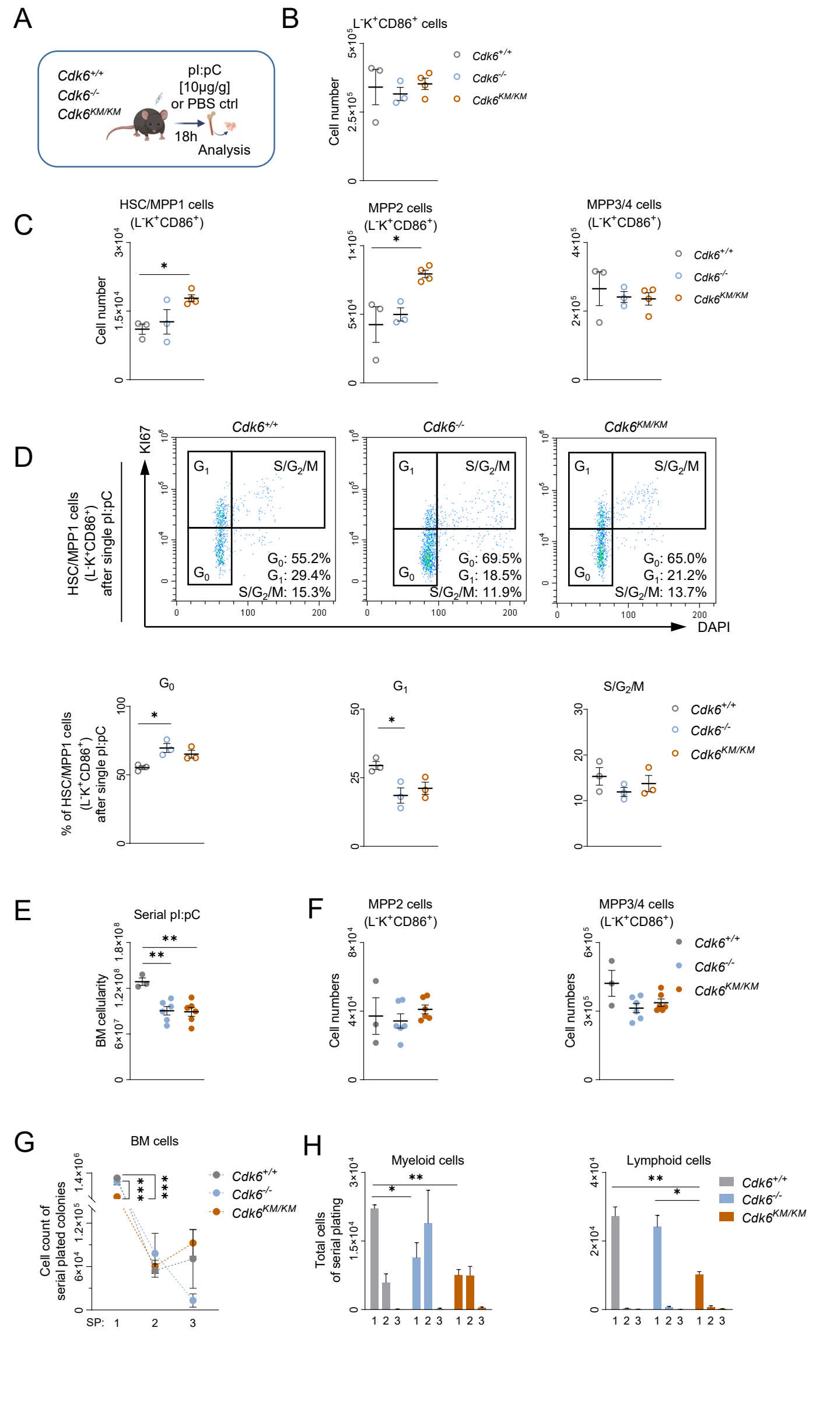

A
CDK6 [MFI minus unstained]
Cdk6+/+
Cdk6-/-
Cdk6KM/KM
B
HSC/MPP1 cells
C
Lineage depleted BM
CDK6
HSC70
CDK6 [rel. protein level]
D
BM cellularity
Cell number
E
LSK cells
% Cells
F
% LSK cells
HSC
MPP1
MPP2
MPP3/4
G
Mecom
Mcm3
Il7r
Dntt
Mki67
Ccnb2
Apoe
Id2
Ctsg
Tcf7
UMAP_1
UMAP_2
H
Clustersizes
Log2FC relative to Cdk6+/+
HSPC
Rep
Lym 1
Lym 2
Myel 1
Lym 3
Myel 2
Lym 4
Lym 5
Cycle 1
Cycle 2
I
KI67
DAPI
G1
S/G2/M
G0: 62.4%
G1:25.8%
S/G2/M: 12.0%
G0: 70.8%
G1:23.4%
S/G2/M: 5.8%
G0: 68.2%
G1:26.2%
S/G2/M: 5.6%
% HSC/MPP1 cells
J
LSK cells
HSCs
Cell number [log10]
Days

Supp Fig 1

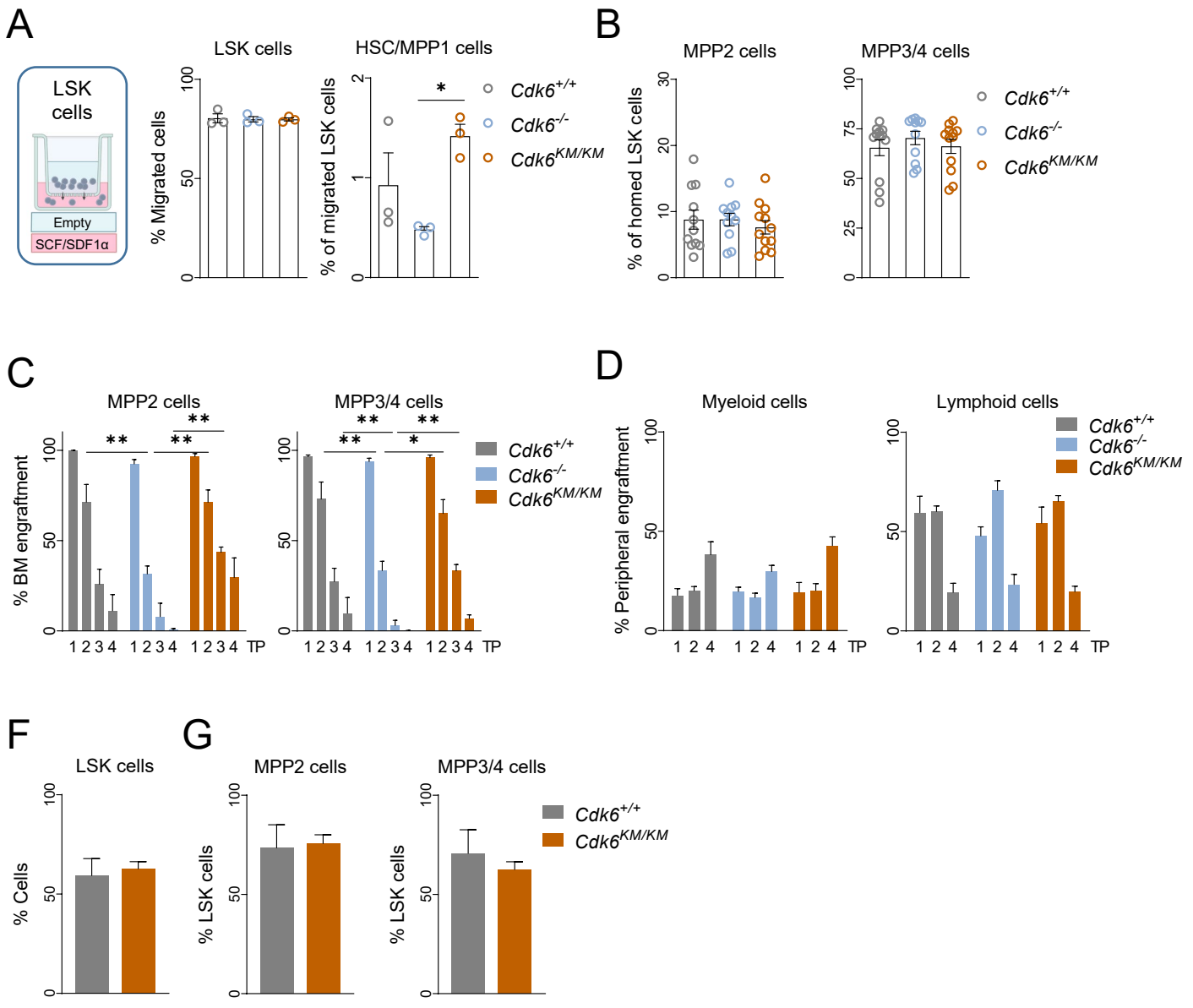
K


Flt3
Cd69
Atpif1
Cd48
Dntt
Cd34
Mllt3
Pdzk1ip1
Mecom
Mpo
Apoe
Mki67
Irf7
Stat1
UMAP_1
UMAP_2

Supp Fig 2

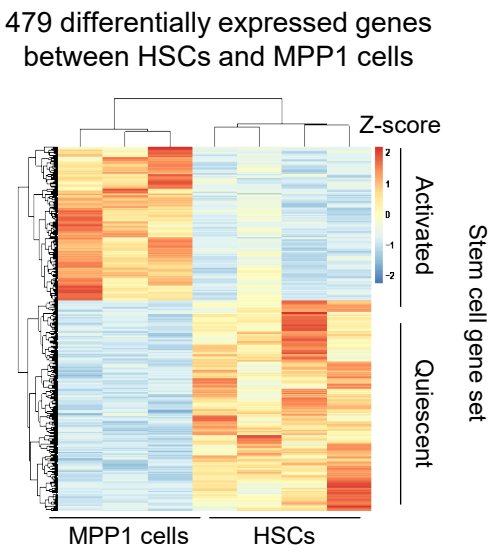

A
Cdk6+/+
Cdk6-/-
Cdk6KM/KM
pI:pC
[10µg/g]
or PBS ctrl
18h
Analysis
B
L-K+CD86+ cells
Cell number
Cdk6+/+
Cdk6-/-
Cdk6KM/KM
C
HSC/MPP1 cells
(L-K+CD86+)
MPP2 cells
(L-K+CD86+)
MPP3/4 cells
(L-K+CD86+)
Cell number
D
Cdk6+/+
Cdk6-/-
Cdk6KM/KM
KI67
DAPI
HSC/MPP1 cells
(L-K+CD86+)
after single pI:pC
G1
S/G2/M
G0
G0: 55.2%
G1: 29.4%
S/G2/M: 15.3%
G0: 69.5%
G1: 18.5%
S/G2/M: 11.9%
G0: 65.0%
G1: 21.2%
S/G2/M: 13.7%
% of HSC/MPP1 cells
(L-K+CD86+)
after single pI:pC
G0
G1
S/G2/M
E
Serial pI:pC
BM cellularity
F
MPP2 cells
(L-K+CD86+)
MPP3/4 cells
(L-K+CD86+)
Cell numbers
G
BM cells
Cell count of
serial plated colonies
SP: 1 2 3
H
Myeloid cells
Lymphoid cells
Total cells
of serial plating
1 2 3 1 2 3 1 2 3

Supp Fig 3

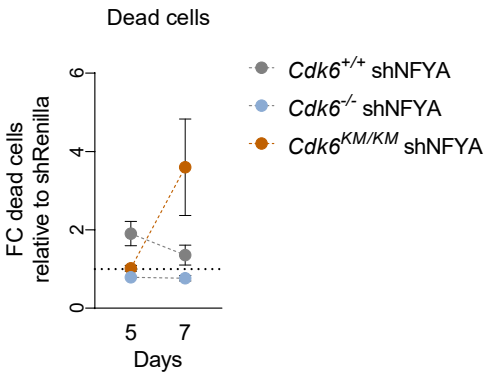

A
LSK cells
Empty
SCF/SDF1α
LSK cells
% Migrated cells
HSC/MPP1 cells
% of migrated LSK cells
Cdk6+/+
Cdk6-/-
Cdk6KM/KM
B
MPP2 cells
% of homed LSK cells
MPP3/4 cells
C
MPP2 cells
MPP3/4 cells
% BM engraftment
1 2 3 4 1 2 3 4 1 2 3 4 TP
D
Myeloid cells
Lymphoid cells
% Peripheral engraftment
1 2 4 1 2 4 1 2 4 TP
F
LSK cells
% Cells
G
MPP2 cells
MPP3/4 cells
% LSK cells

Supp Fig 4

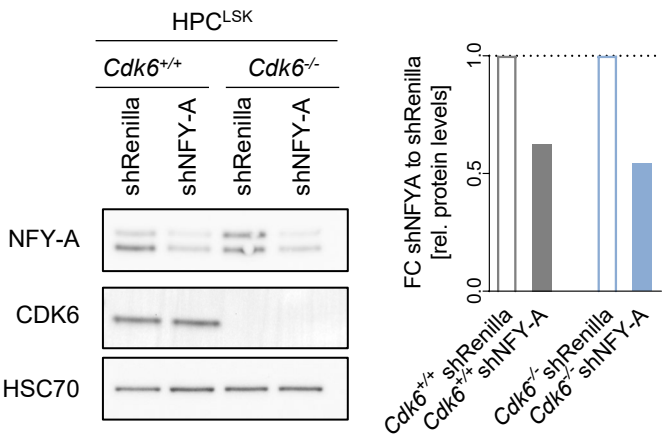
A



B

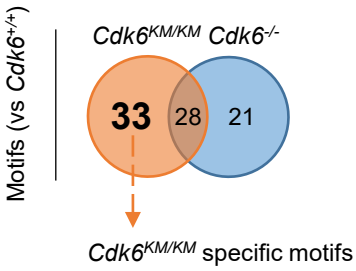


C

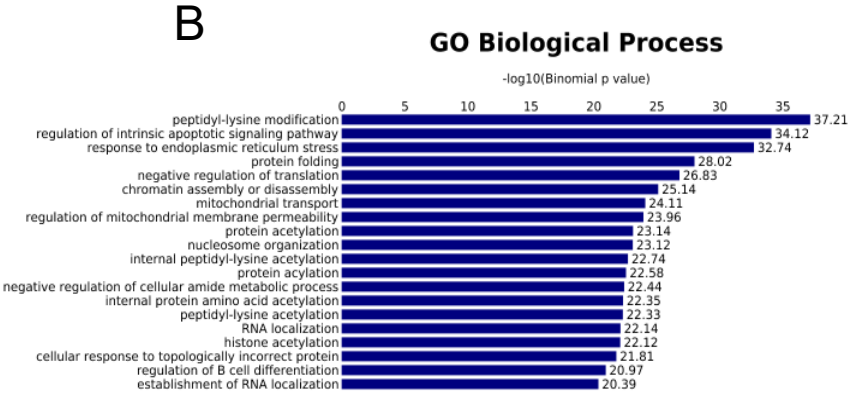


Supp Fig 5

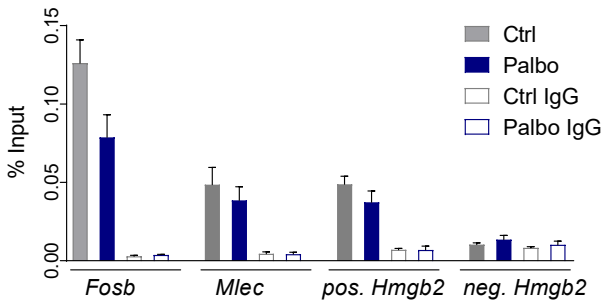
A



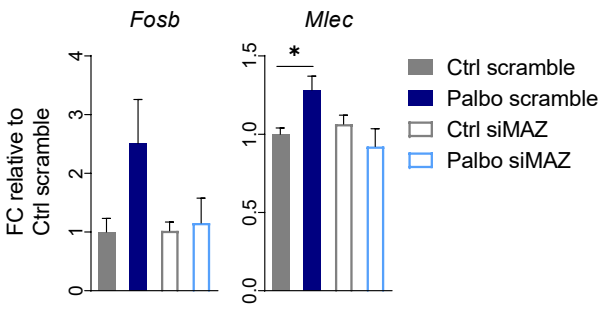
B



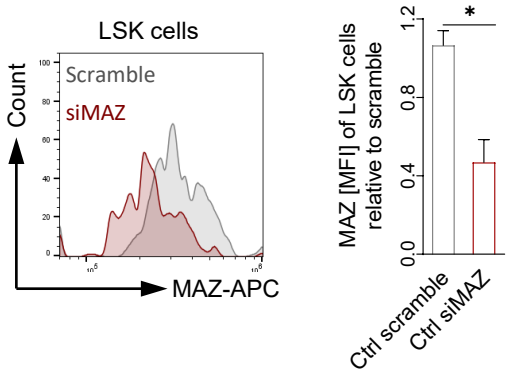
C



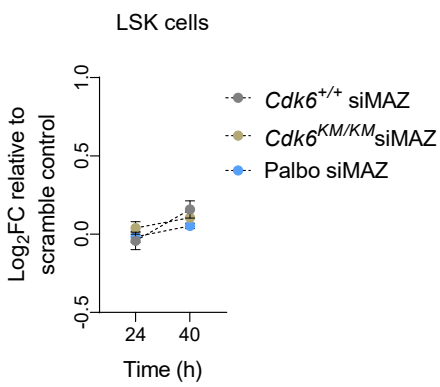
D



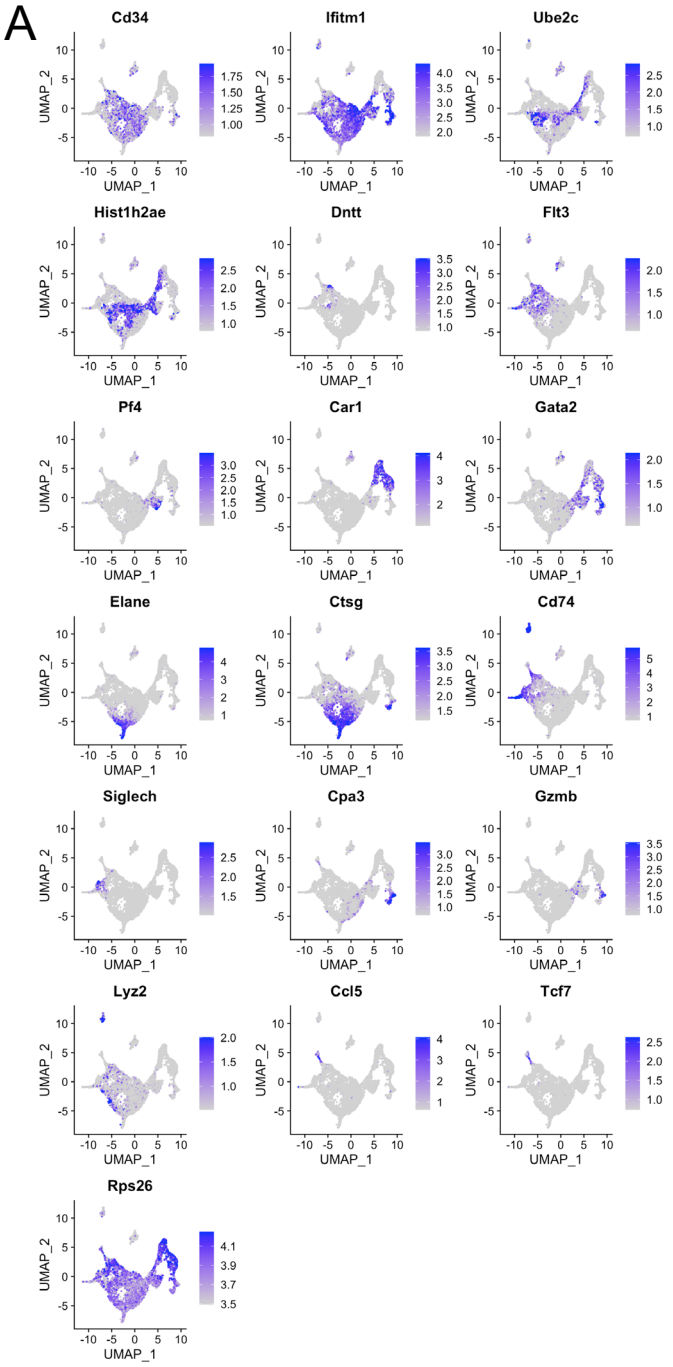
E

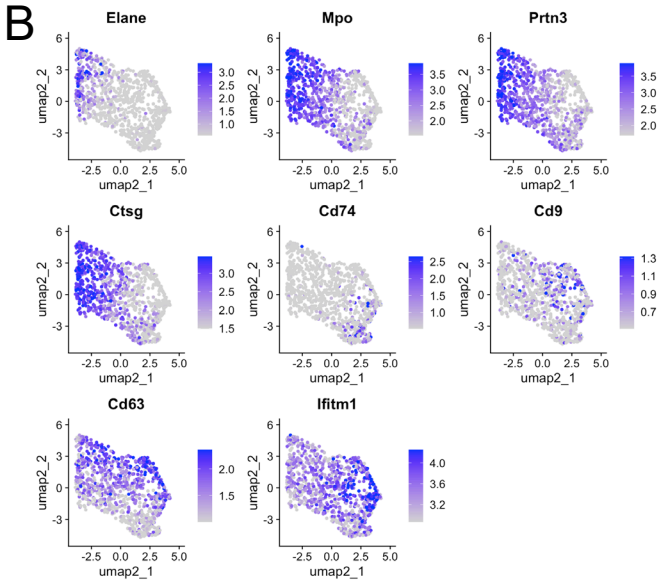


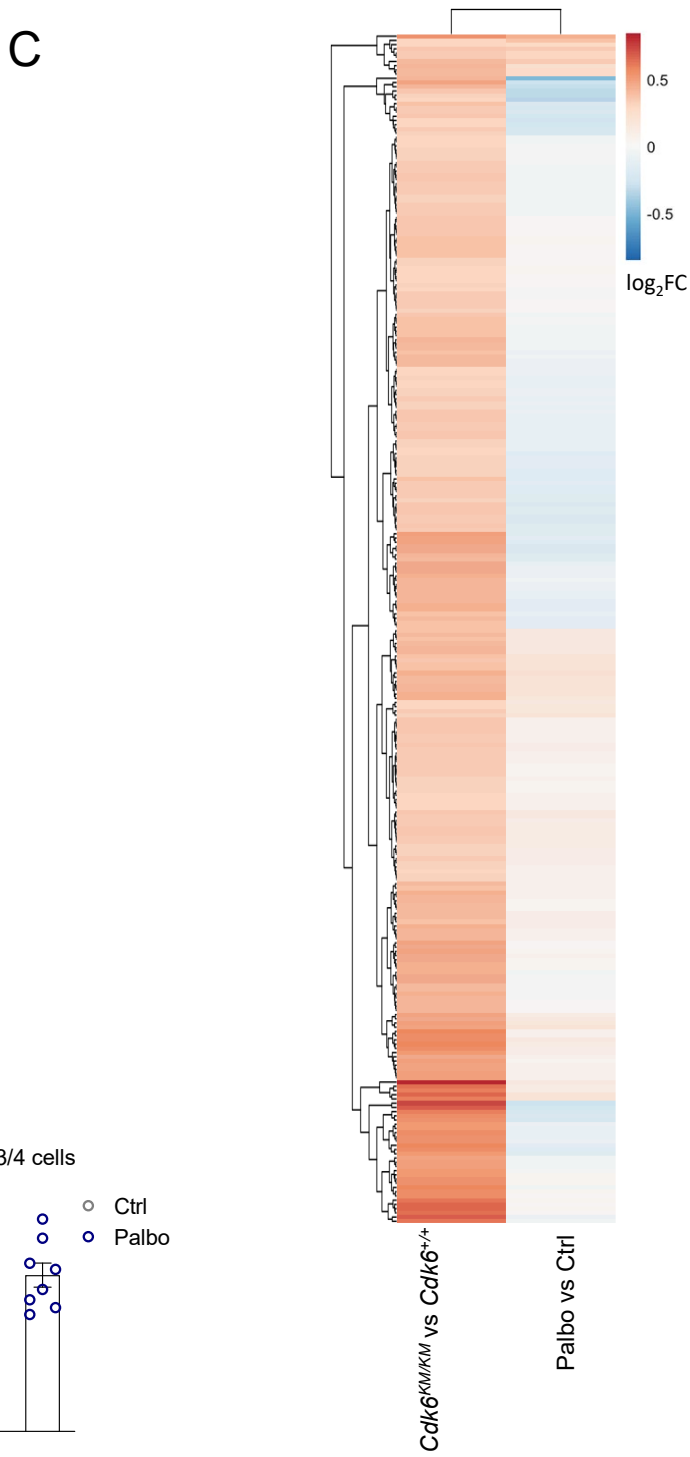
F

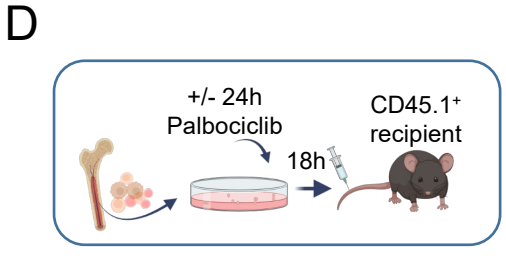


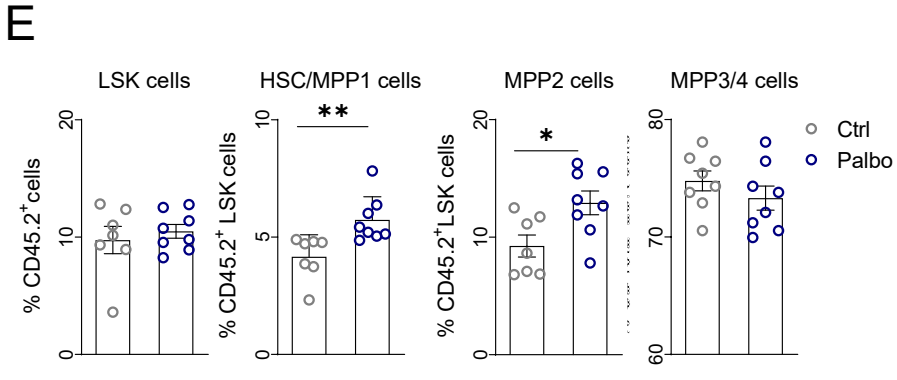
Supp Fig 6

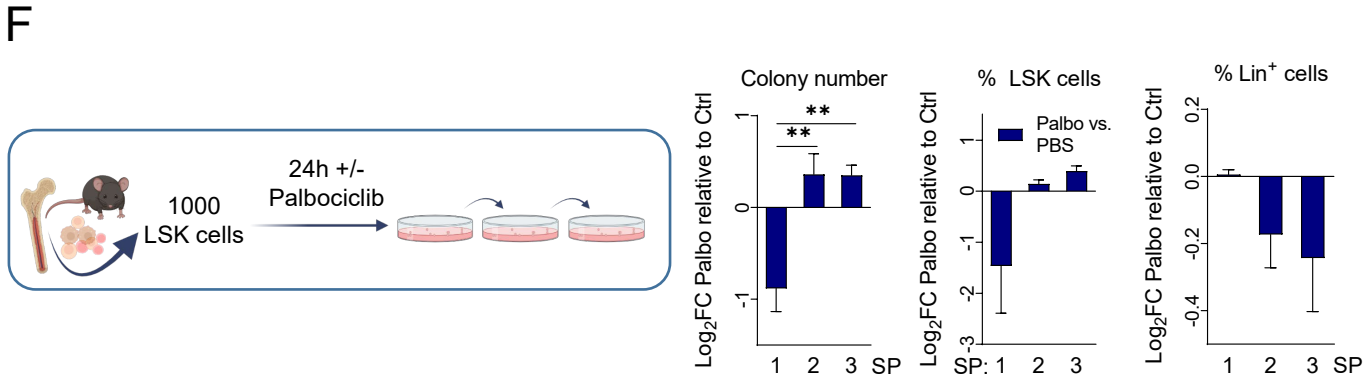

A
Cd34
Ifitm1
Ube2c
Hist1h2ae
Dntt
Flt3
Pf4
Car1
Gata2
Elane
Ctsg
Cd74
Siglech
Cpa3
Gzmb
Lyz2
Ccl5
Tcf7
Rps26
UMAP_1
UMAP_2


B
Elane
Mpo
Prtn3
Ctsg
Cd74
Cd9
Cd63
Ifitm1
umap2_1
umap2_2

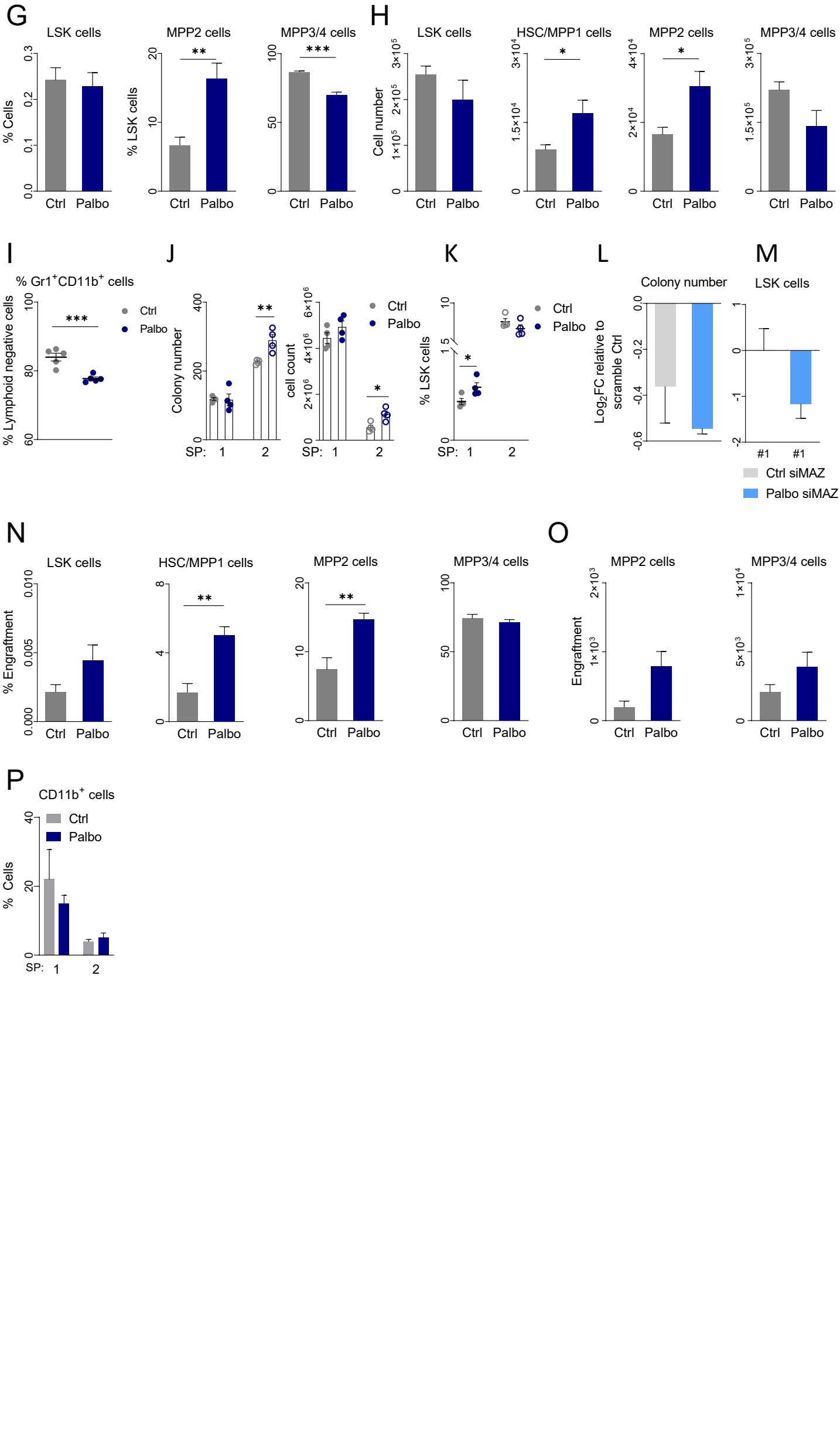

C
log2FC
Cdk6KM/KM vs Cdk6+/+
Palbo vs Ctrl


D
+/- 24h
Palbociclib
18h
CD45.1+
recipient


E
LSK cells
HSC/MPP1 cells
MPP2 cells
MPP3/4 cells
% CD45.2+cells
% CD45.2+ LSK cells
Ctrl
Palbo


F
1000
LSK cells
24h +/-
Palbociclib
Colony number
% LSK cells
% Lin+ cells
Log2FC Palbo relative to Ctrl
Palbo vs.
PBS
SP

Supp Fig 6


G
LSK cells
% Cells
Ctrl
Palbo
MPP2 cells
% LSK cells
**
MPP3/4 cells
***
H
LSK cells
Cell number
HSC/MPP1 cells
*
MPP2 cells
*
MPP3/4 cells
I
% Gr1+CD11b+ cells
% Lymphoid negative cells
***
J
Colony number
**
cell count
*
SP: 1 2
K
% LSK cells
L
Colony number
Log2FC relative to scramble Ctrl
M
LSK cells
#1 #1
Ctrl siMAZ
Palbo siMAZ
N
LSK cells
% Engraftment
HSC/MPP1 cells
MPP2 cells
MPP3/4 cells
O
MPP2 cells
Engraftment
MPP3/4 cells
P
CD11b+ cells
% Cells
SP: 1 2