Differential transcriptional control of hematopoiesis in congenital and cyclic neutropenia patients harboring *ELANE* mutations

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Supplemental information

Supplemental Methods

scRNA sequencing

Two healthy donor samples were chosen with a $CD34^+$ cells purity of 94.4% (HD92) and 99.4% (HD84). Each sample was thawed in 1 ml complete growth medium, RPMI 10% FBS (Gibco, Life Technologies) and dropped into a 50 ml conical tube. The cells were then diluted by 1:1 incremental volume additions of complete growth media, five times in total. Media was added with a speed of 1 ml/3-5 sec while swirling the tube. Cells were collected by centrifugation (300 relative centrifugal force (RCF) for 5 min), and the supernatant was removed, leaving 1 ml for cell resuspension. The resulting suspension was washed with room temperature PBS (Gibco, Life Technologies) and 0.04 % BSA (Sigma). The supernatant was decanted without disturbing the pellet and resuspended in 1 ml PBS, and 0.04 % BSA. Cells were assessed for viability using trypan blue on a disposable hemocytometer (C-Chip, NanoEnTec) and adjusted to a final concentration of 1,200 cells/ul. To prepare the library, the single-cell suspension was loaded into the Chromium system (10X Genomics), targeting 9,000 cell recovery. Next, barcoded sequencing libraries were generated using the Chromium Single Cell 3′ v3.1 Reagent Kit (10X Genomics) according to the manufacturer protocol. The libraries were indexed and sequenced on an Illumina NovaSeq 6000 PE150, with a minimum of 80 Gb of raw data per sample. Fastq files were analyzed by running the cellranger pipeline version 5.0.0 (10X Genomics). The output of the cellranger pipeline (unique molecular identified (UMI) count matrix) was analyzed in R package Seurat v.4.3.0 (1). To filter out cells with high proportion of mitochondrial reads or reads covering a single most expressed gene – sample's 85th percentile of those two metrics were used as upper threshold to include cells in analysis. Upper threshold for cell's total read counts and cell's number of detected genes was sample's mean plus 2 standard deviations of those metrics. Lower threshold was set to 500 of total reads or total number of genes detected per cell. Expression data was normalized and dataset dimensionality reduced using Seurat's default settings. Sample-specific bias was compensated using R package harmony v0.1.1 (2). Cells clusters were determined based on first 15 principal components. Cell type were assigned based on clusters distinct gene signatures.

Generation of iPSCs of CyN patients

For two CyN patients, 5 x 10^5 peripheral blood or bone marrow mononuclear cells (PBMNCs) were seeded in 1 ml $CD34^+$ cell expansion medium: Stemline II, 10 % FCS, 1 % Penicillin/Streptomycin, 1 % GlutaMAX, and cytokines (20 ng/ml IL-6, 50 ng/ml SCF, 20 ng/ml TPO, 50 ng/ml FLT3, 20 ng/ml IL-3) in a 24-well plate. After 2 days, cells were transferred to RetroNectin®(Clontech)-coated 12-well plate together with lentiviral supernatant containing lentivirus particles for Oct4, Sox2 and Klf4 (OSK) at a multiplicity of infection (MOI) of 2.5. Cells were kept in culture in $CD34^+$ cell expansion medium: Stemline II, 10 % FCS, 1 % Penicillin/Streptomycin, 1 % GlutaMAX, and cytokines (20 ng/ml IL-6, 50 ng/ml SCF, 20 ng/ml TPO, 50 ng/ml FLT3, 20 ng/ml IL-3) until day 6. Additionally, 2 mM valproic

acid and 50 µg/ml vitamin C were added to the culture every second day. On day 6, the cells were transferred onto the SNL feeder cells. $CD34^+$ cells expansion medium was gradually changed to iPSCs culture medium (hiPS medium: D-MEM F12 supplemented with 20 % KnockOut SR, NEAA, 1% L-Glutamine, 0.1 mM 2-Mercapto Ethanol, 1 % Penicillin/Streptomycin and 30 ng/ml bFGF). First iPSCs colonies appeared approximately three weeks after initiation of reprogramming. For one CyN patient, fibroblasts were transduced with OSK-RFP lentivirus. Three to five days after transduction, the red signal appeared in transfected cells, and iPSC colonies were generated within 8-10 days after plating on SNL feeder cells.

iPSC pluripotency testing

The pluripotency potential of the newly generated iPSC lines was tested by analysis of the expression of the pluripotency surface markers SSEA4 and TRA-1-60 measured by flow cytometry, as well as, mRNA expression levels of SOX2, NANOG, and DMNT3A using quantitative RT-PCR. The differentiation potential of iPSCs into three germ layers was estimated with STEMdiff™ Trilineage Differentiation Kit (STEMCELL Technologies, Cat# 05230) and Human Three Germ Layer 3-Color Immunocytochemistry Kit (R&D, Cat SC022). Using this kit, ectodermal and endodermal differentiation was investigated. Mesodermal differentiation was assessed by hematopoietic (mesodermal origin) cell production by these iPSC lines, as described in the next section.

EB-based hematopoietic differentiation of iPSCs

Geltrex™-coated plates were used to plate hiPSCs for 5 days until they became confluent. HiPSCs were then detached after incubation with PBS/EDTA (0.02%) for 5-7 min. The induction of EBs started by spinning down 20,000 cells/EB in 96-well plates containing APEL serum-free differentiation medium (STEMCELL Technologies) supplemented with 10 ng/ml bFGF and ROCK inhibitor (Tocris). Next day, mesoderm induction was initiated by adding 40 ng/ml of BMP4. Two days later, the EBs were transferred to a new Geltrex-coated 6-well-plate (10 EBs/well) in hematopoietic stem cell differentiation medium (APEL medium supplemented with 40 ng/ml VEGF, 50 ng/ml SCF, and 50 ng/ml IL-3). Medium was changed 3 days later to neutrophil differentiation medium (APEL medium supplemented with 50 ng/ml IL3 and 10 ng/ml G-CSF) and refreshed every 3 days with 2 ml neutrophil differentiation medium. Suspension cells were harvested and analyzed by flow cytometry on day 14 and day 28.

Flow cytometry analysis of iPSC-derived hematopoietic cells

5 x 10^4 suspension cells were collected from the hematopoietic iPSC-differention culture and analyzed by flow cytometry at day 14 and day 28. For detection of hematopoietic stem and progenitor cells and myeloid progenitors at day 14, cells were stained with mouse anti-human antibodies: CD33-BV421 (BioLegend), CD34-PeCy7 (BD), KDR-AF647 (BL), CD43-PE (BD), CD41a-FITC (BD), CD235a-FITC (BD), CD45-BV510 (BioLegend), 7-AAD (BD). For HD and CyN, cells at day 14 were alternatively stained with the following anti-human antibodies: CD38-BV510 (BioLegend), CD34-PE-Cy7 (BD), CD90-BV785 (BioLegend), CD45RA-Alexa700 (BD), CD49f-BV421 (BioLegend),

CD133-PE (BioLegend), CD202b (Tie2)-APC (Miltenyi Biotec), and 7-ADD (BD). For detection of mature myeloid cells at day 28, cells were stained with the following mouse anti-human antibodies: CD15-PE (BD), CD16-FITC (BD), CD14-APC-H7 (BD), CD45-BV510 (BL), CD33-BV-421 (BL) and 7-AAD (BD). Anti-mouse IgGk beads (BD) were used for compensation. Samples were analyzed using a BD FACSCanto II and FlowJo V10.

Lentiviral transduction and hematopoietic differentiation of human CD34$^+$ HSPCs

2.5-5x10^5 CD34$^+$ cells/ml from three CN patients were cultured for 24 hours in medium: StemSpan SFEM II medium (STEMCELL Technologies) supplemented with 100 ng/ml FLT3L, 100 ng/ml SCF, 100 ng/ml TPO, 20 ng/ml IL3, 35 nM UM171 and 1% Penicillin/Streptomycin. The following day, cells were resuspended in stimulation media containing 5 mg/ml of protamine sulfate and concentrated lentivirus particles at a MOI of 50 and transferred to RetroNectin®-coated plates. After 24 hours, cells were collected and resuspended in fresh stimulation medium for 48 additional hours. After transduction, 2 x 10^5 cells/ml were cultured for 7 days in RPMI 1640 GlutaMAX supplemented with 10 % FBS, 1 % Penicillin/Streptomycin, 5 ng/ml SCF, 5 ng/ml IL-3, 5 ng/ml GM-CSF, and 1 ng/ml G-CSF. The medium was exchanged every second day. On day 7, the medium was replaced by RPMI 1640 GlutaMAX medium supplemented with 10 % FBS, 1 % Penicillin/Streptomycin, and 1 ng/ml G-CSF. The medium was exchanged every second day. On day 14, cells were analyzed by morphological assessment of May-Grunwald-Giemsa stained cytospin slides or flow cytometry analysis. Cytospin slides were prepared by centrifugation of 30,000 cells at 250 G for 3 min. For the detection of mature myeloid cells at day 14, 50.000 cells were stained with mouse anti-human antibodies: CD16-BV605 (BD), CD45-BV510 (BioLegend), CD33 BV-421 (BioLegend), CD15 BV785 (BD), and CD11b APC-Cy7 (BD). Anti-mouse IgGk beads (BD) were used for compensation. Samples were analyzed using BD LSRFortessa and FlowJo V10.

Quantitative RT-PCR

RNA was isolated using RNeasy Mini Kit (Qiagen). cDNA was prepared from 0.5 µg of total RNA using Omniscript RT Kit (Qiagen). qPCR was performed using SYBR Green qPCR master mix (Roche) and Light Cycler 480 (Roche). Gene expression was calculated using ΔΔCt approach. Target gene expression was normalized to β-actin as a housekeeping gene. Primer sequences are shown in **Suppl. Table 2**.

Analysis of the NE protein by ELISA

Neutrophil elastase protein levels in plasma were measured in duplicates in appropriately diluted samples with a NE-specific enzyme-linked immunoabsorbent assay (ELISA) kit (Hycult Biotechnology, Catalog Nr. HK319-01). The detection limit of the assay was 0.4 ng/mL of NE.

Peripheral blood absolute neutrophil counts (ANC)

ANCs were measured in the EDTA capillary peripheral blood samples using a Cobas integrated

hematology analyzer (Roche Diagnostics).

Statistics

Statistical analysis was performed using Prism 9.0 from GraphPad.

Supplemental Tables.

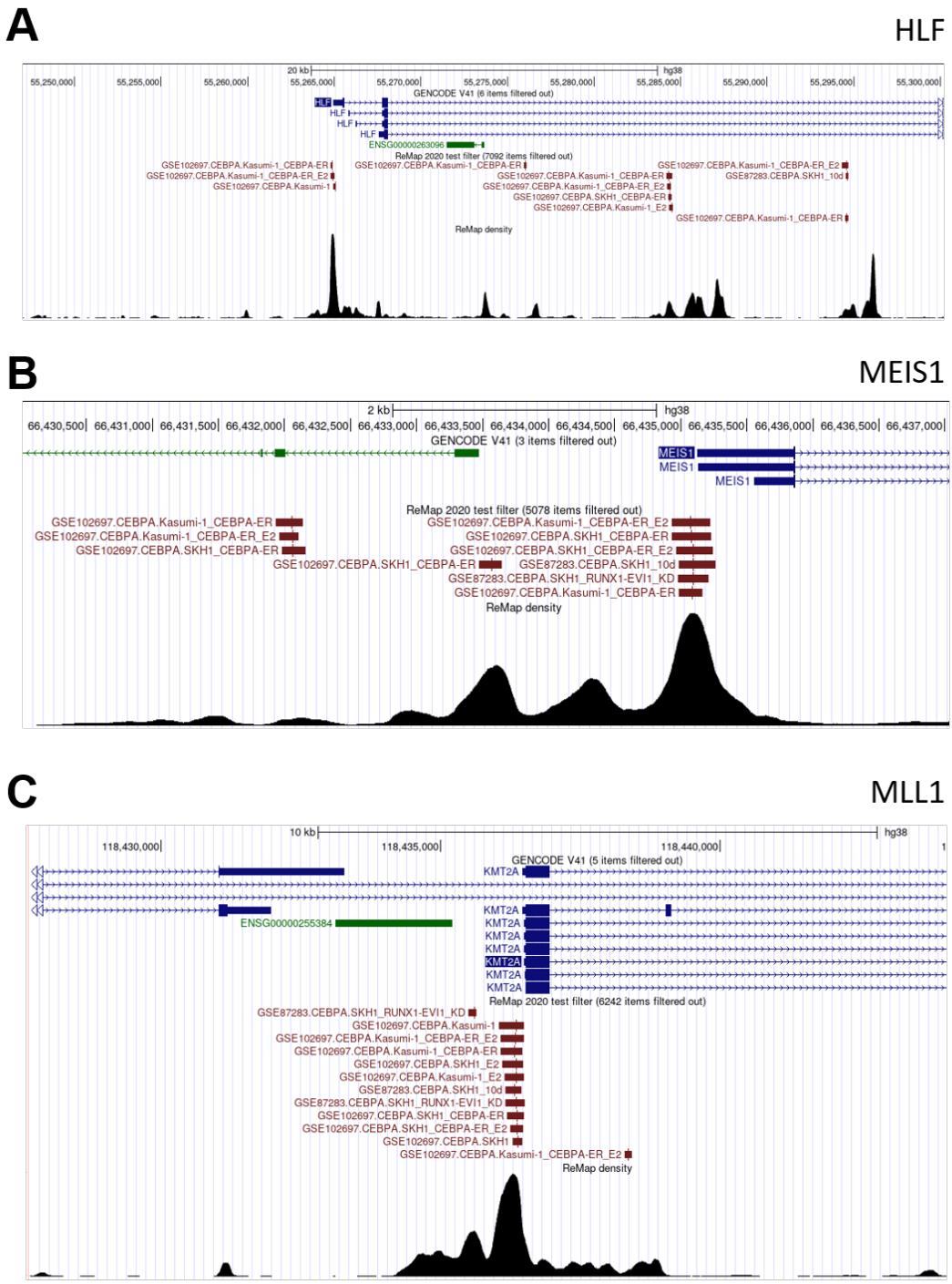
Supplemental Table 1. Patients characteristics.

Patient	**CN/CyN**	**Gender**	**Year of birth**	***ELANE* mutation position, NP_001963**	**G-CSF dose**	**Somatic *CSF3R* mutations, NP_000751.1**
Pt.1	CyN	f	1989	p.V190_F199del	1,97 µg/kg/d	p.Q743* 0,43%
Pt.2	CyN	m	1993	p.V190_F199del	1,4 µg/kg/d	None
Pt.3	CyN	m	1997	p.V190_F199del	1,4 µg/kg/d	None
Pt.4	CyN	m	1981	p.V101M	1,08 µg/kg/d	None
Pt.5	CyN	f	2005	p.V190_F199del	6 µg/kg/d	None
Pt. 6	CyN	F	1978	p.W241L	0,9 µg/kg/d	p.M222T, % NA
Pt. 7	CyN	m	1992	p.R220Q	2,5 µg/kg/d	None
Pt. 8	CyN	m	1989	p.W241G	0.7 µg/kg/d	None
Pt. 9	CyN	f	1956	p.W241G	0,6 µg/kg/d	None
Pt. 10	CyN	m	1963	p.V190_F199del	1,47 µg/kg/d	None
Pt. 11	CyN	f	2006	p.V190_F199del	1,03 µg/kg/d	None
Pt. 12	CyN	f	2010	p.V190_F199del	1,5 µg/kg/d	p.Q739* 1,67%
Pt. 13	CyN	f	2000	p.V190_F199del	1,5 µg/kg/d	p.Q739* 1,09%; p.Q749* 18,81%
Pt. 14	CN	m	1972	p.R103P	3 µg/kg/d	NA
Pt. 15	CN		2003	p.R103P	10,5 µg/kg/d	p,Q739* 2,5%; p,Q754* 0,68%; p,Q749* 0,65%
Pt.16	CN	m	1954	p.I120F	3 µg/kg/d	NA
Pt. 17	CN	f	1989	p.I120F	1,5 µg/kg/d	p.Q730* 1%
Pt. 18	CN	m	1988	p.I120F	1,6 µg/kg/d	p.Q743* 28,8%; p.Q749* 1,36%
Pt. 19	CN	m	1967	p.V190_F199del	1,75 µg/kg/d	NA
Pt. 20	CN	m	2002	p.A57V	150 µg/kg/d	NA
Pt. 21	CN	m	1991	p.C151Y	4,8 µg/kg/d	NA
Pt. 22	CN	m	1996	p.G214R	30 µg/kg/d	p.Q770*, % NA
Pt. 23	CN	f	2017	p.A57V	55 µg/kg/d	None
Pt. 24	CN	f	2005	p.G210W	6,59 µg/kg/d	p.Q741* 6,8%
Pt. 25	CN	f	2009	p.S126L	4,35 µg/kg/d	p.Q749* 26,20%
Pt. 26	CN	f	2010	p.S126L	1,83 µg/kg/d	p.Q749* 0,14 %

Supplemental Table 2. Primer sequences used for qRT-PCR

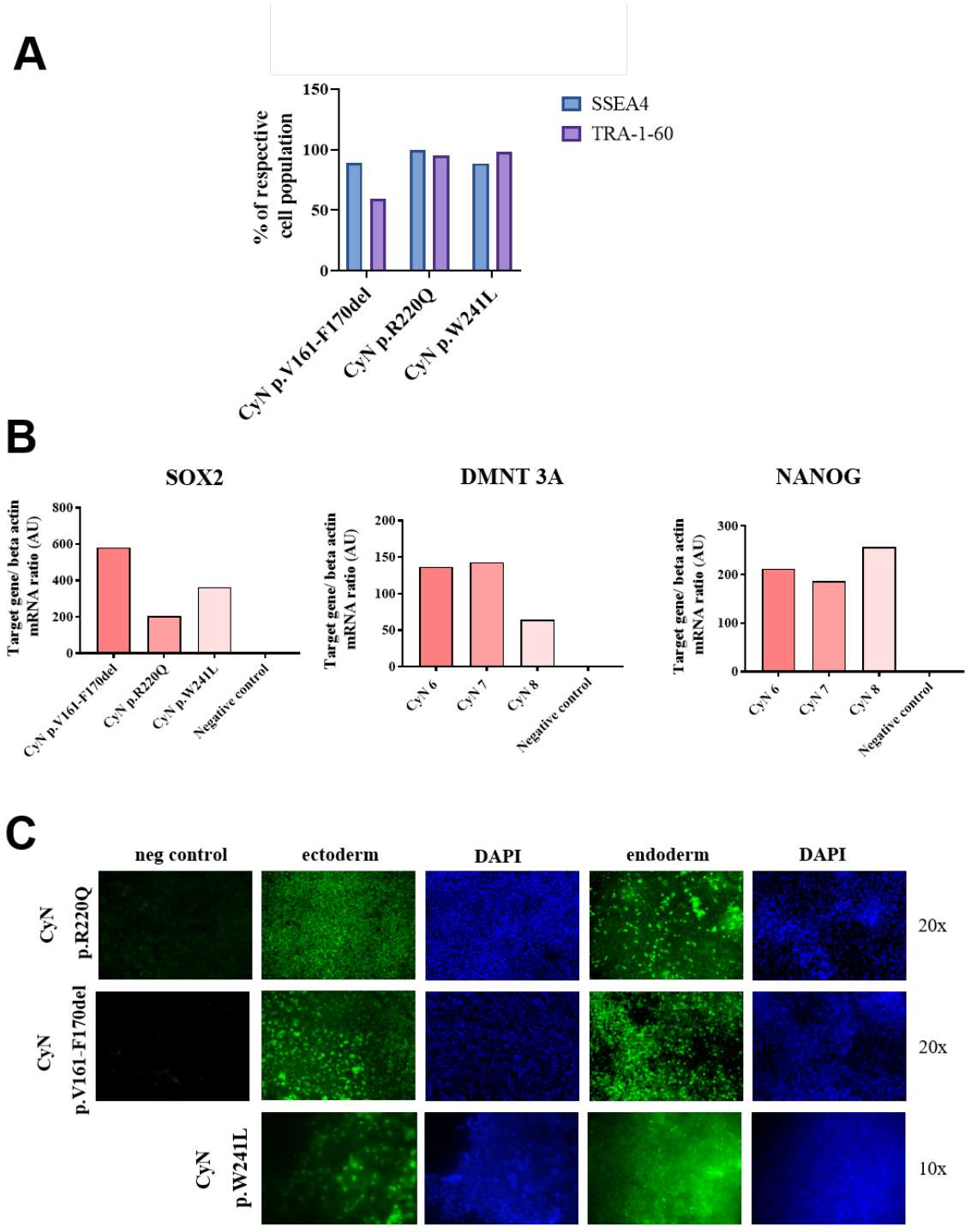
Gene of interest	Primer sequence (5´→ 3')
MEIS1_q_F	TACCCGCACACAGCTCATAC
MEIS1_q_R	CATTGAATGACTCTGACGAGCA
HLF_q_F	GGTCCCAGTGGGTTATGAGC
HLF_q_R	TCAGGGATGAAGACTTTGCGA
HOXA9_q_F	GGCAGGTACATGCGCTCC
HOXA9_q_R	ACCACAAGCATAGTCAGTCAGG
C/EBPA - _q_F	CCGGACTTGGTGCGTCTAAG
C/EBPA - _q_R	AGGCATTGGAGCGGTGAGT
MLL1_q_F	GGTA AGCTCTTTTTCAAGGGCCAGT
MLL1_q_R	CAGAAGATGAGATGTATGAGA
DMNT3A_q_F	ATAAGTCGAAGGTGCGTCGT
DMNT3A_q_R	GGCAACATCTGAAGCCATTT
NANOG_q_F	WTCACACGGAGACTGTCTCTC
NANOG_q_R	RVGAACACAGTTCTGGTCTTCTG
SOX2_q_F	TTCACATGTCCCAGCACTACCAGA
SOX2_q_R	TCACATGTGTGAGAGGGGCAGTGTGC

Supplemental Figures



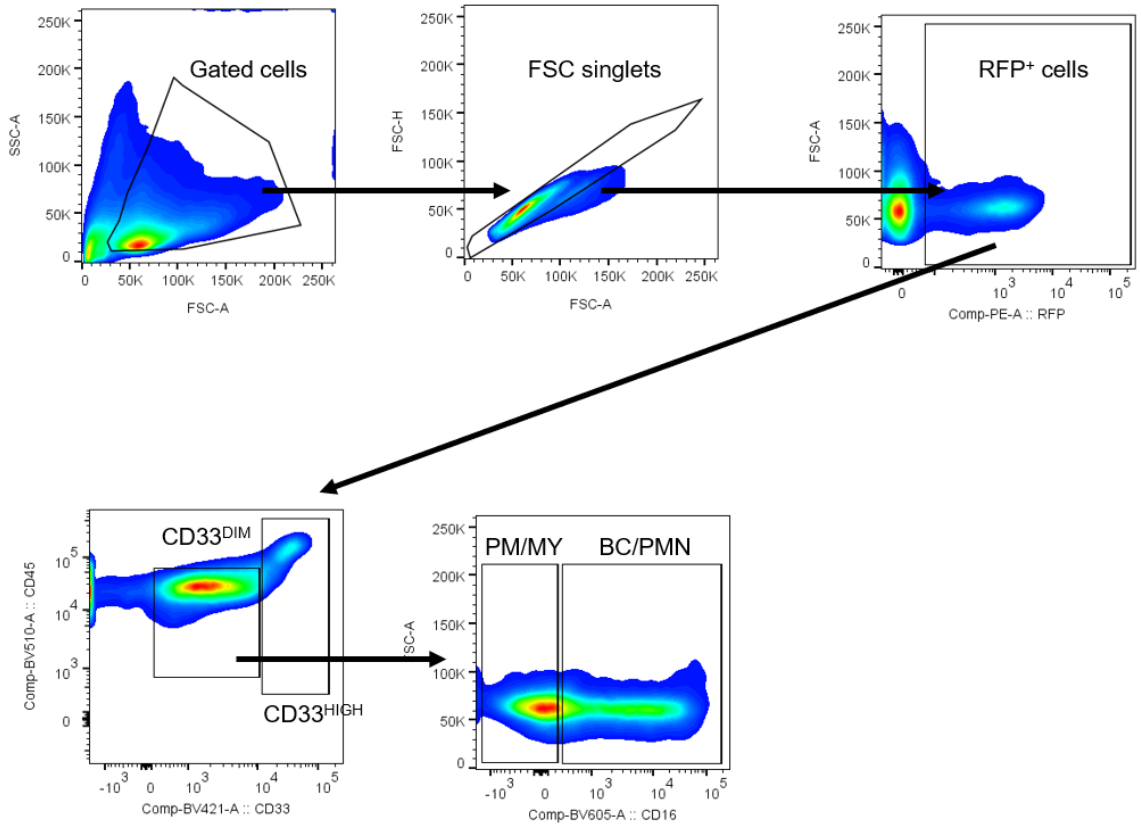
Supplementary figure 1. C/EBPα binding sites on the regulatory regions of *HLF*, *MEIS1* and *MLL*

(A-C) C/EBPα binding sites on promoter regions of (**A**) *HLF*, (**B**) *MEIS1*, and (**C**) *MLL1 (KMT2A)* were analyzed in the UCSC Genome Browser (https://genome-euro.ucsc.edu). Data from the ReMap Atlas of regulatory regions (https://doi.org/10.1093/nar/gkz945) were added to the UCSC Genome Browser and queried against the following cell datasets: bone marrow, CD14, CD34, HL-60, K-562, Kasumi-1, KG-1, lymphocyte, macrophage, MOLM-13, MOLM-14, MOLT-3, monocyte, NB4, SKH1, and SKNO1. Only tracks with peaks at *HLF*, *MEIS1*, and *MLL1 (KMT2A)* promoters are shown. For some cell datasets, C/EBPα ChIP seq results were not available.



Supplementary figure 2. Characterization of CyN iPSC clones

(**A**) Flow cytometry analysis of pluripotency-specific surface markers (Tra1-60 and SSEA4) in indicated CyN iPSC clones. (**B**) Relative mRNA expression of the indicated pluripotency-specific genes in CyN iPSC clones to $CD34^{+}$ cells measured by qRT-PCR. Data were normalized to β-actin. (**C**) Representative images of immunofluorescence staining of ectoderm (anti-Tubulin III, Sigma; 1:200) and endoderm (anti-Sox17, R&D; 1:10) after three weeks of spontaneous differentiation of iPSCs *via* embryoid body system evaluated on Observer Z1 Microscope (Zeiss) and ImageJ Software (NIH). Mesoderm differentiation ability was confirmed by hematopoietic differentiation (see Figure 3).



Supplementary figure 3. Representative FACS gating strategy

(A) Gating strategy of mature myeloid cells after 14 days in liquid culture differentiation. GMP/MB are $RFP^{+}CD45^{+}CD33^{HIGH}$ cells, PM/MY are $RFP^{+}CD33^{DIM}CD16^{-}$ cells and BC/PMN are $RFP^{+}CD45^{+}CD33^{DIM}CD16^{+}$ cells.

References

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