ANKRD26 is a new regulator of type I cytokine receptor signaling in normal and pathological hematopoiesis

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Received: September 5, 2022.
Accepted: February 7, 2023.
Early view: February 16, 2023.

https://doi.org/10.3324/haematol.2022.282049

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Running title: ANKRD26 regulates cytokine-mediated signaling

SUPPLEMENTAL MATERIAL AND METHODS

Study approval

Peripheral blood samples from patients and healthy subjects, and cord-blood samples were collected after informed written consent and obtained in accordance with the Declaration of Helsinki. The study was approved by the Comité de Protection des Personnes CPP N°2020T2-02 and by AP-HP, Hôpital Saint-Louis, Unité de Thérapie Cellulaire, CRB-Banque de Sang de Cordon, Paris, France, N° of authorization: AC-2016-2759.

The NSG mice were provided from Charles River Laboratories. Animal experiments were performed in accordance with 2010/63/UE European legislation and decree n°2013-118 of French legislation and recorded under protocol number APAFIS# 2016-008-7175. Mice were housed in pre-clinical platform of Gustave Roussy Animal Facilities PFEP (Villejuif, France) (ministerial approval n° 94-076-11).

Plasmid constructions

The full cDNA of human ANKRD26 was amplified by PCR from the pFN21a_ANKRD26_Halotag plasmid, using Phusion High-Fidelity Polymerase (Thermo Scientific) and ANKRD26_pEF6/V5-His primers. The PCR product was digested with DpnI enzyme (Thermo Scientific) for 1h at 37°C, to remove the wild-type template plasmid, purified using GeneJET Gel Extraction Kit (Thermo Fisher) and cloned into the linearized pEF6/V5-His TOPO vector using the InFusion kit (Clontech), following the manufacturer's instructions. Then, the ANKRD26_V5-His sequence was amplified by ANKRD26_pRRL primers and cloned into the linearized pRRL_EF1a-MCS-PGK_hygromycin B or pRRL_EF1a-MCS-PGK_Cherry lentiviral vector. Alternatively, ANKRD26 cDNA was amplified from the pFN21a_ANKRD26_Halotag plasmid using FLAG-ANKRD26 primers and cloned into the linearized pRRL_EF1a-MCS-PGK_hygromycin B lentiviral vector. The HA_G-CSFR cDNA was amplified by RT-PCR with primers containing HA sequence and cloned into the MIGR retroviral plasmid. Plasmids were obtained after transformation of XL_10 Gold ultracompetent cells and DNA purification with (NucleoBond Xtra Midi kit, Macherey-Nagel), and constructions were verified by sequencing. Lentiviral vectors encoding either for short hairpin RNAs against ANKRD26 (sinPRRL-H1-shANK-PGK-mCherry or sinPRRL-H1-shANK-PGK-GFP) or a scramble sequence (sinPRRL-H1-shSCR-PGK-mCherry or sinPRRL-H1-shSCR-PGK-GFP) were previously described[10]. Two different shANK (shANK_1 and

shANK_2)[10] giving similar results were used and the results are presented as a mean of both. A retrovirus encoding for wild type Jak2 was used (pREX- Jak2_IRES-CD4) was used for PLA assay. Two different shRNA against CCNI2 (shCCNI2_1 and shCCNI2_2 were cloned) into the sinPRRL-H1-PGK-GFP vector (sinPRRL-H1_CCNI2_PGK-GFP).

Lenti- and Retroviral production

For lentiviral production, HEK293T cells were seeded to achieve 70% confluence at the time of transfection, typically $1x10^6$ cells per T175 flask seeded three days before. The cells were co-transfected with the following plasmids: pCMV, pMD2G, and the plasmid of interest. For retroviral production, HEK293EBNA cells were co-transfected with the following plasmids: GAG-POL, VSV-G, and the plasmid of interest. Transfection was carried out using JetPrime reagents following the manufacturer's instructions. The viral supernatant was collected 48 and 72 hours after transfection, concentrated by ultracentrifugation at 22K rpm for 1h30, and stored at -80°C.

Primary cell culture

$CD34^+$ cells were isolated from umbilical cord blood or peripheral blood by positive selection using immunomagnetic bead cell-sorting system (AutoMacs; Miltenyi Biotec) and cultured in Iscove modified Dulbecco medium with penicillin/streptomycin/glutamine, alpha-thioglycerol, bovine serum albumin, a mixture of sonicated lipids and insulin-transferrin containing SCF (25 ng/mL) and TPO (10 ng/mL), or G-CSF (20 ng/mL), IL-3 (10 ng/mL) and SCF (25 ng/mL) or apo-transferrin instead of insulin-transferrin, EPO (1 U), IL-3 (10 ng/mL) and SCF (25 ng/mL).

Cell lines

Human UT7 cells were maintained in MEMα medium supplemented with 10% fetal calf serum, 1% PenStrep, 1% L-Glutamine, and in the presence of 5 ng/mL of GM-CSF. They were transduced with lentiviral vectors encoding either for short hairpin RNAs against ANKRD26 or a scramble sequence, as previously described[10], and isolated via cell sorting 48 hours later, based on the reporter gene expression. Similarly, UT7 cells were transduced with a lentiviral vector encoding for the full-length ANKRD26 cDNA expressed under the EF1a promoter and a hygromycin B resistance gene expressed under PGK promoter (pRRL-EF1a-ANKRD26/PGK-hygromycin B) and were selected using 400 µg/mL of antibiotic.

Murine Ba/F3 cells were maintained in DMEM medium supplemented with 10% fetal calf serum, 5% Wehi, 1% PenStrep, 1% L-Glutamine, and in the presence of 5% WEHI-conditioned medium. Ba/F3 were transduced with the retroviral vector encoding for wild-type human HA_MPL, HA_EPOR or HA_G_CSFR and the lentiviral vector pRRL_EF1a-ANKRD26/PGK-hygromycin B or the empty vector pRRL_EF1a-MCS_PGK-hygromycin B.
Human Gamma-2A cell line without expression of endogenous JAK2 and Human embryonic kidney HEK293T cell lines were maintained in DMEM medium supplemented with 10% fetal calf serum, 1% PenStrep, 1% L-Glutamine.
All cells were cultured in a humidified incubator at 37°C with 5% CO2.

iPSCs expansion

iPSCs were maintained on Essential 8 or Essential 8 Flex medium (Gibco), on plates coated with N-truncated Human recombinant vitronectin (Gibco). Cell passage was routinely performed using a solution of EDTA 500 M in PBS 1X, or TrypLE 1X (Gibco). A Mycoplasma screening was routinely performed, accordingly to the manufacturer instructions (Sigma). Cells were kept in culture for a limited number of passages, to prevent the surge of karyotype and genomic anomalies. The characterization of iPSCs is described in supplemental material and method section.

iPSC characterization

qRT-PCR analysis of pluripotency markers

The expression of self-renewal stem cell markers was validated by qRT-PCR analysis. qRT-PCR was assessed by the DDCt method on Rotor-Gene Q (Qiagen) using the Maxima SYBR Green qPCR Master Mix (ThermoFisher Scientific). GAPDH was selected as a housekeeping gene and data were normalized to its expression. Statistical analysis was performed using REST (Relative Expression Software Tool) software. The expression of self-renewal stem cell markers of ANK1 and ANK2 cells was compared to hESCs RC17 (Rosalin Cells, Edinburg UK) and hiPSCs CTR2#6 and C1[31].

Immunofluorescence analysis of pluripotency markers

The expression of self-renewal stem cell markers was validated by immunofluorescence analysis performed on cells 48h after thawing. Cells were immunostained at 4 °C overnight with primary antibodies, and with appropriate secondary antibodies for 1 hour at RT; nuclei

were counterstained with Hoechst 33258. The images were acquired using a Leica DMI4000B inverted microscope linked to a DFC360FX or to a DFC280 camera (Leica Microsystems).

Karyotype Analysis

iPS-cells were treated on the 4th day after split for 3 hours with 0.1 μg/ml Colcemid. A total of 32 to 38 metaphases were analyzed and 13-15 total metaphases were karyotyped by QFQ-banding: q-Bands by Fluorescence using Quinacrine and with Fluorescence Microscope Olympus CHB (BX63) with quinacrine mustard filter and CCD camera Bright-Field Microscope"GenASIs" Software, version 8.1.0.47741; Applied Spectral Imaging.

Short Tandem Repeat (STR) Testing Report

The amplification was performed by Polymerase chain reaction (PCR) for each nine autosomal STR molecular markers (D21S11, D7S820, CSF1PO, TH01, D13S317, D16S539, vWA, TPOX, D5S818) along with the gender-determining marker Amelogenin with Promega GenePrint 10 Kit following manufacturer's recommended protocol. Appropriate positive and negative amplification controls were used as kit recommended guidelines. The amplified products were analyzed on an ABI Prism® 3730xl Genetic Analyzer using an Internal Lane Standard 600 (Promega). Data generated was analyzed using GeneMapper® Software version 4.0 (Applied Biosystems) following manufacturer's instructions.

Teratoma formation assay

iPS-cells ($1x10^6$ cells) were resuspended in Essential 8 (140 L) and Geltrex (60 L) to a final volume of 200 L and injected intramuscularly in NSG mice. Mice were monitored and euthanized when the size of the tumoral mass was visibly affecting the animal motility and behavior. Teratomas were excised, fixed and embedded in paraffin, while the corresponding sections were stained with hematoxylin, eosin and safranin.

iPSCs hematopoietic differentiation

Clumps of pluripotent cells were seeded on Geltrex (Gibco)-coated plates, in presence of E8 medium, at day -1. The departing cell concentration was adjusted for each cell line and was comprised in the 10-20% confluence range. At Day 0, cells were transferred in a xeno-free medium based on StemPro-34 SFM (Gibco), supplemented with Penicillin/Streptomycin 1% v/v (Gibco), L-Glutamine 1% v/v (Gibco), 1-Thioglycerol 0.04 mg/mL (Sigma) and ascorbic acid 50 μg/mL (Sigma). This medium was retained for the entire experiment and supplemented with different cytokines and growth factors, accordingly to the following schedule: Days 0 – 2:

BMP4 (10 ng/mL), VEGF (50 ng/mL) and CHIR99021 (2 M). Days 2-4: BMP4 (10 ng/mL), VEGF (50 ng/mL) and FGF2 (20 ng/mL). Days 4 – 6: VEGF (15 ng/mL) and FGF2 (5 ng/mL). Day 6: VEGF (50 ng/mL), FGF2 (50 ng/mL), SCF (50 ng/mL) and FLT3L (5 ng/mL). Days 7-10: VEGF (50 ng/mL), FGF2 (50 ng/mL), SCF (50 ng/mL), FLT3L (5 ng/mL), TPO (50 ng/mL) and IL-6 (10 ng/mL). Days 10-20: SCF (50 ng/mL), G-CSF (25 ng/mL), and IL-3 (10 ng/mL).

Clonogenic potential of primary cells in semi-solid culture

Methylcellulose culture assay: hematopoietic progenitors ($CD34^+CD43^+$ for iPSC and $CD34^+$ for primary cells) were plated in triplicate at different cell densities ($1x10^3$ cells/plate for primary cells, $3x10^3$ cells/plate for iPSC-derived cells) in human methylcellulose medium H4434 (STEMCELL technologies), containing recombinant human cytokines and scored for the presence of colonies 14 days after. When indicated, $CD34^+$ progenitor cells transduced with lentiviral vectors encoding for short hairpin RNAs against ANKRD26 (PRRL_H1-shANK/PGK-GFP) or a scramble sequence (PRRL_H1-shSCR/PGK-GFP), were cultured for 2 days in presence of SCF (25 ng/mL), FLT3L (10 ng/mL), TPO (10 ng/mL), IL-6 (10 ng/mL), IL-3 (10 ng/mL). $CD34^+GFP^+$ cells were then sorted on a BD Influx instrument and seeded on triplicate at $1x10^3$ cells/mL in methylcellulose H4230 (STEMCELL technologies) supplemented with 25 ng/mL SCF alone or with different doses of EPO (1, 0.1, 0.01 U/mL).

Fibrin clot assay: To assess the CFU-MK potential, $CD34^+GFP^+$ or $CD34^+Cherry^+$ sorted cells were seeded at 1.5 x 10^3 cells/mL in triplicate in fibrin clot medium, as previously described[29]. Different concentrations of TPO (0.01 ng/mL, 0.1 ng/mL, 1 ng/mL, 10 ng/mL) were added to 25 ng/mL SCF; plates with only SCF and no cytokines were also prepared. CFU-MK colonies were scored after 10 days, using an indirect alkaline phosphatase–based immunostaining technique based on CD41a detection by monoclonal antibody.

Proliferation assay in liquid medium

UT7/HA_MPL shANK/shSCR cells were washed three times in phosphate-buffered saline (PBS 1X) and seeded in MEMα with the indicated concentrations of TPO or Eltrombopag and 5 ng/mL of GM-CSF as control. $5x10^4$ cells per condition were plated in triplicate and manually assessed every day for 4 days, performing a Trypan blue exclusion and using a Malassez hemocytometer. The same protocol was used to measure the proliferation of UT7/HA_G-CSFR shANK/shSCR cells and UT7/HA_EPOR shANK/shSCR cells, using the appropriate cytokine and GM-CSF as positive control.

Primary cells ($CD34^+$) or iPS-derived progenitors ($CD34^+CD43^+$ sorted at day 14 of culture) were seeded at a cell density of $5x10^4$ cells/well in a 12-wells plate, in serum-free medium containing G-CSF (25 ng/mL), SCF (20 ng/mL) and IL-3 (10 ng/mL) for granulocytic lineage and EPO (1U/mL), SCF (20 ng/mL) and IL-3 (10 ng/mL) for erythroid lineage. Alternatively, iPS-derived $CD34^+CD43^+$ progenitors were sorted at day 14 of culture, transduced with shSCR or shCCNI2 encoding lentiviruses, sorted at day 16 on GFP^+ and $1x10^4$ cells/well were seeded in a 48-well plate. Cell numbers were quantified at the indicated days. The proliferation curves are reported relative to the number of seeded cells.

MGG staining

Cells were centrifuged on slides for 5 minutes at 500 rpm, on a Cytospin 2 centrifuge (Shandon). Slides were stained with May-Grünwald Giemsa (MGG); at least 200 cells per slide were scored. Images were obtained using a Leica DMRB microscope, using a 63x magnification objective. The % of myeloblasts, promyelocytes, myelocytes, metamyelocytes and PNN (based on the morphology) was calculated after counting under the microscope 200 cells corresponding to all these five subtypes.

Proplatelet formation assay: $CD41^+CD42^+GFP^+$ or $CD41^+CD42^+$ cells selected on hygromycin B were sorted at day 10 of culture and seeded at a cell density of $5x10^3$ cells/well in a 96-wells plate, in a serum-free medium containing TPO (10 ng/mL) and SCF (25 ng/mL). Proplatelet-forming cells were scored after 4-5 days by enumeration of no less than 200 cells per well using an inverted microscope (Carl Zeiss), at a 200x magnification. A proplatelet-forming megakaryocyte was considered as a cell displaying at least one cytoplasmic process with a clearly defined constriction area. Each condition was examined in triplicate.

Receptor surface expression and internalization assay

UT7 cells expressing respectively HA_MPL, HA_G-CSFR or HA_EPOR and transduced either with shSCR or shANK were stained with an anti-MPL or anti-HA (Miltenyi) antibody and analyzed on a LSRFortessa flow cytometer (BD).

For internalization assay, Ba/F3-HA_MPL, Ba/F3-HA_MPL-ANKRD26_V5, Ba/F3-HA_EPOR, Ba/F3-HA_EPOR-ANKRD26_V5, Ba/F3-HA_G_CSFR and Ba/F3-HA_G_CSFR-ANKRD26_V5 cells ($2x10^5$ cells per time point) were washed twice in PBS 1x and cytokine-deprived overnight in IMDM medium supplemented with 1,5% bovine serum albumin (BSA). The following days, cells were stimulated with 50 ng/mL TPO, 1 U/mL RPO

or 20 ng/mL G-CSF for 15/30/60 min, a non-stimulated condition was used as T=0. At the end of the stimulation, cells were washed in cold PBS supplemented with 0,5% BSA, incubated with a PE-conjugated anti-CD110 antibody (MPL-PE), an APC-conjugated anti-HA antibody (HA-APC) or a matched IgG for 30 min on ice, and then analyzed on a Canto X flow cytometer (BD). The median fluorescence intensity (MFI) was analyzed.

Flow Cytometry

Single cell suspensions were stained with monoclonal antibodies directly coupled to their respective fluorochromes. A list of the used antibodies, included the isotype control, is available, with the respective clone names (Supplementary Table 4). Cells were incubated with antibodies at 4 °C for at least 30 minutes. Cells were washed before and after incubation in PBS 1X and analyzed with a BD Canto II or BD LSRFortessa cytometers (BD). Cell Sorting was routinely used for hematopoietic cells purification and performed on Influx, ARIA III or ARIA Fusion cell sorters (BD).

Western blot analysis

Signaling studies were performed on UT7 cell lines and iPS-derived cells

UT7 cells expressing respectively HA_MPL, HA_G-CSFR and HA_EPOR and transduced with shSCR or shANK were washed three times in PBS 1X and cytokine-deprived overnight in MEMα medium supplemented with 5% FCS.

Ba/F3 cells expressing respectively HA_MPL, HA_G-CSFR and HA_EPOR and transduced with pRRL_EF1a-MCS-PGK_hygromycin B (empty vector, EV) or pRRL_EF1a-ANKRD26-PGK_hygromycin B (selected by 400 µg/mL Hygromycin B) were washed three times in PBS 1X and cytokine-deprived overnight in IMDM medium supplemented with 1% BSA.

Re-stimulation was done with TPO/G-CSF/EPO for the indicated times and doses at 37°C. Next, cells were washed in ice-cold PBS to block stimulation. Total cell lysates were obtained by addition of 2X Laemmli buffer supplemented with DTT 0.1 M.

iPS-derived cells: cells were washed three times in PBS 1X and starved overnight in serum-free medium, deprived of insulin-transferrin-selenium. At least $4x10^5$ cells per condition were plated. Re-stimulation was done with 25 ng/mL of G-CSF at different time points. Cells were then harvested in ice-cold PBS 1X, washed three times in clod PBS 1X, immediately lysed in ice-cold Laemmli buffer containing DTT 0.1 M, protease inhibitor cOmplete (5X) (cOmplete™, Mini Protease Inhibitor Cocktail, Roche), phosphatase inhibitor cocktail 3 (25X), sodium orthovanadate 50 mM, sodium fluoride 500 mM and PMSF 100 mM.

Lysates were then sonicated and heated at 95°C for 5 minutes. Proteins were resolved by SDS-PAGE and then transferred onto a nitrocellulose membrane. After blocking in 5% BSA in TBS-0.1% Tween20 (TBS-T) for 1 hour, the membranes were incubated with rabbit primary antibodies (anti–phospho-STAT5 (Y705), anti-STAT5, anti–phospho-STAT3 (Y705), anti-STAT3, anti–phospho-AKT (S473), anti-AKT, anti–phospho-ERK1/2, anti-ERK1/2 (Cell Signaling Technologies), diluted 1:1000 in blocking buffer overnight at 4°C with gentle shaking. For detection, the membranes were washed and incubated with secondary HRP-conjugated antibodies, diluted 1:5000 in blocking buffer. Band detection was performed by enhanced chemiluminescence system (ECL or SuperSignal West Pico Plus kit; Life Technologies) using Image Quant LAS 4000 (GE Healthcare)/iBright FL1500 (Thermo Fisher Scientific). The quantification was by ImageJ2 software.

Co-immunoprecipitation

HEK293T cells were transiently transfected with a pMX-IRES-GFP vector encoding respectively for HA_MPL, HA_EPOR and HA_G-CSFR, and with a pEF6/V5-His TOPO vector encoding for ANKRD26, using JetPrime reagents. After 72 hours, 1-2 x 10^7 cells were pelleted by centrifugation and lysed in a buffer containing 50 mM TrisHCl pH 7.5, 150 mM NaCl, 1% NP40, supplemented with fresh protease inhibitors (cOmplete™, Mini Protease Inhibitor Cocktail, Roche) and phosphatase inhibitors (1 mM Na3VO4, 10 mM NaF). Lysates were cleared by centrifugation at 10,000 g for 15 min at 4°C and then incubated with primary antibodies (anti-TPOR 06-944, Millipore; anti-V5 R960-25, Invitrogen; anti-HA ab9110, Abcam) overnight at 4°C with gentle shaking. As no specific antibodies are available for EPOR and G-CSFR, anti-HA was used in these two cases. Next, protein A/G Plus agarose beads (sc-2003, Santa Cruz Biotechnology) were added for 2 hours at 4 °C. Beads were washed once with lysis buffer and once with PBS, and samples were boiled in 2X Laemmli sample buffer supplemented with DTT 0.1 M. Samples were then analyzed by western blotting as described above.

Proximity ligation assay (PLA)

Parental UT7 cells were transduced with FLAG_ANKRD26_Hygromycin and HA_MPL_GFP encoding lenti- or retroviruses, sorted on GFP^+ and cultured in presence of 400 μg/mL Hygromycin B (Sigma Aldrich). Non-transduced cells were used as negative controls.

Alternatively, adherent Gamma-2A cells were transiently transfected with FLAG_ANKRD26_Hygromycin, HA_MPL_GFP and Jak2_CD4 lenti- or retroviruses or corresponding empty vectors by using TransIT®-293 transfection reagent (Mirus). First, the cells were transfected with FLAG_ANKRD26_Hygromycin lentivirus or corresponding empty vector and cultured in presence of 400 µg/mL of Hygromycin B (Sigma Aldrich). Then, cells were transfected with HA_MPL_GFP and/or Jak2_CD4 retroviruses. Two days after second transfection, cells were stained with anti-CD4-APC antibody and $CD4^+GFP^+$ cells were sorted. After sorting, UT7 or Gamma-2A cells were cytocentrifuged (5 min at 500 rpm) to Poly-L-Lysine slides (Thermo Fisher Scientific). Cells were fixed with 4% paraformaldehyde in for 10 min, then permeabilized in PBS 1x, containing 0.2% Triton X-100 for 5 minutes. Fixed cells were incubated overnight with anti-FLAG (F7425, rabbit, MilliporeSigma, dilution 1:400) and anti-HA (901513, mouse, BioLegend, dilution 1:200) antibodies. Proximity ligation assays were performed according to the manufacturer's instructions (Duolink, Sigma Aldrich) using oligonucleotide-coupled secondary antibodies against mouse and rabbit primary antibodies (PLA probes). Only when a pair of PLA probes has bound two primary antibodies in close proximity (<40 nm), a red fluorescent spot is generated as a result of the hybridization and circularization of fluorescently labeled oligonucleotides during the amplification reaction. No fluorescence was detected when the cells were incubated in the presence of the two complementary oligonucleotides and the kit components, but without primary antibodies. Images were acquired under a Confocal Leica SP8 laser-scanning microscope, with a 63x/1.4 numeric aperture oil objective (Leica Microsystem). Slides analyzed for a presence of red dots were scored by enumeration of at least 100 cells per slide. Image analysis was performed with the LASX software. For each experiment at least 10 cells per condition (for UT7) and 40 cells per condition (for Gamma-2A) were analyzed for the number of spots using Image J2 software.

Whole-transcriptome RNA-seq and analysis

Cells were sorted and flash-frozen at -80 °C. RNA extraction and purification were performed with the RNeasy Mini Kit (Qiagen). The RNA integrity (RNA Integrity Score≥7.0) was checked on the Agilent 2100 Bioanalyzer (Agilent) and quantity was determined using Qubit (Invitrogen). SureSelect Automated Strand Specific RNA Library Preparation Kit was used according to manufacturer's instructions with the Bravo Platform. Briefly, 50 to 200ng of total RNA sample was used for poly-A mRNA selection using oligo(dT) beads and subjected to thermal mRNA fragmentation. The fragmented mRNA samples were subjected to cDNA synthesis and were further converted into double stranded DNA using the reagents supplied in

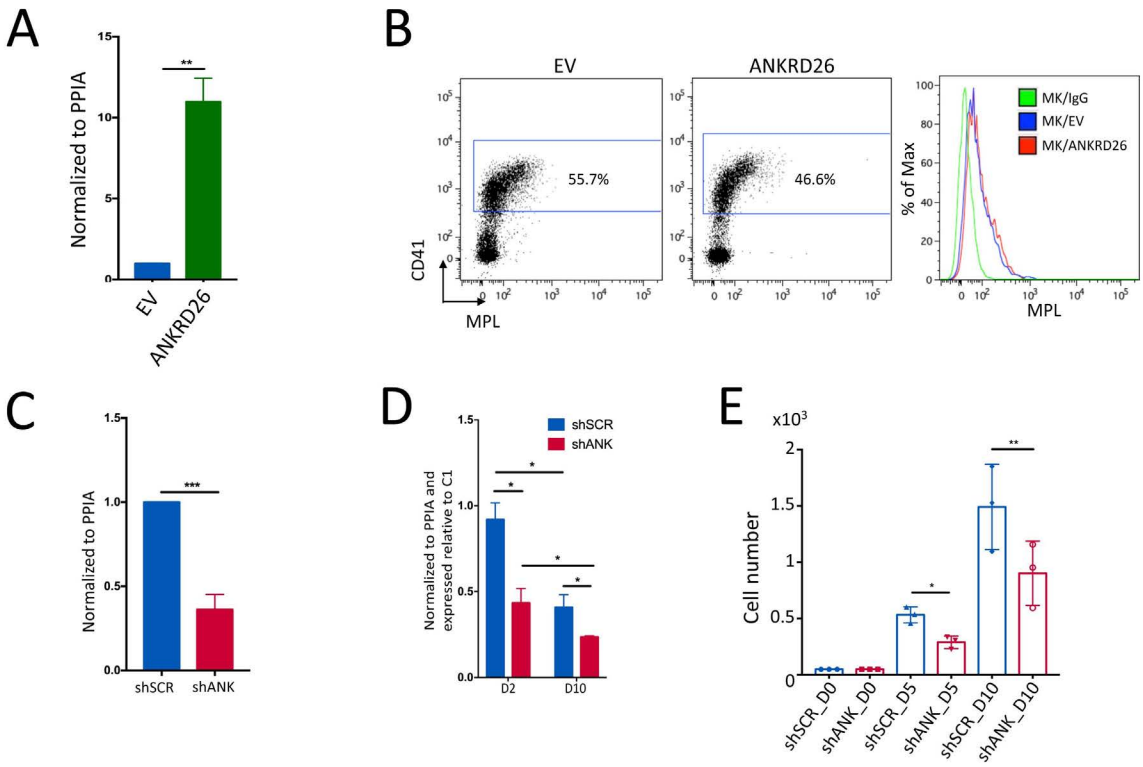
the kit, and the resulting dsDNA was used for library preparation. The final libraries were bar-coded, purified, pooled together in equal concentration and subjected to paired-end sequencing on Novaseq-6000 sequencer (Illumina).
Quality controls were assessed on raw fastq with RseqQC and FastQC. Remaining rRNA were filtered out with BWA, on UCSC's HG19. Filtered reads trimming was performed with Trimmomatic using official TrueSeq adapter list, filtering qualities lower than 20 on a sliding window of size 6, and a minimum length of 40 bases. Remaining reads were mapped with TopHat2 against UCSU's HG19. The insert length deducted in previous RSeQC step, and the realignment edition distance parameter set to 0 were used as optional parameters. Counts were performed over Samtool-sorted bam with HTSeqCount. Rows full of zeros and non-genic annotations from HTSeqCount were removed through in-house bash scripts. The differential analysis was performed with DEseq2, using VST normalization method and default GLM model. Quality control graphs and results were performed with in-house R-scripts (available on request). The data were submitted to EGA under the number EGAD00001008023.
GSEA was performed using the software GSEA (v4.1.0).

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

RNA was purified using the TRIzol™ Reagent (Invitrogen), RNeasy Plus Micro or Mini Kit, accordingly to the cell number and the manufacturer instructions (Qiagen). Complementary DNA synthesis was performed with the Super Script II cDNA synthesis Kit or the Super Script Vilo cDNA synthesis kit (Thermo Fisher), accordingly to the amount of extracted RNA. Real Time PCRs were performed using the Takara Bio SYBR Premix Ex Taq (Clontech) reaction mix, on the 7500 Real Time PCR system (Applied Biosystems). Gene expression was assessed by comparative CT method, using PPIA or HPRT as reference genes.

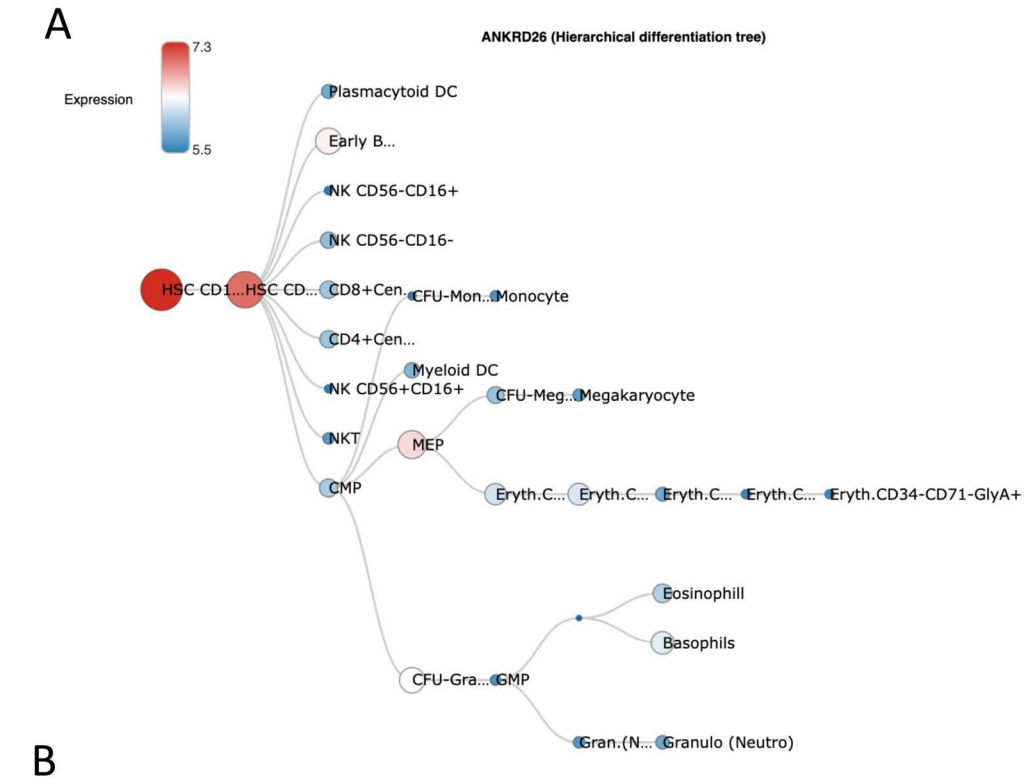
Supplementary Figures

Supplementary Figure 1



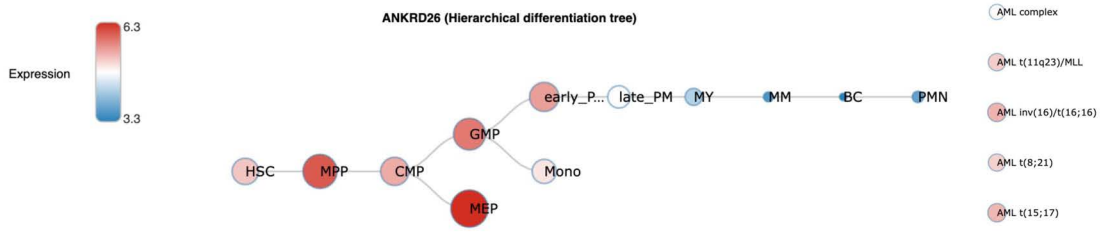
Supplementary Figure 1. Quantification of ANKRD26 transcripts.
A) *ANKRD26* transcript level in cord-blood (CB)-CD34$^+$ cells transduced with empty lentivirus (EV) or lentivirus encoding ANKRD26_V5 (ANKRD26) and Cherry. CD34$^+$Cherry$^+$ cells were sorted at day 2 post-transduction. *ANKRD26* transcript level was measured by qRT-PCR and normalized to *PPIA* housekeeping gene. B) Primary CD34$^+$ cells were transduced with empty lentivirus (EV) or lentivirus encoding *ANKRD26_V5* (ANKRD26) and gene resistant to hygromycin B and culture for 10 days in the presence of SCF and TPO. MPL level at megakaryocytes (CD41$^+$ cells) cell surface was not affected by ANKRD26 overexpression. C, D) CB-CD34$^+$ or adult CD34$^+$ hematopoietic progenitors were transduced with lentiviruses encoding shSCR (control) or shANK (shANK_1 or shANK_2) and GFP. CD34$^+$GFP$^+$ cells were sorted at day 2 post-transduction. C) *ANKRD26* transcript level was measured in CB-CD34$^+$GFP$^+$ cells sorted at day 2 post-transduction. D) *ANKRD26* transcript level was measured in adult-CD34$^+$GFP$^+$ cells sorted at day 2 post-transduction (D2) and in megakaryocytes at day 10 (D10) of culture in presence of TPO and SCF.
ANKRD26 level was measured by qRT-PCR and normalized to *PPIA* housekeeping gene. Shown are averages of 3 independent experiments as mean±SD. In A, the results are presented relative to EV. *P<0.05; **P<0.01; ***P<0.005, paired t-test.

Supplementary Figure 2



B

From: https://servers.binf.ku.dk/bloodspot/?gene=ANKRD26&dataset=DMAP

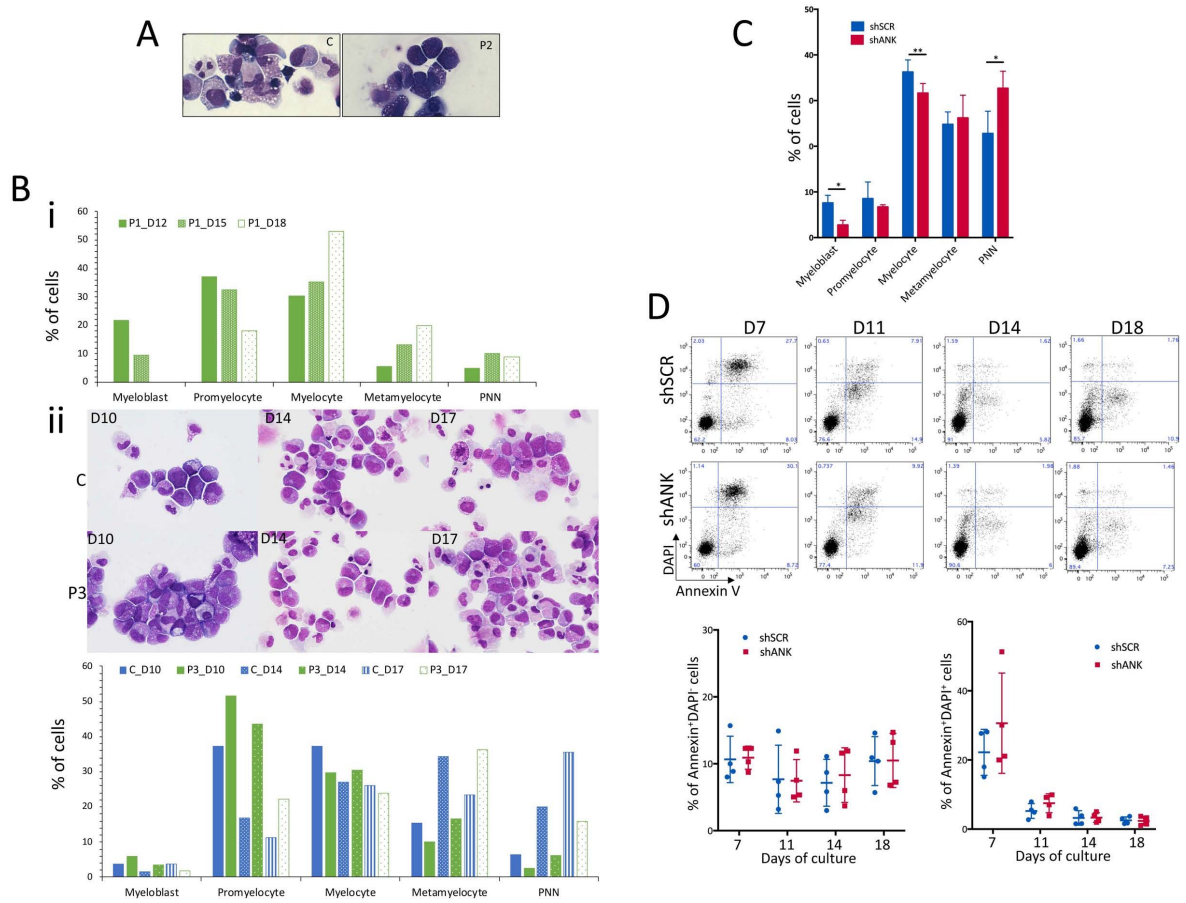


From: https://servers.binf.ku.dk/bloodspot/?gene=ANKRD26&dataset=normal_human_v2_with_AMLs

Supplementary Figure 2. ANKRD26 expression level through hematopoietic differentiation.

The data are extracted from BloodSpot database (https://servers.binf.ku.dk/bloodspot/). ANKRD26 is highly expressed in hematopoietic stem cells (HSC) and multipotent progenitors (MPP) (A, B), and its expression level decreased through megakaryocyte (A), erythrocyte (A) and granulocyte (A, B) differentiation. ANKRD26 is expressed in different AML subtypes (B).

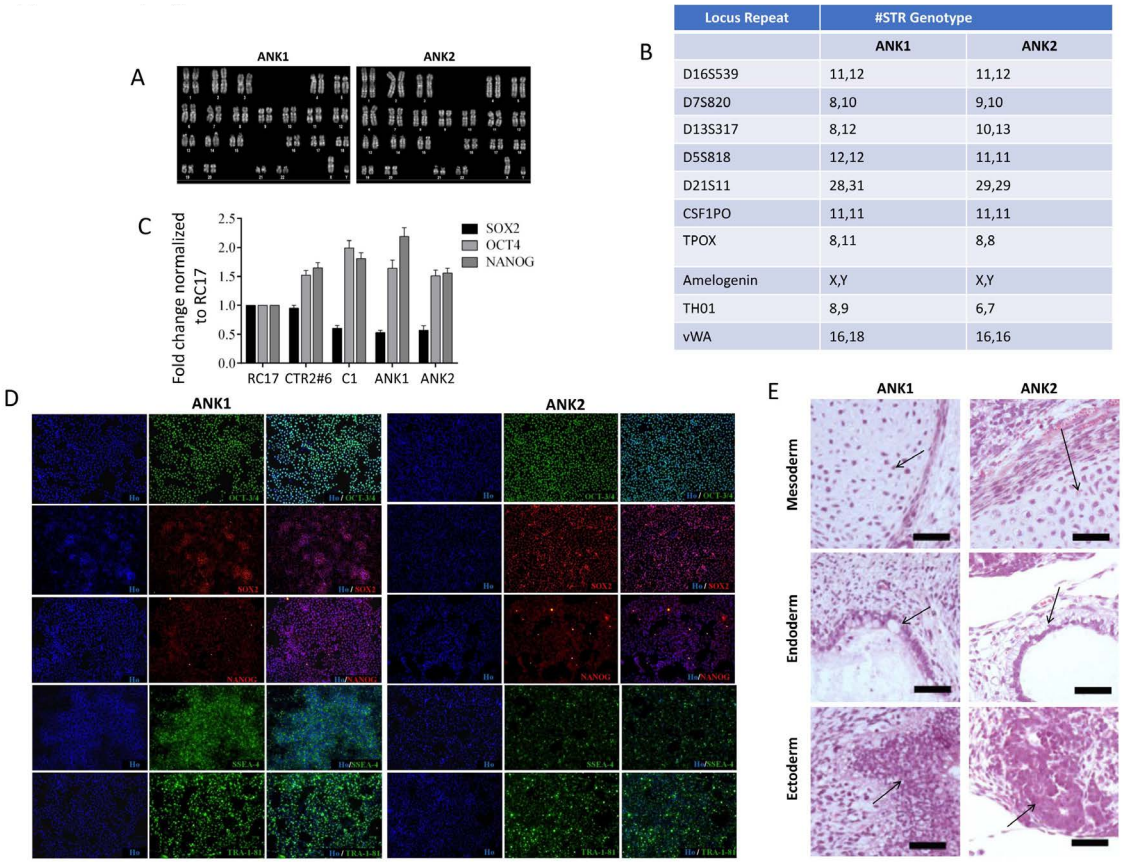
Supplementary Figure 3



Supplementary Figure 3. ANKRD26 levels affect granulocyte maturation, but not apoptosis.

A) MGG staining of patient 2 (P2) and 1 control samples at day 15 of culture. B) Kinetics of granulocyte differentiation for one control and two patient (P1 and P3) samples. MGG staining and analysis of the proportion of myeloblasts, promyelocytes, myelocytes, metamyelocytes and PNN was performed at different days of culture of $CD34^+$ cells in presence of G-CSF, IL3 and SCF. The kinetics for patient 1 (P1) at days 12 (D12), 15 (D15) and 18 (D18) of culture is shown on the top panel (i). MGG staining corresponding to control (C) and patient 3 (P3) samples is shown in the middle panel (ii) and the kinetics for P3 and C at days 10 (D10), 14 (D14) and 17 (D17) of culture is shown on the bottom panel (ii). The figure shows that ANKRD26 overexpression induces a delay, but not a blockage of granulocytic differentiation *in vitro* in THC2 patients. C-D) $CD34^+$ cells were transduced with lentiviruses encoding respectively shSCR and shANK (shANK_1/shANK_2), and were sorted 2 days after transduction. They were grown in liquid medium in presence of 25 ng/mL SCF, 10 ng/mL IL-3 and 20 ng/mL G-CSF. C) MGG staining performed at day 14 of culture showed an accelerated differentiation of shANK-transduced cells. Results are shown as mean±SEM, *P<0.05; **P<0.01; paired t-test. D) Effect of ANKRD26 inhibition on apoptosis in granulocytic lineage. Apoptosis (Annexin-V^+DAPI^- and Annexin-V^+DAPI^+) was measured at different days of culture. The upper panel: the FACS analysis is shown for one of 3 experiments. The lower panel: the averages of 3 independent experiments are shown as mean±SEM, *P<0.05; **P<0.01; paired t-test.

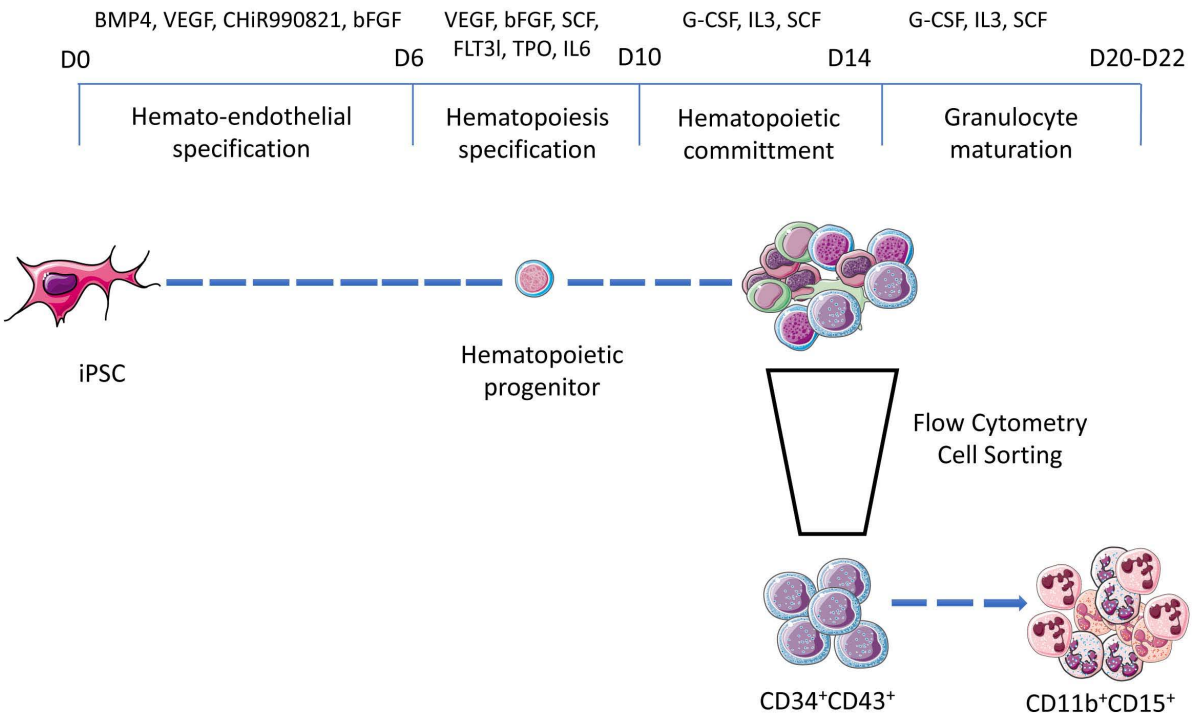
Supplementary Figure 4.



Locus Repeat	#STR Genotype	
	ANK1	ANK2
D16S539	11,12	11,12
D7S820	8,10	9,10
D13S317	8,12	10,13
D5S818	12,12	11,11
D21S11	28,31	29,29
CSF1PO	11,11	11,11
TPOX	8,11	8,8
Amelogenin	X,Y	X,Y
TH01	8,9	6,7
vWA	16,18	16,16

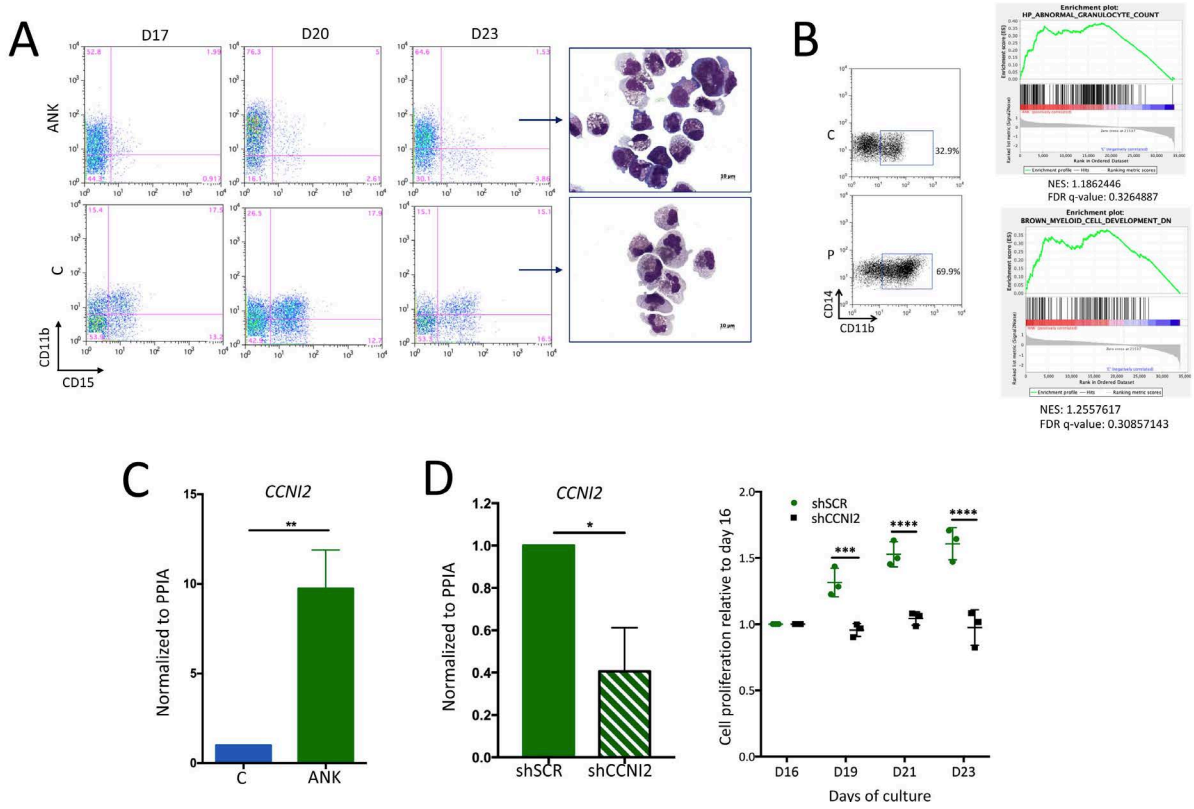
Supplementary Figure 4. Characterization of iPSC ANK1 and ANK2 lines. A) Karyotype analysis showing normal 46, XY karyotype for both ANK1 and ANK2 iPSC lines. 32 to 38 total metaphases were analyzed and 13-15 total metaphases were karyotyped by Q-binding method with resolution of 350 bands. B) Short Tandem Repeat (STR) Testing Report. C-D) Analysis of pluripotency markers. C) qRT-PCR analysis of *SOX2*, *OCT4* and *NANOG* transcripts relative to *GAPDH* housekeeping gene and normalized to control line hESCs RC17 cell line. D) Immunofluorescence analysis of pluripotency markers OCT3/4, SOX2, NANOG, SSEA-4, TRA-1-81. E) *In vivo* differentiation of iPSC ANK1 and ANK2 lines into 3 germ layers; mesoderm, endoderm and ectoderm.

Supplementary Figure 5



Supplementary Figure 5. Schema of granulocyte differentiation protocol. D indicates days of culture.

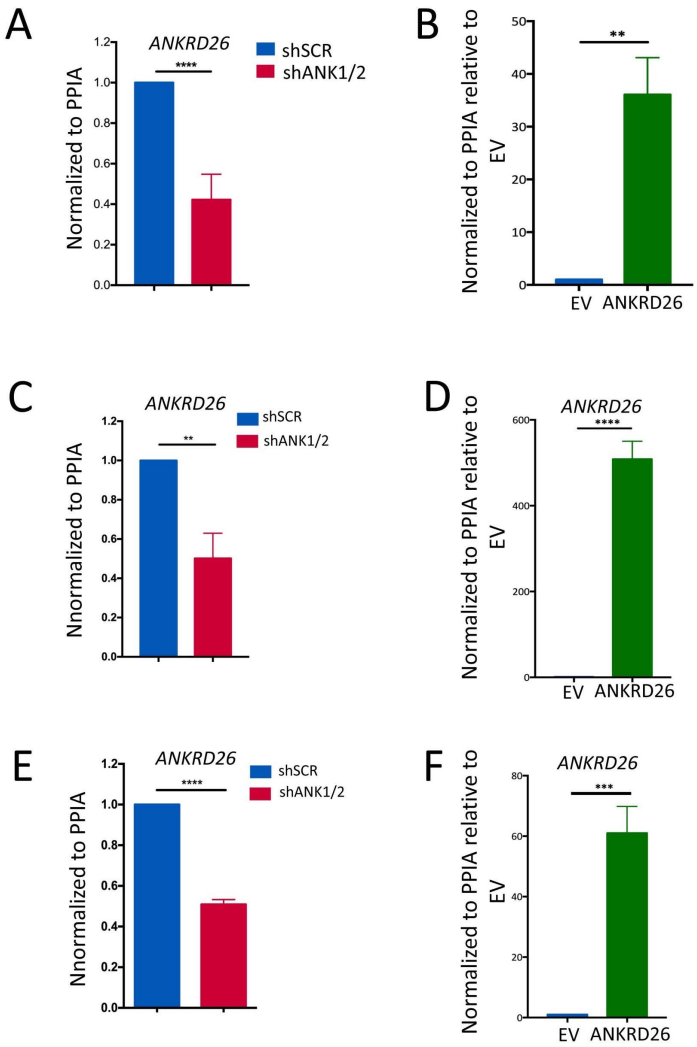
Supplementary Figure 6



Supplementary Figure 6. Increased ANKRD26 expression level enhances proliferation of granulocyte progenitors in an iPSC model.

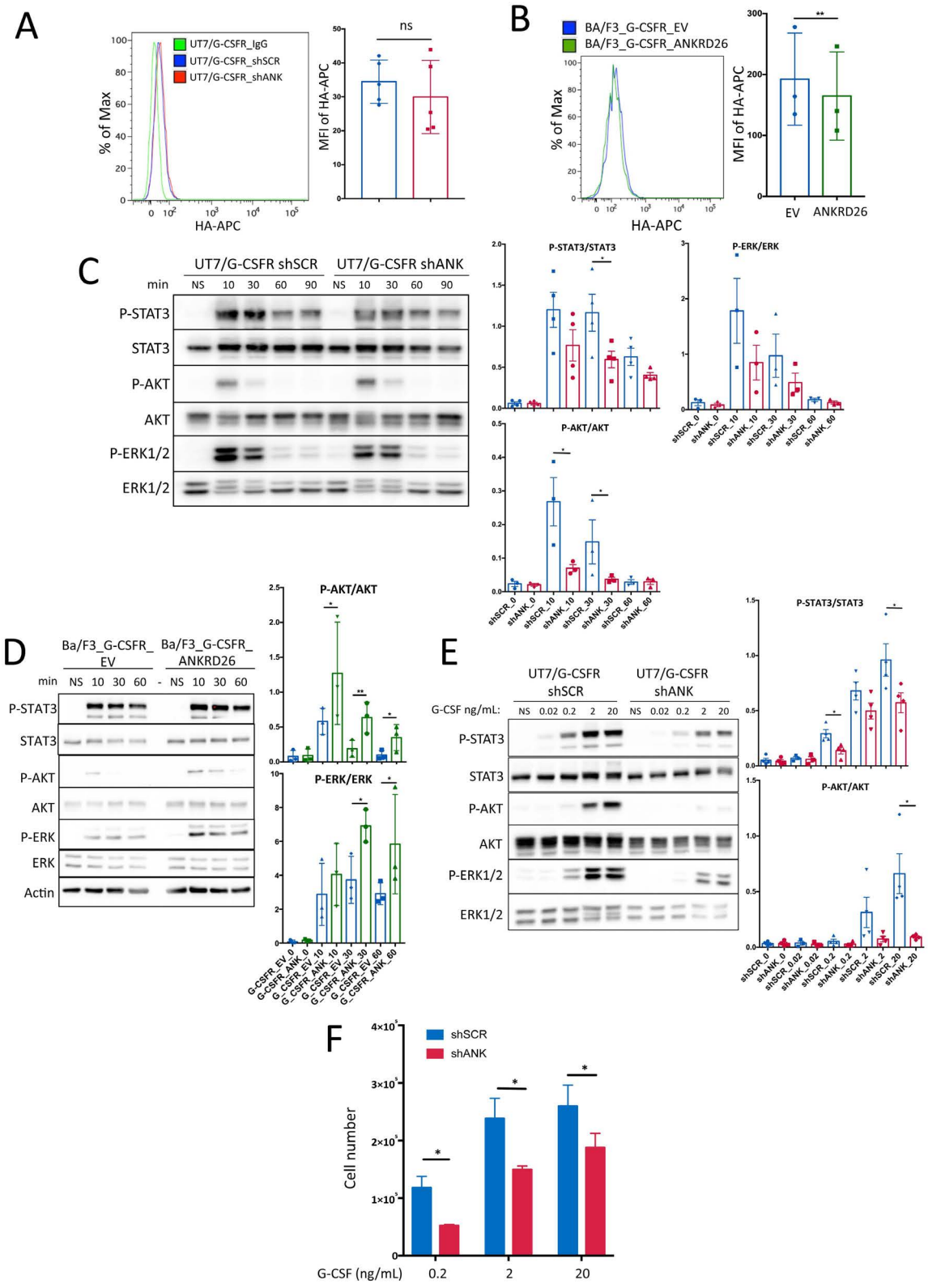
A) FACS analysis of $CD34^+CD43^+$ progenitors derived from patient (ANK) and control (C) iPSC differentiated as granulocytes in the presence of G-CSF, SCF and IL-3 at different days of culture. MGG staining of $CD11b^+CD15^+CD14^-$ cells at day 23 is shown on the right. B) RNA-seq assay was performed on $CD43^+CD11b^+CD14^-$ progenitors sorted at day 16 of culture. FACS analysis of sorted cells is shown on he left panel. On right panel are shown GSEA analysis for HP_ABNORMAL_GRANUCOLYTE_COUNT (HP:0032309) and for BROWN_MYELOID_CELL_DEVELOPMENT_DN in ANK versus Control iPSC. C) qRT-PCR analysis confirmed the CCNI2 up-regulation in patient $CD43^+CD11b^+CD14^-$ cells sorted at day 16 of culture. Shown are the averages of 3 independent experiments as mean±SD, **P<0.01; unpaired t-test. D) $CD34^+CD43^+$ cells derived from patient iPSC (D14 of culture) were transduced with lentiviruses encoding shSCR and shCCNI2, sorted on GFP^+ 2 days after transduction (Day 16 of culture) and cultured for 7 days in the presence of G-CSF, SCF and IL-3. CCNI2 inhibition measured by qRT-PCR (*P<0.05, unpaired t-test) (left panel) led to inhibition of proliferation (right panel) confirming that CCNI2 up-regulation in patient cells contributes to the increased proliferation rate. Shown are the averages of 3 independent experiments (n=1 for shCCNI2_1 and n=2 for shCCNI2_2) as mean±SD, ***P<0.005; ****P<0.001; 2-way ANOVA with multiple comparisons.

Supplementary Figure 7



Supplementary Figure 7: Down- and up-regulation of ANKRD26 in UT7 and Ba/F3 cells. UT7 cells expressing MPL (A), G-CSFR (C) or EPOR ((E) were transduced with lentivirus harboring control scramble shRNA (shSCR) and shANKRD26 (shANK) respectively. Ba/F3 cells expressing MPL (B), G-CSFR (D) or EPOR (F) were transduced with empty lentivirus (EV) or lentivirus encoding *ANKRD26* cDNA (ANKRD26). *ANKRD26* transcript level was measured by qRT-PCR and normalized to *PPIA* housekeeping gene. Shown are averages of 3 independent experiments as mean±SD (2 with shANK1_1 and 1 with shANK_2). **P<0.01; ****P<0.001; ns: non-significant, t-test with Mann Whitney correction.

Supplementary Figure 8

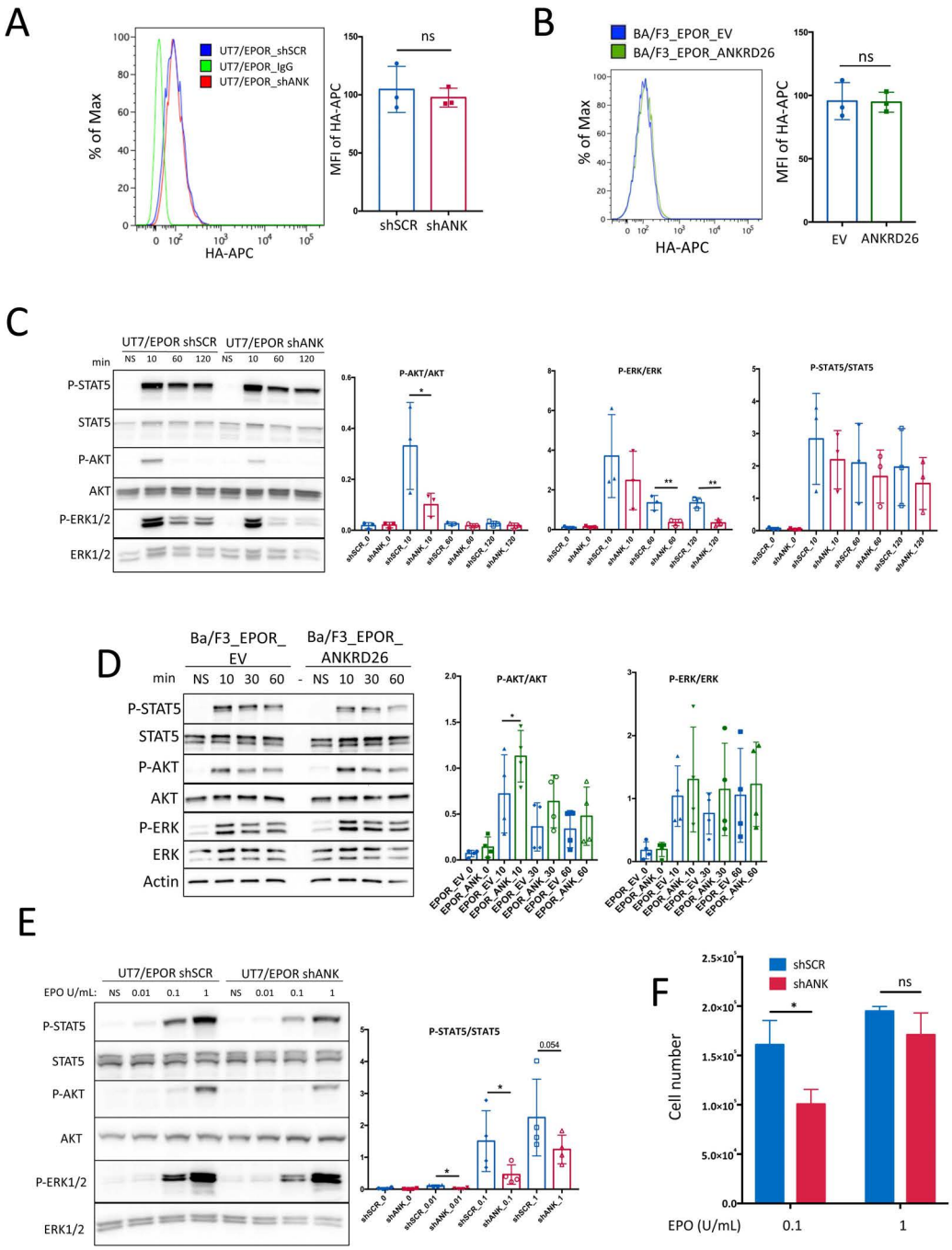


Supplementary Figure 8. Higher ANKRD26 leads to the increased G-CSF/G-CSFR signaling.

UT7 or Ba/F3 cell lines expressing G-CSFR were transduced with the lentiviruses harboring control scramble shRNA (shSCR), shANKRD26 (shANK), *ANKRD26* cDNA or empty virus (EV). A, B) Down-regulation (A) or upregulation (B) of ANKRD26 expression level did not

affect the expression of G-CSFR measured with anti-HA antibody. The receptor levels are presented as median fluorescence intensity (MFI) at the cell surface. Shown are averages of 3 independent experiments as mean±SD (2 with shANK1_1 and 1 with shANK_2). **P<0.01; ****P<0.001; ns: non-significant, t-test with Mann Whitney correction. C-E) One of at least three independents Western Blot (WB) analyses on signaling proteins in Ba/F3 and UT7 cells, at different times post-stimulation with 20 ng/mL of G-CSF (C-D), and different G-CSF doses (E) at 10 min. The histograms show quantification of the WBs representing averages of 3 or 4 independent experiments as mean±SD. *P<0.05; **P<0.01; paired t-test. F) Number of UT7/G-CSFR cells measured at day 4 of culture, with 3 different doses of G-CSF are shown as mean±SEM of three independent experiments, *P<0.05, t-test with Mann-Whitney correction was used.

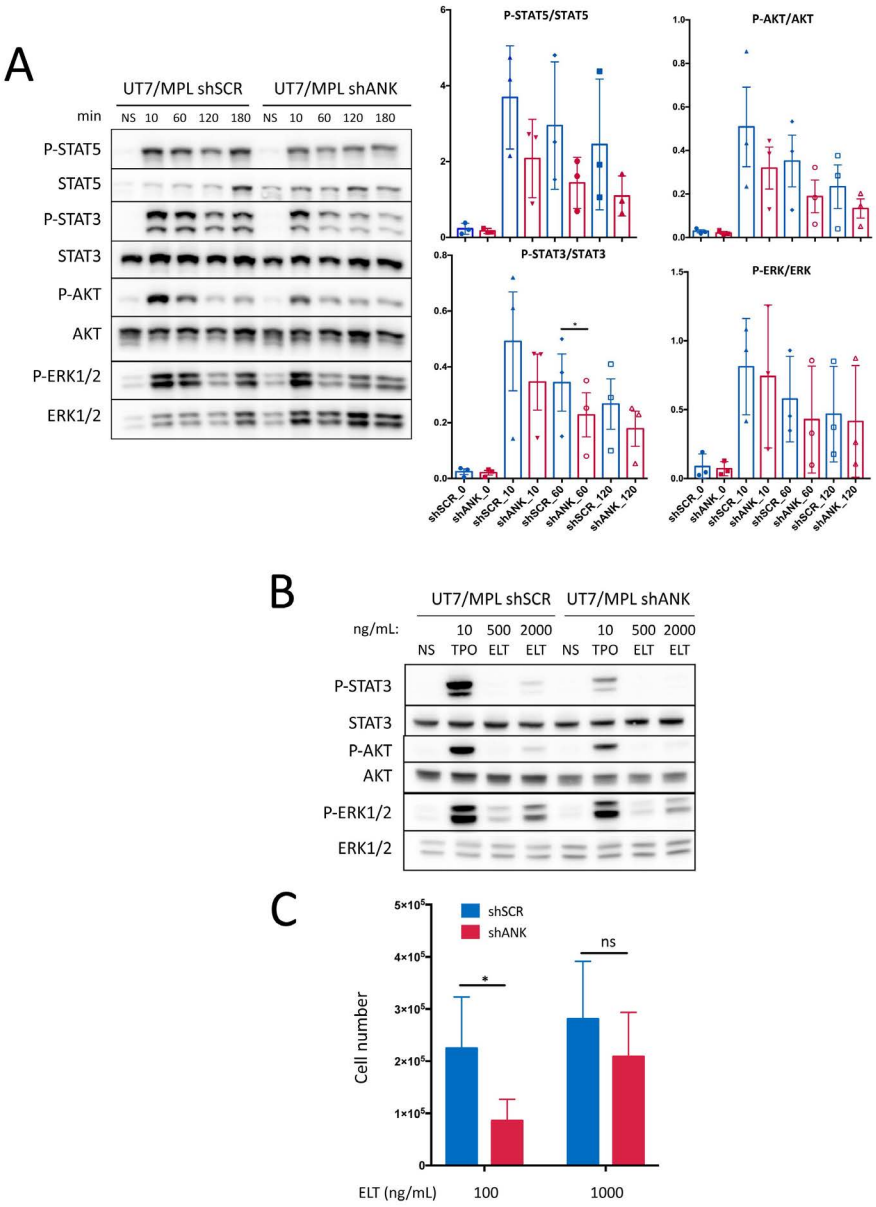
Supplementary Figure 9



Supplementary Figure 9. Higher ANKRD26 leads to the increased EPO/EPOR signaling. UT7 or Ba/F3 cell lines expressing EPOR were transduced with the lentiviruses harboring control scramble shRNA (shSCR), shANKRD26 (shANK), *ANKRD26* cDNA or empty virus (EV). A-B) Down-regulation (A) or upregulation (B) of ANKRD26 expression level did not affect the expression of EPOR measured with anti-HA antibody. The receptor levels are presented as median fluorescence intensity (MFI) at the cell surface. Shown are averages of 3 independent experiments as mean±SD (2 with shANK1_1 and 1 with shANK_2). **P<0.01; ****P<0.001; ns: non-significant, t-test with Mann Whitney correction.C-E) One of at least three independents Western Blot (WB) analyses on signaling proteins in Ba/F3 and UT7 cells, at different times post-stimulation with 1 U/mL of EPO (C-D), and different EPO doses (E) at 10 min. The histograms show quantification of the WBs representing averages of 3 or 4

independent experiments as mean±SD. *P<0.05; **P<0.01; paired t-test. F) Number of UT7/EPOR cells measured at day 4 of culture, with 2 different doses of EPO are shown as mean±SEM of three independent experiments, *P<0.05, ns: non-significant, t-test with Mann-Whitney correction was used.

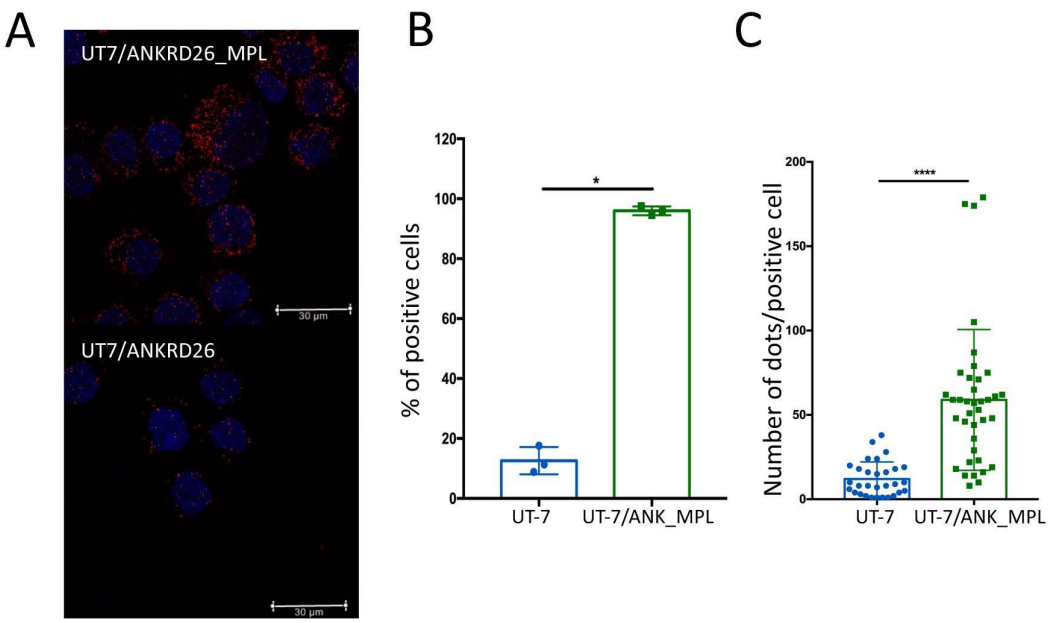
Supplementary Figure 10.



Supplementary Figure 10. Higher ANKRD26 leads to the increased TPO/MPL and ELT/MPL signaling.

A) HA_MPL expressing UT7 cell line (UT7/MPL) was transduced with the lentiviruses harboring control scramble shRNA (shSCR) or shANK (shANK_1 or shANK_2). Western blot analysis and quantification of TPO induced signaling in UT7/HA_MPL (UT7/MPL) cells transduced with shSCR or shANK at different times post-stimulation of cells with 10 ng/mL of TPO. The histograms show quantification of the WBs representing averages of 3 independent experiments as mean±SEM. *P<0.05; paired t-test. B) Western blot analysis at different doses of Eltrombopag (ELT) at 10 min of stimulation showing that higher ANKRD26 expression (shSCR) leads to a hypersensitivity of MPL to ELT. C) Cell number of UT7/MPL cells measured at day 4 of culture with 2 different ELT doses is shown as mean±SEM of 3 independent experiments (2 with shANK_1 and 1 with shANK_2), *P<0.05, ns: non-significant, paired t-test with was used.

Supplementary Figure 11



Supplementary Figure 11. ANKRD26 and MPL interaction

Representative pictures of proximity ligation assay for the ANKRD26 and MPL interaction in UT7 cell line. FLAG_ANKRD26 (ANK) and HA_MPL (MPL) were overexpressed in UT7 cell line. Monoclonal anti-FLAG antibody was used for ANKRD26 and polyclonal anti-HA for MPL. Left panel: the representative pictures (red staining) represent the interaction between ANKRD26 and MPL, scale bar = 30 µm. Middle panel: data represent the mean±SD of 3 independent experiments. Right panel: at least 40 positive cells were analyzed for each condition. *P<0.05, ****P<0.001, t-test with Mann Whitney correction.

Supplementary Table 1. List of primers, antibodies and other products used

Primer	Sequence	Experiment
ANKRD26_F	CTATGTCAGAGGCTTCACTGGAG	qPCR
ANKRD26_R	CTCAGCACATCTGACAGCTTCTG	qPCR
PPIA_F	GTCAACCCCACCGTGTTCTT	qPCR
PPIA_R	CTGCTGTCTTTGGGACCTTGT	qPCR
SOX2_F	GACAGAGCCCATTTTCTCCA	qPCR
SOX2_R	AAATCCTGTCCTCCCATTCC	qPCR
NANOG_F	TATGCCTGTGATTTGTGGGC	qPCR
NANOG_R	GTTTGCCTTTGGGACTGGTG	qPCR
OCT-4_F	AGAGGATCACCCTGGGATAT	qPCR
OCT-4_R	CGCCGGTTACAGAACCACAC	qPCR
GAPDH_F	TGTTCGACAGTCAGCCGCAT	qPCR
GAPDH_R	TAAAAGCAGCCCTGGTGACC	qPCR
CCNI2_F	TGACTATATTCCATGCCCTGGTG	qPCR
CCNI2_R	AGTGTGGAGCCCTTGAACTG	qPCR
seq_ANKRD26_R1	AGCCATTCTTCCTGAGCAAA	sequencing
seq_ANKRD26_R2	TTGTGGGAAGGTTTTGGAAG	sequencing
seq_ANKRD26_F3	CTTCCAAAACCTTCCCACAA	sequencing
seq_ANKRD26_R4	AGTTGCCTCTGAGCGTCATT	sequencing
seq_ANKRD26_R5	TGCAGTCTCCAGTTCTGCTG	sequencing
seq_ANKRD26_F6	TTACAGGCACAAGCAGCATC	sequencing
seq_ANKRD26_R7	GCATCATCCAGTTGTTGTCG	sequencing
ANKRD26_pEF6/V5-His_F	TACCGAGCTCGGATCCCACCATGAAGAAGATTTTTAGTAAG	InFusion cloning
ANKRD26_pEF6/V5-His_R	GCCACTGTGCTGGATGATCATATAATTTTTCTTTAAAACC	InFusion cloning
PRRL_ANK_F	TCCTAGGCCTACGCGCCACCATGAAGAAGATTTTTAGT	InFusion cloning
PRRL_ANK_R	GCATGCCGGCACGCGTCAATGGTGATGGTGATGATGA	InFusion cloning
Infusion_FLAG_ANKRD26_3e_F	TCCTAGGCCTACGCGTCCACCATGGACTACAAGGACGACGATGACAAGAAGAAGATTTTTAGTAAGAAG	InFusion cloning
Infusion_FLAG_ANKRD26_2e_R	GCATGCCGGCACGCGTTCAGATCATATAATTTTTCTTTAAAACCTGTAC	InFusion cloning
shCCNI2_1	TACCTGCATTGCGCCACAATT	Lentiviral cloning
shCCNI2_2	CTGGACTTCTTGACTATATTC	Lentiviral cloning

Antibody	Source	Clone
CD34-PE	BD Biosciences	581
CD34-PE-Cy7	Biolegend	581
CD43-APC	BD Biosciences	1G10
CD11b-PE	BD Biosciences	ICRF44
CD14-APC-H7	BD Biosciences	M5E2
CD15-V450	BD Biosciences	HI98
CD45-FITC	BD Biosciences	HI30
Oct3/4	Santa Cruz	C-10
Sox2	Millipore	Polyclonal
TRA 1-81	Millipore	TRA-1-81
SSEA4	Millipore	MC-813-70
Nanog	Abcam	Polyclonal
IgM-APC	BD Biosciences	G155-228
IgG1-PE	BD Biosciences	MOPC-21
IgG1-PerCP-Cy™5.5	BD Biosciences	MOPC-21
IgG2a-Alexa Fluor® 647	BD Biosciences	MOPC-173
IgM-Alexa Fluor® 488	Invitrogen	Polyclonal
IgG-Alexa Fluor® 488 F(ab')2 fragment	Invitrogen	Polyclonal
IgG-Alexa Fluor® 568 F(ab')2 fragment	Invitrogen	Polyclonal
CD110-PE	BD Biosciences	1.6.1
CD110-APC	BD Biosciences	1.6.1
CD235/GPA-PE-Cy7	BD Biosciences	GA-R2 (HIR2)
CD36-APC	BD Biosciences	CB38 (NL07)
HA-APC	Miltenyi	GG8-1F3.3.1
STAT3	Cell Signaling Technology	D1A5
STAT5	Cell Signaling Technology	D3N2B
AKT (pan)	Cell Signaling Technology	C67E7
p42/44 MAPK (Erk1/2)	Cell Signaling Technology	Polyclonal
Phospho STAT3 (Y705)	Cell Signaling Technology	D3A7
Phospho STAT5 (Y694)	Cell Signaling Technology	D47E7
Phospho AKT (S473)	Cell Signaling Technology	D9E
Phospho p42/44 MAPK (Erk1/2) (T202/Y204)	Cell Signaling Technology	Polyclonal
Actin	Sigma	AC-15
IgG anti-rabbit H+L Alexa Fluor 633	Invitrogen/Thermo Fisher Scientific	Polyclonal
IgG anti-mouse H+L Alexa Fluor 546	Invitrogen/Thermo Fisher Scientific	Polyclonal
IgG anti-rabbit HRP-linked	Cell Signaling Technology	Polyclonal
IgG anti-mouse HRP-linked	Cell Signaling Technology	Polyclonal
TPOR/c-MPL	Millipore	Polyclonal
FLAG rabbit	Millipore, Sigma	Polyclonal
V5 mouse	Invitrogen	Unknown clone
HA mouse	Biolegend	16B12
CD4-APC	BD Pharmingen	555349

Annexin -PE	BD Pharmingen	5556421
HA rabbit	Abcam	Polyclonal
CD41a-APC	BD Biosciences	HIP8
CD42-PE	BD Biosciences	ALMA.16
Reagent	**Source**	**Identifier**
hBMP4	Peprotech	AF-120-05ET
hVEGF	Peprotech	100-20
hFGF-basic	Peprotech	100-18B
hIL-6	Peprotech	200-06
hEPO	Peprotech	100-64
hG-CSF	Peprotech	300-23
hGM-CSF	Peprotech	300-03
hIL-3	Peprotech	200-03
hTPO	Kyowa Kirin, Tokyo, Japan	/
hFLT3l	Celldex Therapeutics, Inc., Needham, USA	/
hSCF	Biovitrum AB, Stockolm, Sweden	/
CHiR 99021 trihydrochloride	TOCRIS	4953
Y-27632 dihydrochloride	TOCRIS	1254
VTN-N	Gibco/Thermo Fisher Scientific	A14700
Geltrex	Gibco/Thermo Fisher Scientific	A1413202
StemPro®-34 SFM	Gibco/Thermo Fisher Scientific	10639011
E8	Gibco/Thermo Fisher Scientific	A1517001
E8 Flex	Gibco/Thermo Fisher Scientific	A2858501
CytoTune™-iPS 2.0 Sendai Reprogramming Kit	Invitrogen/Thermo Fisher Scientific	A16517
Minimum Essential Medium a	Gibco/Thermo Fisher Scientific	22561021
Fetal Bovine Serum	Hyclone	SV30160.03
1-thioglycerol	Sigma	M6145
cOmplete™ inhibitor cocktail	Sigma/Roche	4693159001
Phosphatase Inhibitor Cocktail 3	Sigma	P0044
PMSF	Sigma	P7626
Sodium Orthovanadate	Sigma	S6508
Sodium Fluoride	Sigma	201154
Duolink® In Situ Red Starter Kit Mouse/Rabbit	Sigma	DUO92101
DMEM	Gibco/Thermo Fisher Scientfic	41966
Trypsin/EDTA 0.05%	Sigma	23500054
TrypIE Express	Gibco/Thermo Fisher Scientfic	12605036
Ascorbic Acid	Gibco/Thermo Fisher Scientfic	A4403
TransIT®-293 Transfection Reagent	Mirus Bio LLC	MIR 2706
Penicillin/Streptomycin	Gibco/Thermo Fisher Scientfic	15140-122
Glutamine	Gibco/Thermo Fisher Scientfic	25030123

Supplementary Table 2

	Mutation	Sex	Family history of malignancy	Platelet count (x10^9/L)	Experiment (Figure)
P1	c. -127delAT	M	No	55	Figure 2A-D, SF3E
P2	c. -127delAT	M	No	56	Figure 2B, D
P3	c.-127A>T	M	No	36	Figures 2B,C 4E, SF3E
P4	c. -118C>A	M	No	45	Figure 2A-C
P5	c.-126T>C	M	No	20	Figure 2A, 4E
P6	c.-116C>T	M	No	69	4E

Supplementary Table 2: List of patient samples used for experiments performed on primary cells

Supplementary Table 3

	Mutation	Sex	Family history of malignancy	Platelet count (x10^9/L)	iPSC cell line
P4	c. -118C>A	M	No	45	ANK1
P7	c. -127delAT	M	No	26	ANK2

Supplementary Table 3: List of patient samples used for iPSC lines generation.

Supplementary Table 4

Upregulated genes		
ID	logFC	p-Value
AC079466.1	3.07	3.39e-08
ADARB2	2.48	3.36e-07
APOL1	2.80	1.27e-07
CCNI2	3.54	2.23e-12
CD2	3.07	3.64e-11
CD3D	1.85	3.84e-06
CES1P1	2.79	1.69e-07
EPS8L3	2.29	5.62e-07
GAPDHP1	2.61	5.34e-10
GIMAP7	2.44	2.30e-07
GPT	2.47	1.30e-06
HLA-DQA1	3.44	9.00e-12
HLA-DQB1	3.66	6.78e-13
IL13	2.48	5.76e-07
NPTX1	2.61	1.66e-07
POF1B	2.42	1.10e-05
RP11-188C12.2	3.18	1.64e-09
RP11-455F5.3	2.41	2.92e-06
RP4-737E23.2	2.33	5.43e-06
SIGLEC12	2.93	3.29e-17
SP7	2.69	2.89e-07
SSXP10	2.39	1.39e-05
SULT1B1	3.04	1.33e-09
TNFRSF18	2.16	6.21e-07

Downreguated genes		
ID	logFC	p-Value
CCDC144NL-AS1	-2.34	1.74e-07
OSMR	-1.87	1.19e-05
ZFP28	-3.48	1.76e-14
ZFP42	-3.43	2.33e-10
ZNF486	-4.35	1.49e-16
ZNF626	-4.01	3.00e-14
ZNF682	-3.08	1.22e-08

Supplementary Table 4. List of up- and down-regulated genes in $CD43^{+}CD11b^{+}CD14^{-}$ progenitors derived from ANK1&2 iPSC lines versus control iPSC lines.

Supplementary Table 5

	Mutation	Sex	Erythrocytosis	Platelet count ($x10^9$/L)	s-EPO (mU/mL)
P8	c. -118C>A	F	No	82	4.7
P9	c. -118C>A	F	No	46	7.07
P10	c.-126T>G	M	No	21	13.7
P11	c. -125T>G	M	Intermittent mild erythrocytosis	35	4.04
P12	c. -125T>G	M	Intermittent mild erythrocytosis	54	3.67
P13	c. -118C>A	M	Chronic mild erythrocytosis	52	2.86
P14	c.-127A>G	M	Chronic mild erythrocytosis	15	3.32

Normal range s-EPO: 3.70-31.00 mU/mL

Supplementary table 5. List of patient samples used for serum EPO (s-EPO) measurement.