SUPPLEMENTAL METHODS

Cell culture

Hematopoietic stem and progenitor cells (HSPCs) (#2M-101C Lonza) were cultured in IMDM (Gibco, life technologies) supplemented with 2.5 % BSA (PAN-Biotech GmbH), 1X ITS-G (Gibco, life technologies), 20 µM β-Mercapoethanol (Acros Organics), 1mM UltraGlutamine-I (Lonza), 50 µg/ml Gentamicin (Acros Organics), 10 µg/ml Ciprofloxacin (Acros Organics), 50 µM 2P-Ascorbic Acid (Sigma-Aldrich), 1 µg/mL Heparin (AppliChem GmbH), 0.5 × Synthechol (Sigma-Aldrich), 50 ng/mL SCF (Immunotools), 50 ng/ml FLT3L (Immunotools), 25 ng/ml TPO (Immunotools) and 35 nM UM171 (Selleckchem).

Ex vivo T-lymphoid differentiation culture assay

LSK cells (Lin- Sca-1+ c-kit+) form a PU.1 knockout mouse model were transduced to stably express a human PU.1 gene including all regulatory elements[1]. For in vitro T-lymphoid coculture, 1x10^4 stromal cells were plated into each well of 24-well plates two days before plating of stem/progenitor cells. Sorted LSK were infected with small-hairpin RNA knockdown of PU.1 asRNA (shPU.1as) or by scrambled control (shControl). Infected stem/progenitor cells were added to the OP9-DL1 stromal cells lines as previously described[2] at Day 0. Cells were cultured in α-MEM (Invitrogen) supplemented with 10% FCS. Flt3-L, SCF and IL-7 (PeproTech). At Day 14-17 cultures of stromal cells and hematopoietic cells were harvested using 0.53 mM EDTA/PBS (pH 7.4). CD45 and DAPI were used to distinguish viable blood cells from OP9-DL1 stroma.

RNA isolation and Northern Analysis

RNA isolation, electrophoresis, transfer and hybridization were carried out as described[3]. Polyadenylated mRNAs were selected according to the MicroPoly(A)Purist^{TM} purification kit (Ambion). Preparation of separate nuclear and cytoplasmic fractions was performed according to the Paris^{TM} kit (Ambion). Northern Quantitative analysis was performed on the Storm Phosphorimager. The antisense-specific probe - mixture of two cloned PCR products are described in Supplemental Table2.

ATAC-seq analysis

Raw .fastq files were adapter trimmed using *Trim Galore!* software (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) and aligned to GRCh37

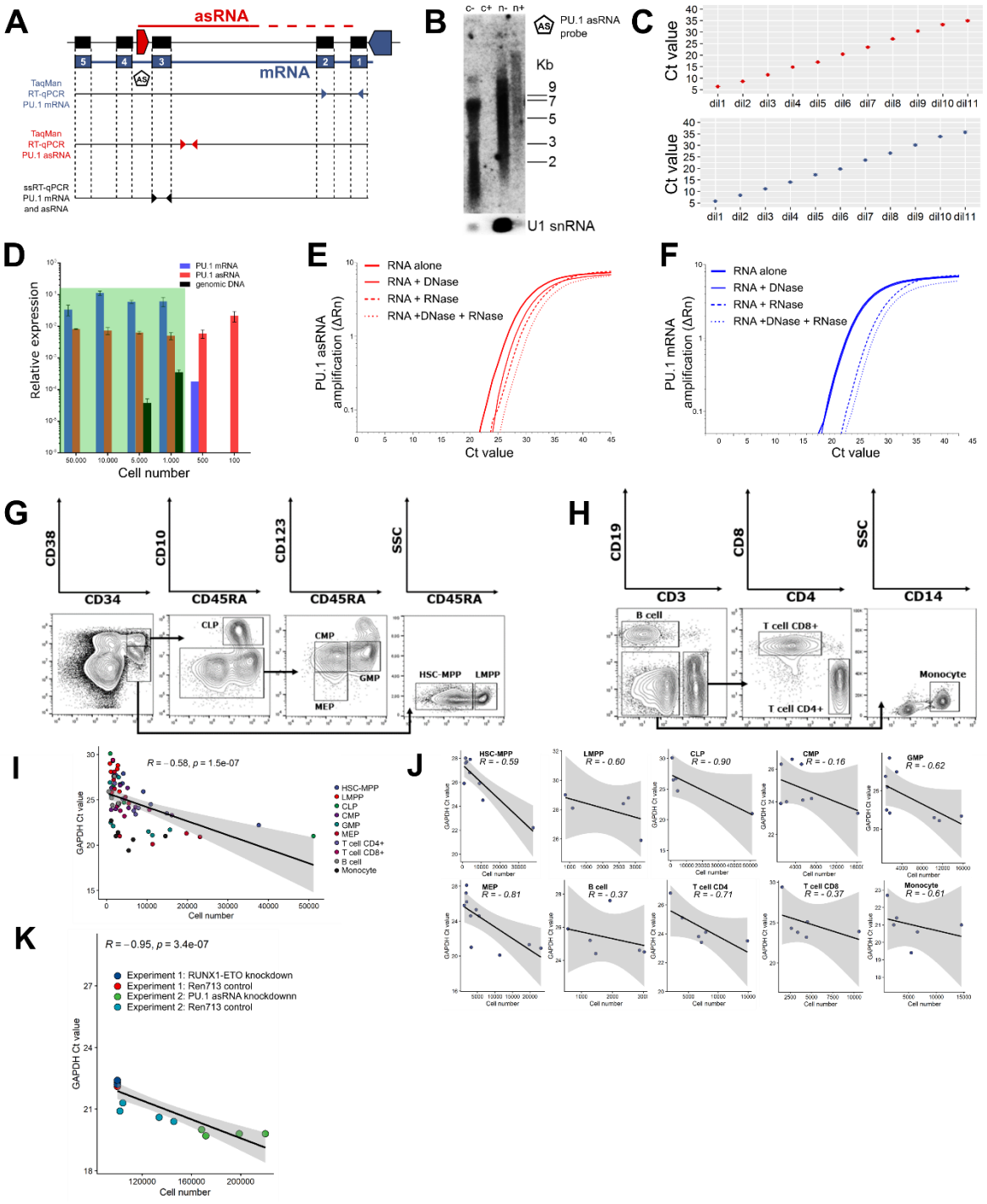
using bowtie2[4]. Reads corresponding to mitochondrial DNA were removed using samtools (samtools view -@ 20 -h $i | grep -v MT | samtools sort)[5]. Picard tools (http://broadinstitute.github.io/picard) were used to mark duplicate reads arising during PCR as artefacts of the library preparation procedure followed by duplicate read and multimapper removal by samtools (samtools view -@ 20 -h -b -q 30 -F 1024). Bigwig files were generated using deepTools, peaks were called by MACS2 and quantified using the R package diffbind[6]. Intensity values were adjusted to the PU.1 -17kb URE; a quantification inferior to 1:8th of both AsPr and PrPr was used as a filter for outliers. Raw ATAC-seq data were deposited in the ArrayExpress database (Accession ID: E-MTAB-9021).

RERERENCES

1. Leddin M, Perrod C, Hoogenkamp M, et al. Two distinct auto-regulatory loops operate at the PU.1 locus in B cells and myeloid cells. *Blood*. 2011;117(10):2827-2838.
2. Schmitt TM, Zúñiga-Pflücker JC. Induction of T cell development from hematopoietic progenitor cells by delta-like-1 in vitro. *Immunity*. 2002;17(6):749-756.
3. Maniatis T, Fritsch EF, Sambrook J. Molecular cloning: a laboratory Manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; 1982.
4. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nat Methods*. 2012;9(4):357-359.
5. Li H. A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. *Bioinformatics*. 2011;27(21):2987-2993.
6. Ross-Innes CS, Stark R, Teschendorff AE, et al. Differential oestrogen receptor binding is associated with clinical outcome in breast cancer. *Nature*. 2012;481(7381):389-393.

SUPPLEMENTAL FIGURES

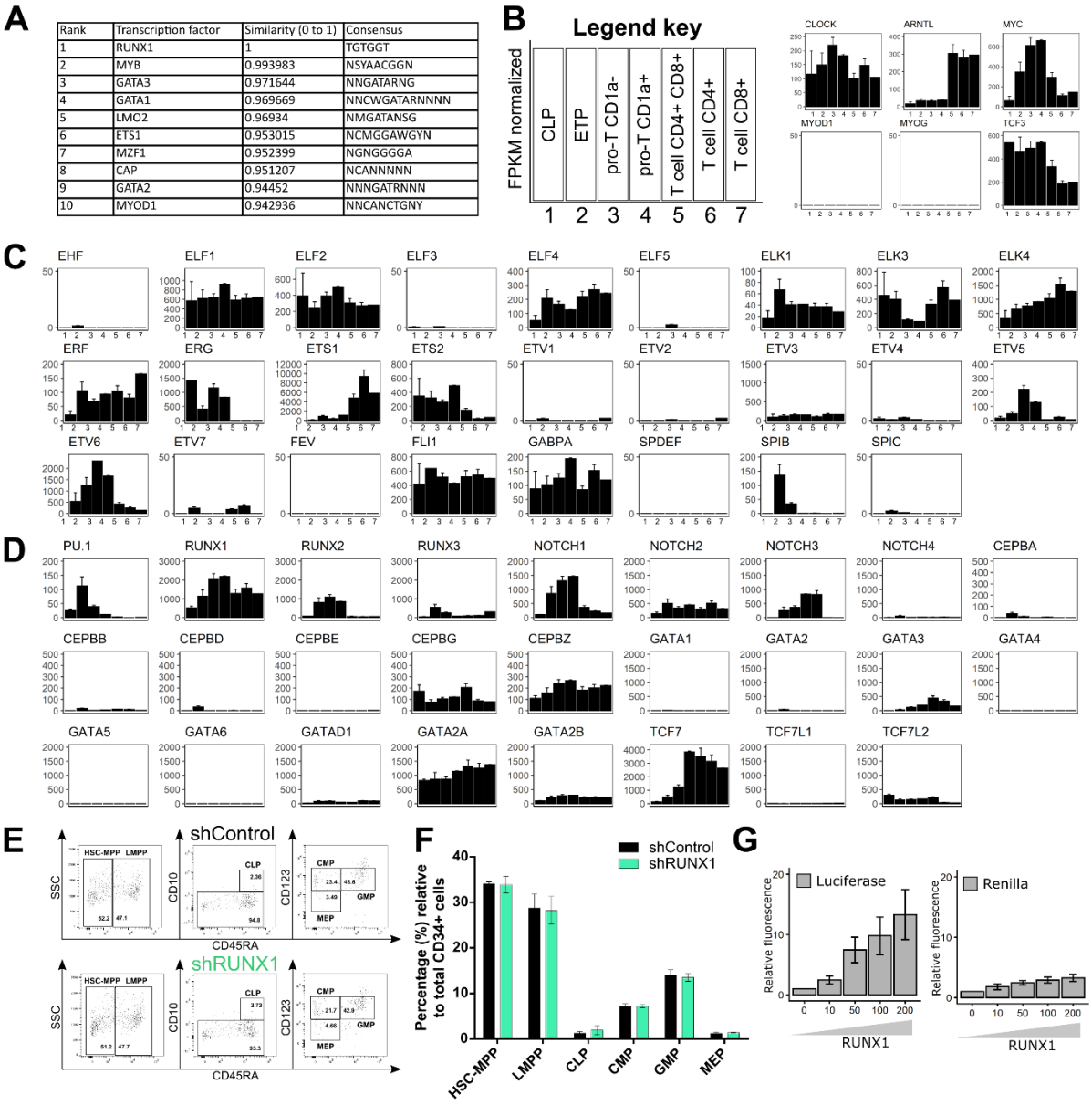
Supplemental Figure 1



Supplemental Figure 1. **PU.1 quantification assay validation, hematopoietic population isolation for PU.1 transcript quantification and hematopoietic transcription factor mobilization during thymic differentiation. (A)** Schematic representation of the PU.1 locus with the antisense promoter (AsPr, red arrow box) and proximal (PrPr, blue arrow box), respectively regulating the transcription of the antisense RNA (asRNA, red line) and the coding

mRNA (blue line, exon number 1-5). Forward and reverse primer (left and right arrows) pair localization each PU.1 transcript quantification using RT-qPCR or strand-specific RT-qPCR (ssRT-qPCR). Black pentagon arrow shows PU.1 asRNA probe for Northern blot analysis. Colors used in this schematic are consistent throughout the figure. **(B)** Northern blot analysis of PU.1 asRNA in HL-60 cell line using PU.1as RNA probe after cytoplasmic (c) or nucleic (n) RNA extraction with (+) or without (-) polyadenylation enrichment. U1 snRNA control probe for nucleic RNA extraction enrichment. Ladder legend for RNA size (Kb, kilobase). **(C)** Titration of PU.1 asRNA amplicon (upper panel) and mRNA (lower panel) using the Taqman RT-qPCR assay. Starting DNA at 0.156ng/µL (dil1, dilution one) is incrementally diluted at a 1:8 ratio (n=2). **(D)** Cell limit determination for PU.1 asRNA and mRNA transcript detection using RT-qPCR assay (green area, lowest cell limit, n=2). **(E and F)** Characterization of **(E)** PU.1 asRNA and **(F)** mRNA transcript quantification with RNase and DNase treatments (n=3) using ssRT-qPCR. **(G and H)** Cell isolation using flow cytometry sorting from healthy donors of **(G)** total bone marrow (HSC-MPP, merged hematopoietic stem cell and multipotent progenitor; CMP, common myeloid progenitor; MEP, megakaryocyte-erythroid progenitor; GMP, granulocyte-macrophage progenitor; LMPP, lymphoid-primed multipotent progenitor; CLP, common lymphoid progenitor) and **(H)** peripheral blood. **(I-K)** Correlation plot between GAPDH housekeeping Ct values from RT-qPCR data and sorted cell number for **(I)** combined hematopoietic stem, progenitor and peripheral blood, **(J)** single populations and **(K)** perturbation experiment in Kasumi-1 cells (Spearman correlation). Data are represented as mean value ± SEM.

Supplemental Figure 2



Rank	Transcription factor	Similarity (0 to 1)	Consensus
1	RUNX1	1	TGTGGT
2	MYB	0.993983	NSYAACGGN
3	GATA3	0.971644	NNGATARNG
4	GATA1	0.969669	NNCWGATARNNNN
5	LMO2	0.96934	NMGATANSG
6	ETS1	0.953015	NCMGGAWGYN
7	MZF1	0.952399	NGNGGGGA
8	CAP	0.951207	NCANNNNN
9	GATA2	0.94452	NNNGATRNNN
10	MYOD1	0.942936	NNCANCTGNY

Supplemental Figure 2. **Early T-lymphoid differentiation regulation by hematopoietic transcription factors. (A)** Ranking for PU.1 antisense promoter (AsPr) transcription factor binding candidates (TFBind) by similarity (0.0-1.0) between a registered sequence for the transcription factor binding sites and the input sequence. **(B-D)** Gene expression by transcript sequencing (RNA-seq) in thymic progenitors and differentiated T cells (ETP, early thymic progenitor) for **(B)** E-box, **(C)** ETS and **(D)** most commonly known hematopoietic transcription factors (RPKM normalized, n=2). **(E)** Gating of cultivated human HSPCs (HSC-MPP, merged hematopoietic stem cell and multipotent progenitor; CMP, common myeloid progenitor; MEP, megakaryocyte-erythroid progenitor; GMP, granulocyte-macrophage progenitor; LMPP, lymphoid-primed multipotent progenitor; CLP, common lymphoid progenitor). Data displayed

as percentage of parent population. **(F)** Hematopoietic populations relative to total CD34+ cells after shRUNX1 (control small-hairpin knockdown against Renilla713, shRen713) in human cultivated HSPCs (n=2). (**G**) Luciferase and Renilla fluorescence measurements relative to 0ng expression plasmid control for PU.1 AsPr Luciferase transactivation in the presence of RUNX1 (n=4). Data are represented as mean value ± SEM.

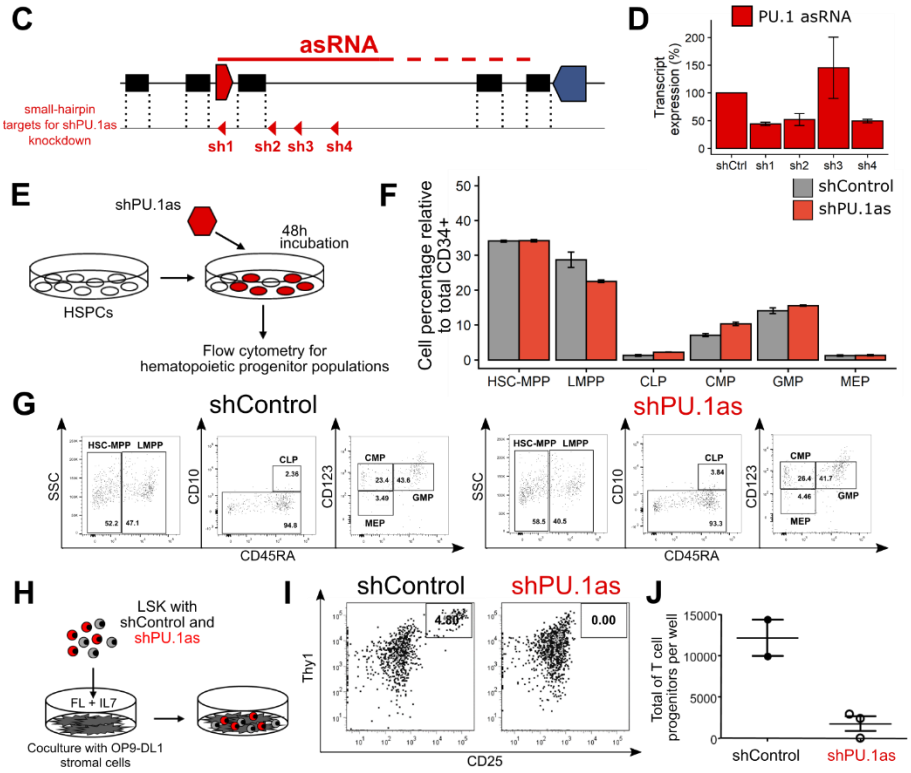
Supplemental Figure 3

A **Patient data for PU.1 transcript expression**

		cohort1	cohort2	cohort3	sum
CBF-AML	t(8;21)(q22;q22)	5	8	5	**36**
	inv(16)(p13;q22)	6	7	5	
NK-AML	FLT3- NPM1-	9	4	5	
	FLT3+ NPM1-	2	1	-	**25**
	FLT3- NPM1+	3	-	-	
	FLT3+ NPM1+	1	-	-	
Healthy bone marrow CD34+		3	6	-	**9**
				total:	**70**

B **Patient data for PU.1 promoter accessibility**

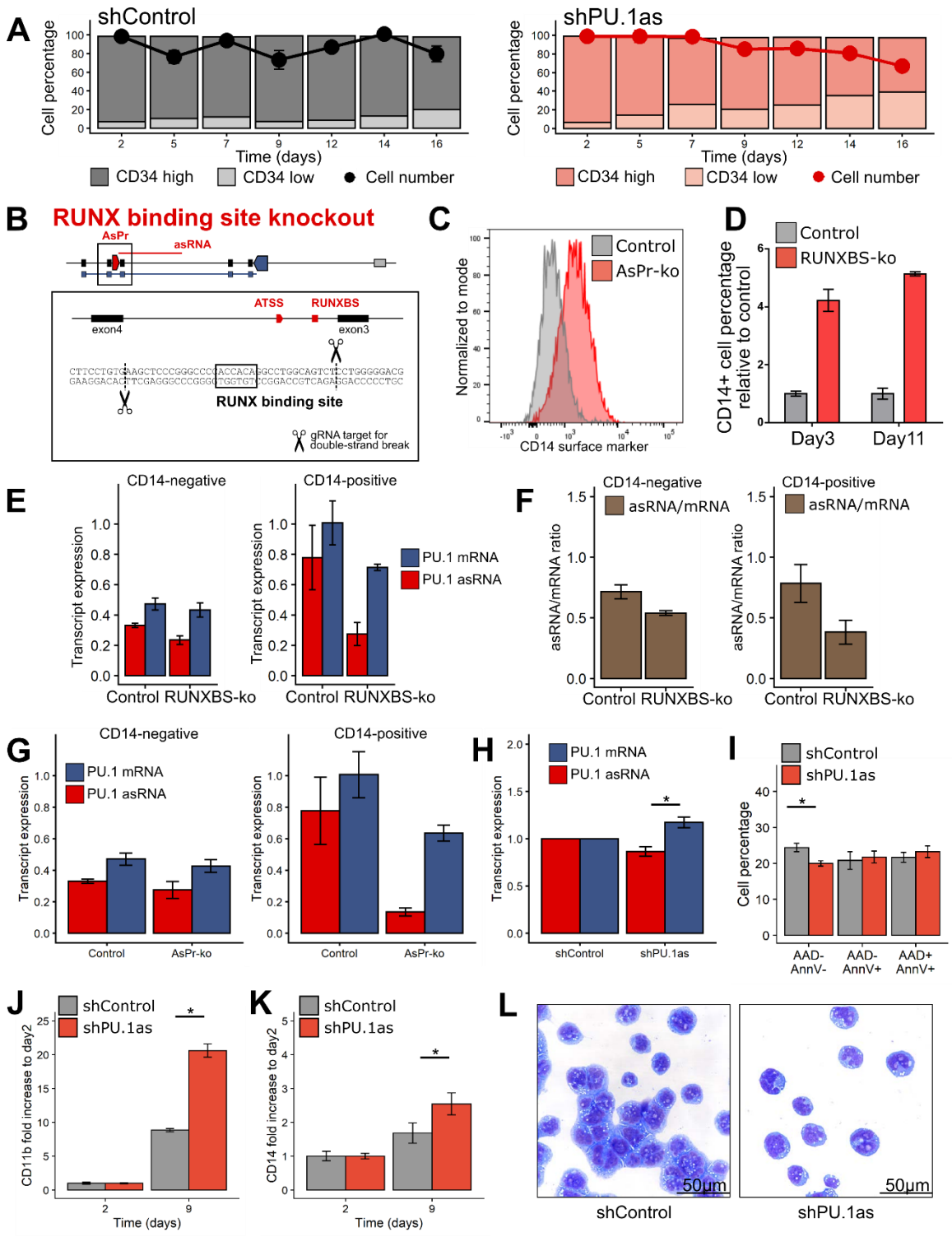
		cohort	sum
CBF-AML	t(8;21)(q22;q22)	3	**5**
	inv(16)(p13;q22)	2	
NK-AML	FLT3+	2	
	FLT3+ NPM1+	7	**14**
	NPM1+	2	
	CEBPα	3	
		total:	**19**



Supplemental Figure 3. **Validation of PU.1 asRNA depletion in vitro and ex vivo. (A)** Core-binding factor AML (CBF-AML) patient group for PU.1 transcript expression analysis contains the t(8;21)(q22;q22) translocation and inv(16)(p13;q22) inversion anomalies, respectively generating RUNX1-ETO and CBFβ-MYH11 fusion proteins, and is subdivided by cohort. Normal karyotype AML (NK-AML) patient group is subdivided by cohort and by mutation (Fms-like tyrosine kinase, FLT3; nucleophosmin, NPM1). Bone marrow (BM) CD34+ group contains healthy CD34-enriched bone marrow samples. **(B)** CBF-AML patient group for promoter accessibility analysis (Assi SA, et al. Nat Genet, 2019) contains the t(8;21)(q22;q22) translocation, inv(16)(p13;q22) inversion anomalies and NK-AML patient group contains FLT3, NPM1 or CCAAT/enhancer-binding protein alpha (CEBPα) mutations. **(C)** Schematic

representation of the PU.1 locus with the antisense promoter (AsPr, red arrow box) regulating antisense RNA (asRNA, red line). Small-hairpin targets 1 to 4 (sh1-4) for lentiviral knockdown of PU.1 asRNA (shPU.1as, red arrows). **(D)** PU.1 asRNA (red) transcript quantification in Kasumi-1 cells after PU.1 asRNA knockdown (n=2). **(E)** Experimental workflow of shRNA knockdown of PU.1 antisense RNA (shPU.1as) in human hematopoietic stem and progenitor cells (HSPCs) followed by fluorescence-activated cell sorting (FACS). **(F)** Hematopoietic populations relative to total CD34+ cells after shPU.1as (control small-hairpin knockdown against Renilla713, shRen713) in human cultivated HSPCs. **(G)** Gating of cultured human HSPCs (HSC-MPP, merged hematopoietic stem cell and multipotent progenitor; CMP, common myeloid progenitor; MEP, megakaryocyte-erythroid progenitor; GMP, granulocyte-macrophage progenitor; LMPP, lymphoid-primed multipotent progenitor; CLP, common lymphoid progenitor). Data displayed as percentage of parent population. **(H)** Experimental workflow of shRNA knockdown of PU.1 antisense RNA (shPU.1as) in humanized PU.1 LSK (Lin- Sca-1+ c-kit+) mouse cells followed by coculture with OP-DL1 stromal cells for T-lymphoid differentiation. **(I)** FACS analysis for Thy1 and CD25 surface marker expression for T-lymphoid differentiation after shPU.1as in LSK cells and coculture with OP9-DL1. **(J)** Absolute count per well of T-lymphoid progenitors after shPU.1as in LSK cells and in vitro coculture with OP9-DL1.

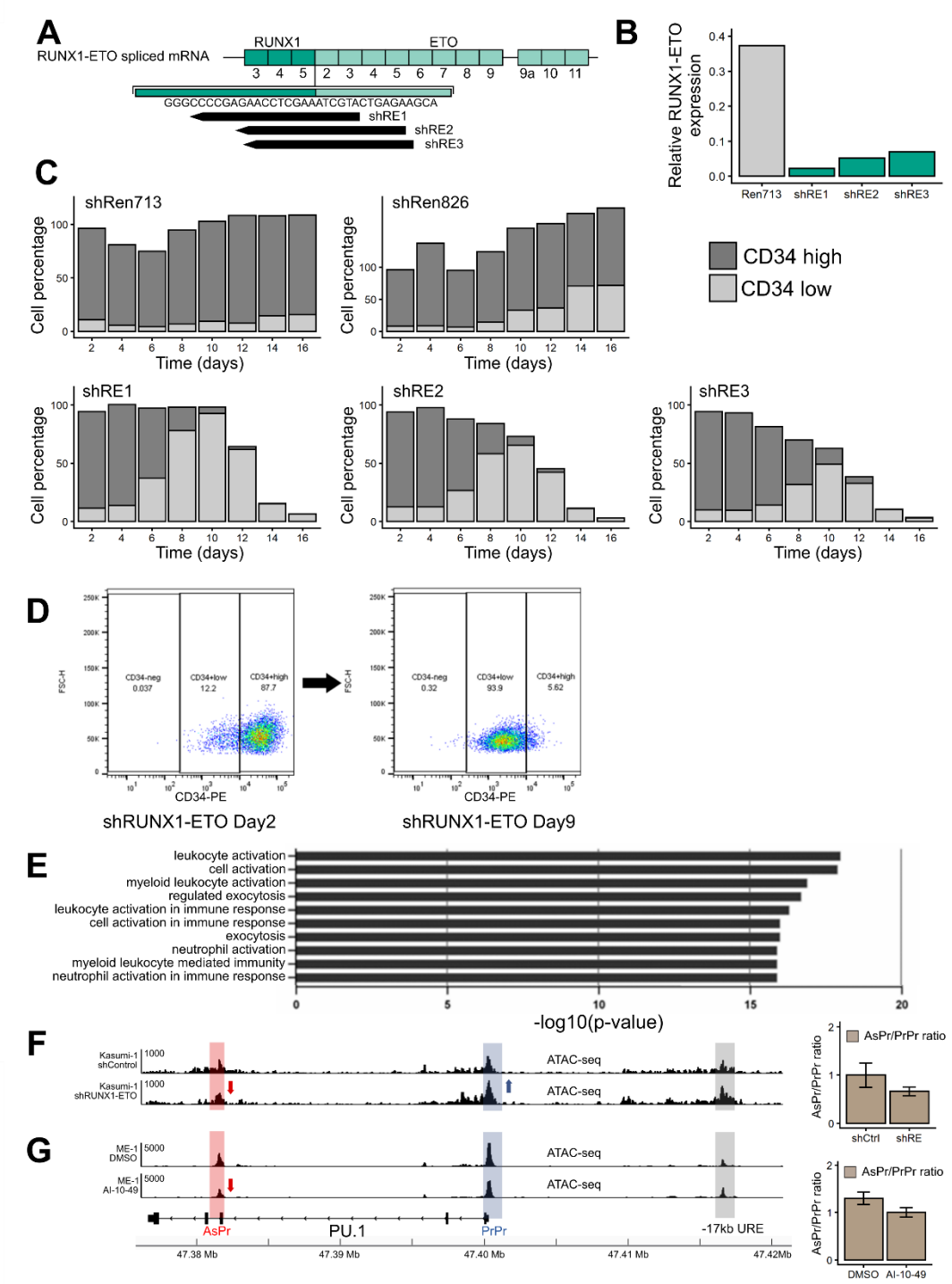
Supplemental Figure 4



Supplemental Figure 4. **PU.1 asRNA function assessment in CBF-AML cell lines. (A)** FACS analysis for CD34 surface marker expression and cell viability kinetics after shPU.1as in Kasumi-1 (n=4). **(B)** Schematic representation of the PU.1 locus with the antisense promoter (AsPr, red arrow box) regulating antisense RNA (asRNA, red line) and the double-strand break locations for CRISPR/Cas9-mediated RUNX binding site (RUNXBS) knockout. Also shown is the antisense transcription start site (ATSS). **(C)** FACS analysis for CD14 surface marker

expression after RUNXBS knockout (RUNXBS-ko) in Kasumi-1 (merged n=4). **(D)** Kinetics of CD14 surface marker expression after RUNXBS-ko in Kasumi-1 (n=4). **(E)** PU.1 asRNA (red) and mRNA (blue) transcript expression after RUNXBS-ko in CD14-negative and CD14-positive Kasumi-1 cells (n=4). **(F)** PU.1 asRNA/mRNA ratio (brown) after RUNXBS-ko in CD14-negative and CD14-positive Kasumi-1 cells (n=4). **(G)** PU.1 asRNA (red) and mRNA (blue) transcript expression after AsPr-ko in CD14-negative and CD14-positive Kasumi-1 cells (n=4). **(H)** PU.1 asRNA and mRNA (blue) transcript quantification in ME-1 cells after shPU.1as with sh4 (n=3). **(I-K)** Flow cytometry after shPU.1as with sh4 target in ME-1 cells for **(I)** cell viability, **(J)** CD11b and **(K)** CD14 surface markers (n=3). **(L)** May Grünwald/Giemsa cytospins for morphology analysis of ME-1 cells after shPU.1as (n=3). Data are represented as mean value ± SEM. *$p < 0.05$, ***$p < 0.001$, Student's t-test.

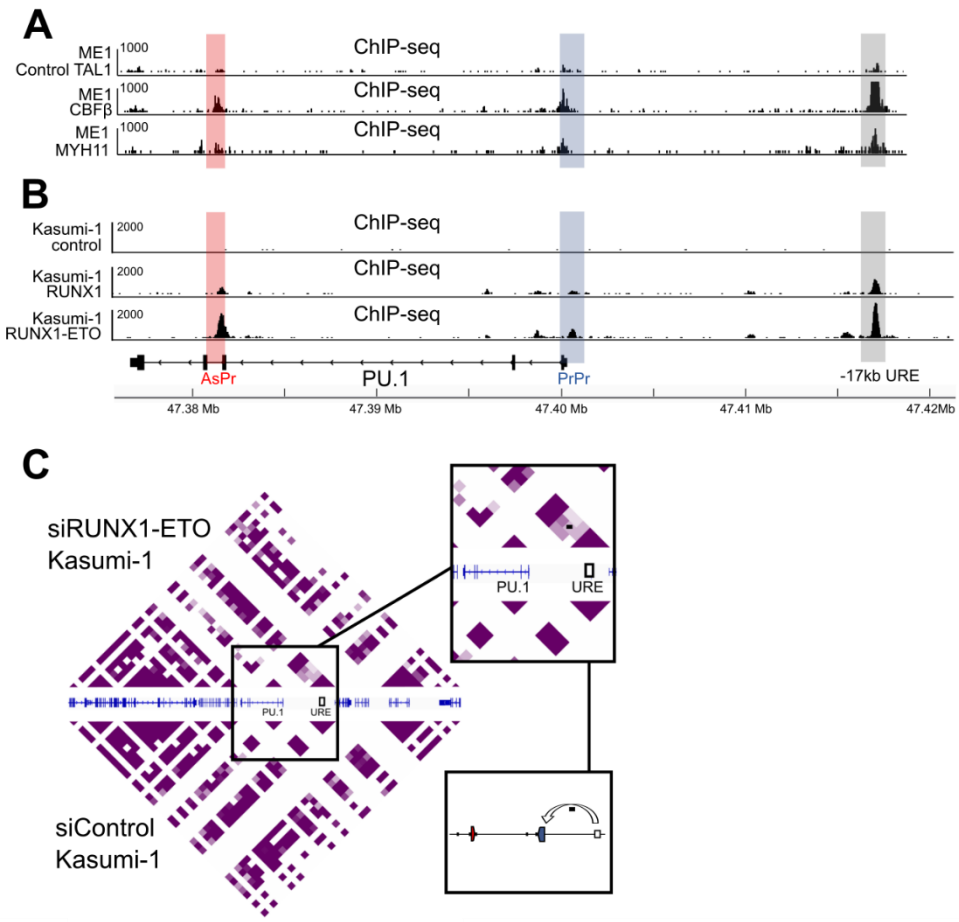
Supplemental Figure 5



Supplemental Figure 5. **RUNX1-ETO depletion by lentiviral small hairpin knockdown prevents cell differentiation and myeloid function. (A)** Scheme of shRUNX1-ETO knockdown targets (shRE1-3, small hairpin RUNX1-ETO knockdown for target 1-3). **(B)** RUNX1-ETO RT-qPCR relative to GAPDH housekeeping gene after RUNX1-ETO

knockdown using each shRE target at day10 in Kasumi-1 cells (single replicate). **(C)** CD34 surface marker kinetics assessed by flow cytometry for two negative viability controls (Renilla713 and 826, shRen713 and shRen826) and three constructs for RUNX1-ETO lentiviral knockdown in Kasumi-1 cells (single replicate). **(D)** Flow cytometry analysis of CD34 surface marker at day2 and day9 after shRUNX1-ETO (shRE) in Kasumi-1 cells (n=2). **(E)** Top 10 Pathway gene ontology analysis using Panther from transcript sequencing (RNA-seq) after shRUNX1-ETO in Kasumi-1 cells (n=3). Differential expression of shRUNX1-ETO Day9 compared to shControl Day2-Day9. **(F and G)** URE-adjusted peak quantification values by ATAC-seq for PU.1 antisense (AsPr, red) and proximal promoter (PrPr, blue) exhibited as AsPr/PrPr ratio for **(F)** RUNX1-ETO knockdown in Kasumi-1 cells (2 days after lentiviral transduction, n=3) and for **(G)** AI-10-49 inhibitor treatment of ME-1 cells (6 hours after treatment, n=2). Data are represented as mean value ± SEM.

Supplemental Figure 6



Supplemental Figure 6. **PU.1 upstream regulatory element is mobilized in early lymphoid differentiation and in PU.1 downregulation by the RUNX1-ETO oncogene. (A)** Chromatin immunoprecipitation sequencing (ChIP-seq) of CBFβ, MYH11 and TAL1 at the PU.1 locus (AsPr, antisense promoter; PrPr, proximal promoter; URE, upstream regulatory element). TAL1 was used as a negative control. **(B)** RUNX1 and RUNX1-ETO ChIP-seq in Kasumi-1 cells (with immunoglobulin G control, IgG). **(C)** Promoter capture chromosomal conformation sequencing (C-HiC) after small-interfering RNA of RUNX-ETO knockdown (siRUNX1-ETO) versus control (siControl). Black dot indicates chromosomal looping of -17kb URE and PrPr. Data are represented as mean value ± SEM.