Experimental
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REGULAR SUBMISSION

Transcriptional regulators CITED2 and PU.1 cooperate in maintaining hematopoietic stem cells

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Reduced expression of the transcription factor PU.1 is frequently associated with development of acute myeloid leukemia (AML), whereas elevated levels of CITED2 (CBP/p300-interacting-transactivator-with-an-ED-rich-tail 2) enhance maintenance of both normal and leukemic hematopoietic stem and progenitor cells (HSPCs). Recent findings indicate that PU.1 and CITED2 act in the same gene regulatory network. We therefore examined a potential synergistic effect of simultaneous PU.1 downregulation and CITED2 upregulation on stem cell biology and AML pathogenesis. We found that simultaneous PU.1/CITED2 deregulation in human CD34$^+$ cord blood (CB) cells, as well as CITED2 upregulation in preleukemic murine PU.1-knockdown (PU.1$^{KD/KD}$) bone marrow cells, significantly increased the maintenance of HSPCs compared with the respective deregulation of either factor alone. Increased replating capacity of PU.1$^{KD/KD}$/CITED2 cells in in vitro assays eventually resulted in outgrowth of transformed cells, while upregulation of CITED2 in PU.1$^{KD/KD}$ cells enhanced their engraftment in in vivo transplantation studies without affecting leukemic transformation. Transcriptional analysis of CD34$^+$ CB cells with combined PU.1/CITED2 alterations revealed a set of differentially expressed genes that highly correlated with gene signatures found in various AML subtypes. These findings illustrate that combined PU.1/CITED2 deregulation induces a transcriptional program that promotes HSPC maintenance, which might be a prerequisite for malignant transformation. © 2019 Published by Elsevier Inc. on behalf of ISEH – Society for Hematology and Stem Cells.

Leukemic transformation has been shown to be a multistep process in which hematopoietic cells acquire multiple mutations/alterations along the differentiation-road that can either influence self-renewing-, differentiation- or proliferation properties of cells [1]. Initial mutations are thought to occur in hematopoietic stem and progenitor cells (HSPCs) which can alter their life-span and/or maintenance and lead to clonal hematopoiesis [2,3]. Additional mutations in such preleukemic HSPCs promote leukemic progression [4]. A key regulator of hematopoiesis is the ETS-family transcription factor PU.1 that is expressed at low levels in HSPCs

[5] and at high levels in the myeloid linage and B-cells [6−8]. PU.1 has crucial functions for both HSPC maintenance [9−14] and differentiation of the myeloid lineage [15,16]. In acute myeloid leukemia (AML), PU.1 expression is frequently found to be disturbed by mutations, translocations, and changes in signal transduction [17−23], which contributes to the accumulation of immature blasts—the characteristic feature of AML. Notably, heterozygous mutations of the *SPI1* gene itself (which is the gene encoding PU.1) are only rarely found in human AML [24,25] and homozygous mutations are not detected at all, which is in line with murine models demonstrating that total absence of PU.1 is not compatible with hematopoiesis, whether it is healthy or pathologic [11,26−29]. To resemble the situation observed in patients, mouse models with reduced PU.1 expression rather than full *Spi1* deletions have become useful models to study AML pathogenesis [12,30−32]. Homozygous deletions of a −14-kb upstream regulatory region (URE) in the Spi1 locus results in 80% reduction of PU.1 expression in murine bone marrow cells, and mice develop AML at approximately 6 months of age. Malignant transformation in PU.1-knockdown mice was found to be recurrently accompanied by chromosomal aberrations [30], indicating that PU.1-low cells are more vulnerable for acquiring additional changes that promote leukemia development. Because PU.1 knockdown mice undergo a preleukemic phase of several months, they can also serve as a model for studying alterations that precede leukemic transformation and identify cooperative factors that accelerate or facilitate transformation. In particular, additional alterations that lead to an increased maintenance of PU.1-low HSPCs could contribute to expansion of a cell pool that is susceptible to mutation acquisition and thereby promote AML development.

We recently demonstrated that PU.1 negatively regulates expression of the transcriptional co-activator CITED2 (CBP/p300-interacting-transactivator-with-an-ED-rich-tail 2) by binding to multiple ETS-binding sites in the CITED2 promoter [33]. CITED2 is a key guardian of hematopoietic stem cell (HSC) maintenance and its deletion in murine HSCs results in increased cell apoptosis, cycling and, consequently, multilineage bone marrow failure [34−36]. Notably, CITED2 has also important functions for the survival of leukemic stem cells [33,37], and pathways that are involved in upregulating CITED2 expression [37−42] are frequently activated in AML. Therefore, we studied the combined de-regulation of PU.1 and CITED2 in normal and leukemic HSPCs.

Here we show that simultaneous upregulation of CITED2 and downregulation of PU.1 in human CD34$^+$ cord blood cells using lentiviral constructs enhance the maintenance of hematopoietic stem and progenitor cells (HSPCs). Similar CITED2 overexpression in preleukemic murine PU.1-knockdown bone marrow cells increased replating capacity and enhanced engrafted cells in transplantation assays, without affecting the transforming event. In summary, our data indicate that combining downregulation of PU.1 and upregulation of CITED2 enhances the lifespan of PU.1-low HSCs, which makes them more prone to full leukemic transformation.

Methods

Isolation of stem and progenitor cells

Neonatal cord blood was derived from healthy full-term pregnancies after informed consent from the obstetrics departments of the Martini Hospital and University Medical Center in Groningen, The Netherlands. Mononuclear cells were isolated by density gradient centrifugation using Lymphoprep (Alere Technologies AS, Oslo, Norway), and CD34$^+$ cells were selected using the MACS CD34 microbead kit on autoMACS (Miltenyi Biotec, Leiden, Netherlands). Lentiviral constructs and transduction procedure are described in the Supplementary Material.

Colony-forming cell assay

Transduced human CD34$^+$ cord blood cells were directly sorted in MethoCult H4230 (StemCell Technologies, Grenoble, France) supplemented with 19% (v/v) Iscove's modified Dulbecco's medium (IMDM), 20 ng/mL interleukin (IL)-3, 20 ng/mL IL-6, 20 ng/mL granulocyte colony-stimulating factor (G-CSF), 20 ng/mL stem cell factor (SCF, Novoprotein), and 1 U/mL erythropoietin (EPO, EPREX). Murine bone marrow (BM) cells were isolated from 8- to 12-week-old B6.J URE$^{-/-}$ mice [30]. Lineage-depleted murine BM cells were transduced as described above, and c-Kit$^+$ cells were directly sorted into MethoCult H4230 (StemCell Technologies) supplemented with 19% (v/v) IMDM (Lonza, Breda, Netherlands), 100 ng/mL murine (m)SCF (PepProtech), 20 ng/mL human GM-CSF, 2 ng/mL mIL-3 (PepProtech). Colonies were scored after 12−14 days of incubation. Subsequently, 50,000 cells were replated and again scored after 12−14 days.

Long-term cultures on stroma

Murine MS5 cells were expanded and cultured as described earlier [43]. Long-term culture-imitating cell (LTC-IC) assays were performed by plating transduced CD34$^+$ cord blood cells in limiting dilutions in the range of 9 to 1000 cells per well on MS5 stromal cells in 96-well plates in LTC medium (α-minimal essential medium [αMEM] supplemented with heat-inactivated 12.5% fetal calf serum [FCS], heat-inactivated 12.5% horse serum [Sigma, Zwijndrecht, Netherlands], 100 U/mL penicillin/streptomycin, 200 mmol/L glutamine, 57.2 μmol/L β-mercaptoethanol [Sigma] and 1 μmol/L hydrocortisone [Sigma]). After 5 weeks, methylcellulose (MethoCult H4230 supplemented with 19% [v/v] IMDM, 20 ng/mL IL-3, 20 ng/mL IL-6, 20 ng/mL G-CSF, 20 ng/mL SCF, and 1 U/mL EPO) was added to the wells. Two weeks later, wells containing colony-forming cells (CFCs) were

scored as positive. LTC-IC frequency was calculated using the L-Calc software. For MS5 co-culture growth curves, 10 −50 × 10^5 transduced CD34$^+$ cord blood cells were plated on MS5 stromal cells in a T25 culture flask in LTC medium supplemented with 20 ng/mL IL-3, G-CSF, and thrombopoietin (TPO). Cultures were demi-depopulated weekly for analysis.

In vivo transplantations into NSG mice
Murine Lin$^-$c-Kit$^+$ BM cells were isolated from 8- to 12-week-old B6.J URE$^{-/-}$ mice by means of lineage depletion (Dynabeads), followed by c-Kit enrichment (MACS). Cells were resuspended in M5300 medium (StemCell Technologies) supplemented with recombinant murine (rm)SCF (50 ng/mL), rmTPO (20 ng/mL), rmIL-3 (25 ng/ml), rmIL-6 (10 ng/mL), and Primocine (anti-mycoplasma agent 2 μL/mL). The next day, the cells were in two subsequent rounds lentivirally transduced with GFP-tagged control or CITED2-overexpressing lentivirus in the presence of 4 ng/ml polybrene. After 2 days, 0.2×10^6 cells for cohort A and 0.5×10^6 cells for cohort B were retro-orbitally injected into NSG mice. Before transplantation, mice were sublethally irradiated (2.0 Gy). Engraftment was analyzed in the peripheral blood (PB) and BM by flow cytometry.

Gene expression profiling
From four independent cord blood batches, CD34$^+$ cells were MACS isolated and transduced with control lentivirus, CITED2-overexpressing lentivirus, a short hairpin (sh)RNA lentivirus against PU.1 or a lentivirus containing a CITED2 overexpression cassette, and a shRNA against PU.1. After 2 days, transduced CD34$^+$ cells were sorted from each transduction group (group 1: control, group 2: CITED2, group 3: shPU.1, group 4: CITED2/shPU.1). Total RNA was isolated using the RNeasy mini kit from Qiagen (Venlo, Netherlands) according to the manufacturer's recommendations. Quantitative polymerase chain reaction (Q-PCR) analysis was used to validate proper overexpression or knockdown of CITED2 and PU.1, respectively. RNA from two cord bloods with similar overexpression or knockdown of CITED2 and PU.1 was pooled within each group, and quality was examined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Genomewide expression analysis was performed on Illumina (Illumina, Inc., San Diego, CA, USA) BeadChip Arrays (Illumina HT12-V4). Typically, 0.5−1 μg of mRNA was used in labeling reactions, and hybridization with the arrays was performed according to the manufacturer's instructions. The expression was quantile normalized using GeneSpring GX software, and from the probe sets that were expressed above background (set to 25), subsequent fold differences were calculated. Genes that indicated a fold change of <2 or >2 were further analyzed. Array data are available at http://www.ncbi.nlm.nih.gov/geo, with the accession code GSE118036. Array data were compared with publically available (http://servers.binf.ku.dk/bloodspot/) gene expression data from 198 AML patients (60 t(8;21), 47 inv (16)/t16;16), 43 t(11q23), and 48 AMLs with complex karyotype) and 18 normal CD34$^+$ subsets.

Immunoblotting
Cell lysates were prepared and the immunoblotting procedure performed as described previously [44]. Primary antibodies for immunoblotting were MRG1 (JA22, Santa Cruz, No. SC-21795), PU.1 (T-21, Santa Cruz, No. SC-352).

Statistical analysis
If not indicated otherwise in figure legends, *p* values were calculated using Student's *t* test.

Results

### Combining PU.1 downregulation with CITED2 upregulation maintains HSCs

To investigate the impact of combined deregulation of PU.1 and CITED2 levels on HSPCs, CD34$^+$ cord blood (CB) cells were isolated and double-transduced with various combinations of lentiviral constructs to achieve either short hairpin-mediated PU.1 downregulation, CITED2 upregulation, or a combination of both. For all conditions, we observed 20%−25% double-transduced cells, and 3 days after transduction, cells were sorted and plated for CFC assays (Figure 1A; Supplementary Figure E1A, B, online only, available at www.exphem.org). Levels of PU.1 reduction and CITED2 overexpression were confirmed by both Q-PCR and Western blot (Figure 1B; Supplementary Figure E1C). The shPU.1-, CITED2-, and shPU1/CITED2-transduced cells provided comparable CFU-GM (colony-forming unit granulocyte−macrophage), BFU-E (burst-forming unit erythroid), and CFU-GEMM (granulocyte, erythroid, monocyte, macrophage) formation compared with control cells (Figure 1C, D). However, replating experiments revealed an increased replating capacity of shPU.1/CITED2 cells that could not be achieved by altering levels of PU.1 or CITED2 only (Figure 1E). Furthermore, we found that the enhanced replating capacity of shPU.1/CITED2 cells is restricted to the CD34$^+$CD38$^-$ fraction (Figure 1F, G), which suggests that it is primarily the more immature fraction of HSPCs that is maintained by simultaneous alterations of CITED2 and PU.1 expression levels.

Simultaneous PU.1 downregulation and CITED2 upregulation increase LTC-IC frequency

To address the question if combined PU.1/CITED2 deregulation can also affect long-term functions of HSPCs, control-, shPU.1-, CITED2-, and shPU1/CITED2-transduced CD34$^+$ CB cells were cultured on an MS5 stromal layer in the presence of cytokines for up to 4 weeks and subsequently plated for CFC assays (Figure 2A). Total cell numbers of shPU.1, CITED2, and shPU1/CITED2 cells in MS5 co-cultures were not different compared with control cells (Figure 2B), indicating that growth factor-induced HSPC expansion was
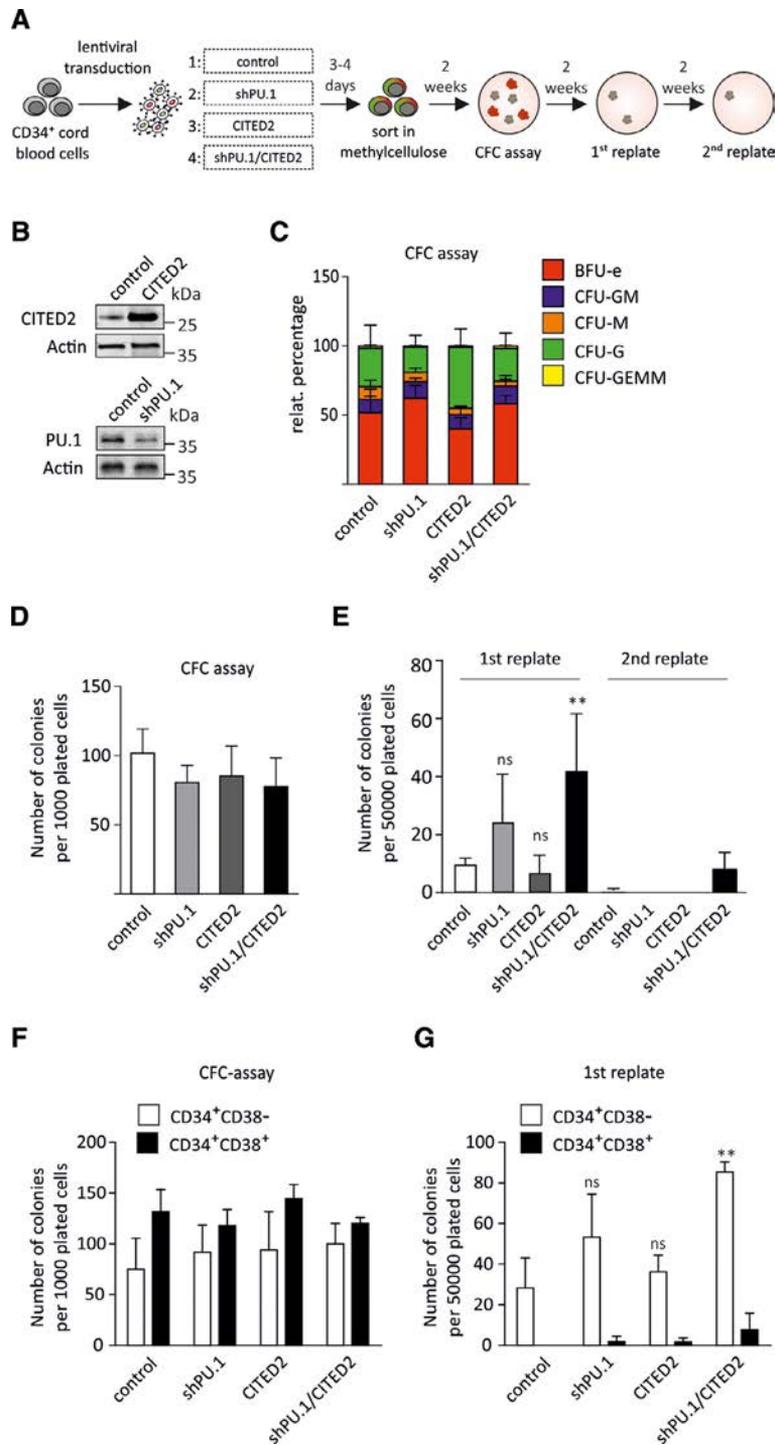
Figure 1. Combining PU.1 downregulation with CITED2 upregulation maintains HSCs. **(A)** Schematic overview of experimental design. Isolated CD34^{+} CB cells were transduced with the indicated combinations of lentiviral constructs, and double-positive cells were sorted for CFC assays **(B)** Downregulation of PU.1 and upregulation of CITED2 by our lentiviral vectors were verified by Western blot in the Molm13 leukemic cell line. **(C, D)** CFC assays performed with transduced CD34^{+} CB cells. Relative percentage of indicated colony types (C) and total number of colonies (D) scored after 14 days. **(E)** Colonies from primary CFC assays were harvested and 50,000 cells were replated. Total numbers of colonies after 14 days are illustrated. **(F, G)** Colony number in CFC assays (F) and replates (G) of transduced CD34^{+}CD38^{−} and CD34^{+}CD38^{+} cell populations. **(D−G)** Error bars indicate the standard deviations of three individual experiments performed in duplicate. Each experiment was performed with CD34^{+} cells from two or three donors. ns = not significant. **$p < 0.01$ compared with control.
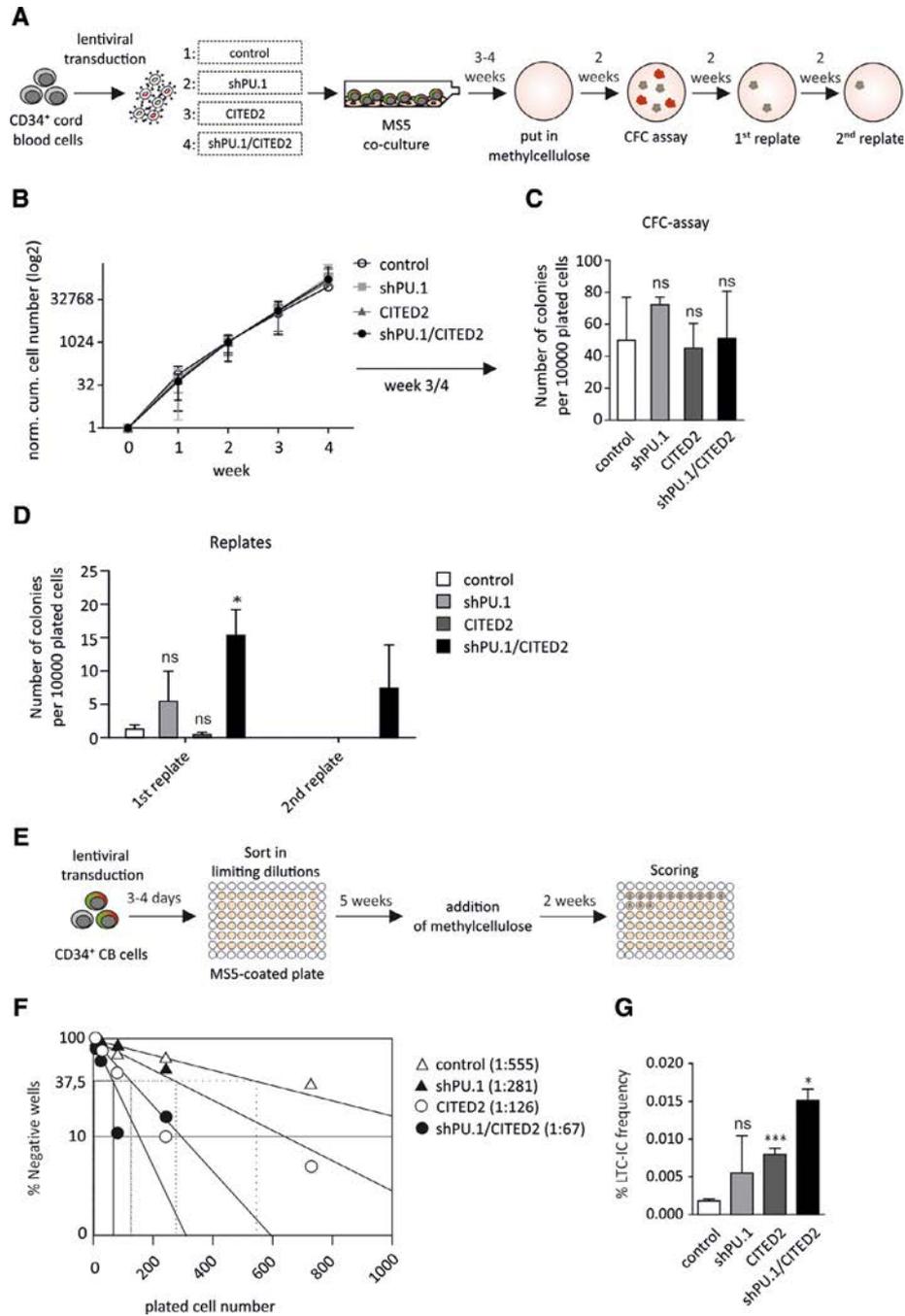
Figure 2. shPU.1/CITED2 cells have the highest LTC-IC frequency. **(A)** Schematic overview of experimental design. CD34$^+$ CB cells were transduced with the indicated lentiviral constructs and plated on an MS5 stromal layer. After 3−4 weeks, CFC assays were performed on cultured cells. **(B)** Growth curve of transduced CD34$^+$ cells cultured on an MS5 stromal layer in Gartner's medium. Error bars indicate the standard deviations of six individual experiments. Each experiment was performed with CB from several donors. **(C)** CFC assays of transduced CB cells that have been cultured as in (B) for 3 or 4 weeks. Error bars indicate the standard deviations of three individual experiments performed in duplicate. ns = not significant. **(D)** First and second replates of cells harvested from CFC assays illustrated in (C). Error bars indicate the standard deviations of three individual experiments performed in duplicate. ns = not significant. *p < 0.05. **(E)** Experimental design of LTC-IC assay. Transduced CD34$^+$ CB cells were sorted in an MS5-coated 96-well plate in limiting dilutions of 9−1000 cells. After 5 weeks, methylcellulose was added, and wells were scored as positive or negative for colony-forming units (CFU) after 14 days to determine LTC-IC frequencies. **(F)** Scoring of one representative LTC-IC experiment performed as described in (E). **(G)** Average LTC-IC frequency of three individual experiments. Each individual experiment was performed with CD34$^+$ cells from several donors. Error bars indicate standard deviations. ns = not significant. *p < 0.05.

not significantly affected by PU.1/CITED2 deregulation. Cells that were plated in methyl cellulose after 3 or 4 weeks of culture formed equal numbers of colonies in CFC assays (Figure 2C); however, replating of CFC assays with shPU1/CITED2 cells resulted in a significantly larger number of colonies compared with all other conditions (Figure 2D). Remarkably, colony formation in the second round of replating was restricted solely to shPU1/CITED2 cells (Figure 2D). The impact of PU.1/CITED2 deregulation on HSPC maintenance was further evaluated by performing LTC-IC assays, in which cells are maintained for 5 weeks on an MS5 stromal layer without additional growth factors prior to reading out their colony-forming capacity (Figure 2E). Whereas PU.1 downregulation did not alter the LTC-IC frequency significantly compared with control cells, upregulation of CITED2 resulted in a 4-fold increase, and combined shPU.1/CITED2 alteration resulted in an 8-fold increase in the LTC-IC frequency (Figure 2F, G). These data indicate that upregulation of CITED2 alone can be sufficient to increase HSPC maintenance under certain conditions. However, if HSPC maintenance is challenged by external signals such as activation of signaling cascades that promote cell proliferation or differentiation, simultaneous downregulation of PU.1 and upregulation of CITED2 can increase the HSPC frequency more effectively.
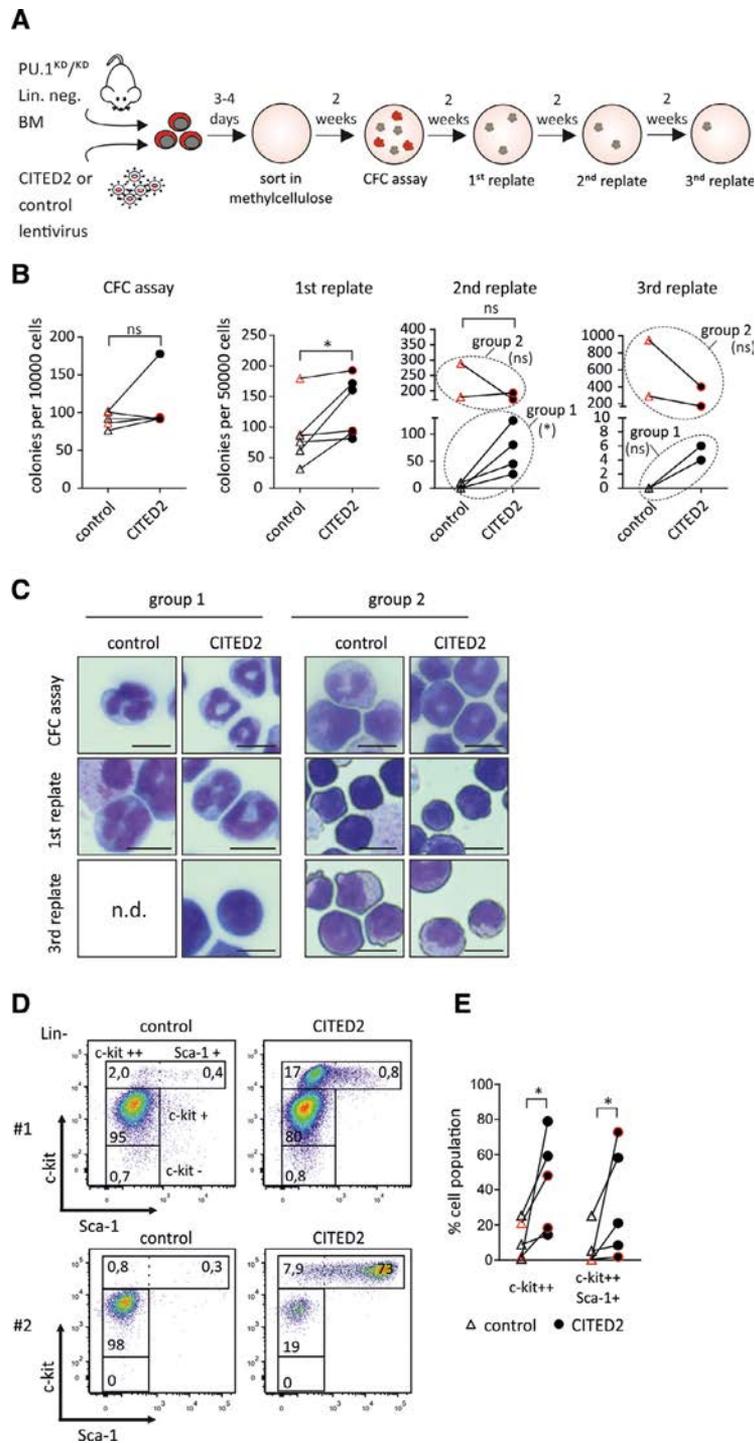
CITED2 overexpression in PU.1$^{KD/KD}$ bone marrow cells enhances the outgrowth of immature cells in in vitro *replating assays*
To confirm and study the effects of CITED2 upregulation in cells with low PU.1 expression with an alternative strategy, we lentivirally overexpressed CITED2 in murine PU.1$^{KD/KD}$ BM cells, which have the potential to transform and are therefore preleukemic [30]. Reduction of PU.1 expression in PU.1$^{KD/KD}$ cells compared with PU.1$^{WT/WT}$ cells was confirmed by Q-PCR (Supplementary Figure E2A, online only, available at www.exphem.org). Lineage-depleted PU.1$^{KD/KD}$ BM cells were isolated from mice in a preleukemic phase ($n = 6$) and control- or CITED2 transduced cells were sorted in methylcellulose to perform CFC assays with subsequent replating (Figure 3A). Similar to the experiments performed with CB cells, we observed comparable numbers of CFCs in control- and CITED2-transduced cells in the primary CFC assay, whereas CITED2 overexpression resulted in significantly more colonies following the first replate ($p < 0.05$; Figure 3B). Phenotypically we did not observe differences between control and CITED2 colonies (Supplementary Figure E2B). Interestingly, in the second and third replates, samples could be divided into two groups based on colony number and replating ability. With cells obtained from 4 mice (group 1), colony

formation could be observed in three rounds of replating, with higher colony numbers in CITED2 than control samples (on average, 69 vs. 5 colonies in second replate, $p = 0.05$; Figure 3B). In group 2, both control- and CITED2-transduced cells gave rise to several hundred colonies even in a third replate (Figure 3B). Notably, groups 1 and 2 had similar transduction efficiencies at the moment of cell sorting. Cytospins of CFCs revealed the presence of mature cells in the first replate of group 1, whereas the third replate was dominated by cells with immature morphology with a number of blast cells (Figure 3C). In contrast, a phenotypically homogenous population of immature blast cells was already present in the first replates of group 2 (Figure 3C), which contained c-Kit^{pos}/Gr-1^{neg} and c-Kit^{pos}/Gr-1^{low} cell populations (Supplementary Figure E2C). By flow cytometric analysis for c-Kit and Sca-1 expression, we observed that colonies from replates of control cells consist mainly of a rather homogenous c-Kit^{low}Sca-1^{neg} cell population (Figure 3D), whereas colonies from CITED2-transduced cells showed a more heterogeneous picture consisting of c-Kit^{low}, c-Kit^{high}, and c-Kit^{high}/Sca-1^{pos} cells (Figure 3D, E). On the basis of these data, we concluded that CITED2 is not required for or additive to leukemic transformation, but potentially supports the outgrowth of phenotypically immature PU.1$^{KD/KD}$ cells, at least for the time frame within which we performed the experiments.

Overexpression of CITED2 in PU.1$^{KD/KD}$ is not sufficient for leukemia initiation in vivo
Next, we questioned whether overexpression of CITED2 in murine PU.1$^{KD/KD}$ cells contributes to leukemia development *in vivo*. Therefore, c-Kit^{pos}-HSPCs were isolated from PU.1$^{KD/KD}$ donor mice and transduced with control or CITED2-overexpressing lentivirus (Supplementary Figure E3A, online only, available at www.exphem.org). Subsequently, transduced cells were transplanted into irradiated NSG recipient mice (Figure 4A). Two independent experiments (referred to as cohorts A and B) were performed with each cohort consisting of 7 mice receiving control-transduced cells and 7 mice receiving CITED2-transduced cells. In both cohorts, we observed a significantly higher percentage of CITED2-GFP donor cells compared with control GFP donor cells in the peripheral blood of recipient mice 11−15 weeks after injection (Figure 4B). However, only modestly higher levels of CITED2-GFP compared with control GFP donor cells were found in the bone marrow of recipient mice after 34 weeks (cohort A) and 24 weeks (cohort B), respectively (Figure 4C); these differences were not significant. In none of the mice were signs of leukemia development observed, as indicated by normal-weight spleens and

Figure 3. Overexpression of CITED2 in murine PU.1$^{KD/KD}$ bone marrow cells maintains stem and progenitor cells prior to transformation. **(A)** Schematic overview of experimental design. Lineage-depleted BM cells derived from PU.1$^{KD/KD}$ mice were transduced with control or CITED2-overexpressing lentivirus, and sorted cells were subjected to CFC assays and subsequent replating. **(B)** Number of colonies in CFC assays and replates performed with PU.1$^{KD/KD}$ cells transduced with control or CITED2 constructs. Data points connected by the *black line* belong to cells isolated from the same mouse. Data from six individual experiments using BM from six individual mice are shown. Samples were separated into two groups based on colony number; group 2 is labeled by a *red border*. *$p < 0.05$. **(C)** May Grunwald/Giemsa staining of cells harvested from CFC assays performed in (B). Scale bars = 10 μm. **(D)** Representative fluorescence-activated cell sorting (FACS) plots indicating c-Kit and Sca-1 expression of lineage-negative control- and CITED2-transduced PU.1$^{KD/KD}$ cells harvested from CFC assay replates shown in (B). Plots of two mice from group 2 are shown (#1, #2). Numbers in gates indicate percentages of c-Kit$^-$, c-Kit$^+$, c-Kit^{++} and c-Kit^{++}/Sca-1$^+$ double-positive cell populations. **(E)** Graph indicating the percentage of c-Kit^{++} and c-Kit^{++}/Sca-1$^+$ cell fractions observed by FACS analysis as described in (D); group 2 samples are labeled by a *red border*. n = 5. *$p < 0.05$.
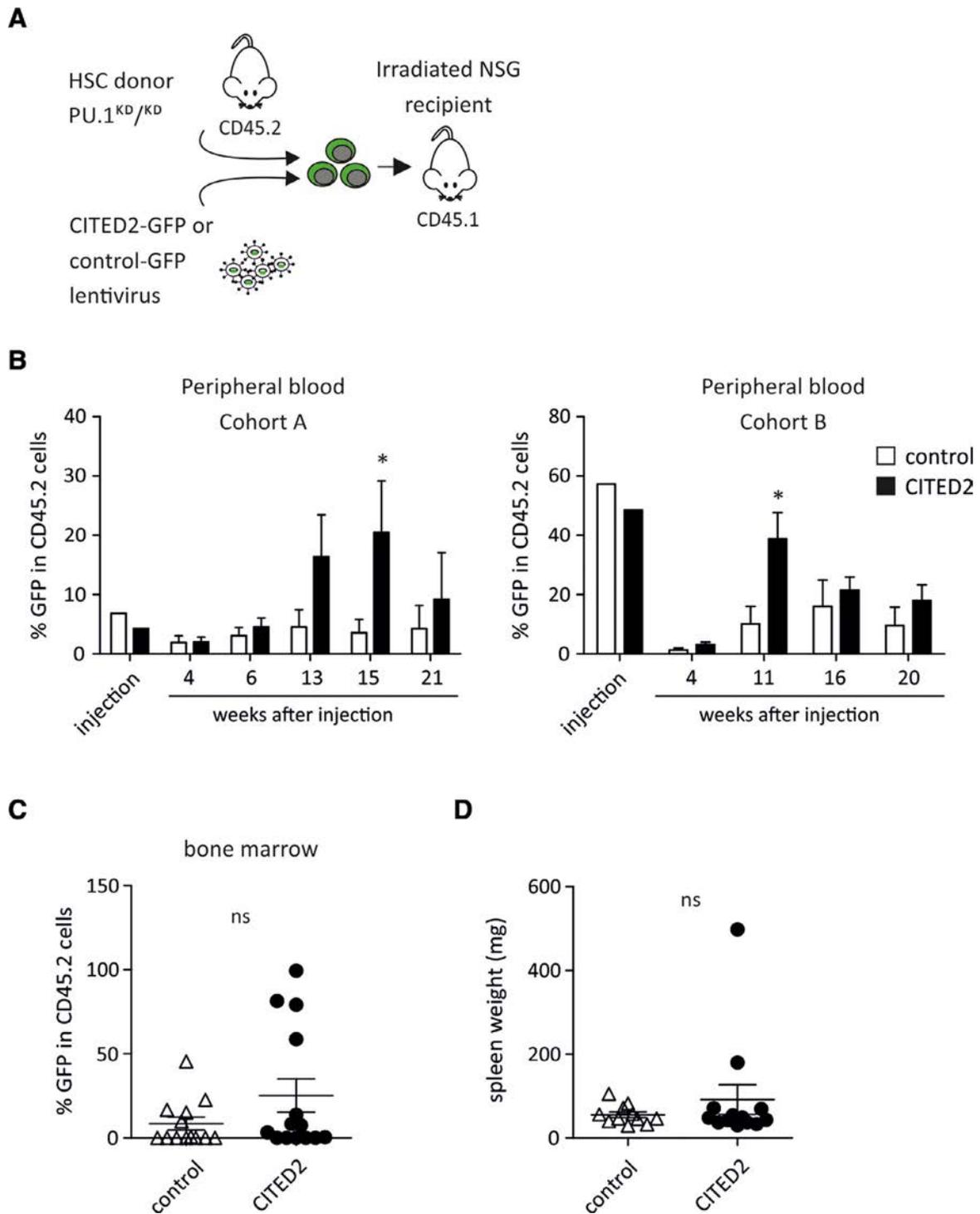
Figure 4. Overexpression of CITED2 in PU.1$^{KD/KD}$ HSCs is not sufficient for leukemic transformation. **(A)** Schematic overview of experimental design. Lin⁻cKit⁺ BM donor cells from PU.1$^{KD/KD}$ mice were transduced with a GFP-tagged control or CITED2 lentivirus and retro-orbitally injected into irradiated NSG recipient mice. Two independent experiments (referred to as cohorts A and B) were performed with 7 control and 7 CITED2 recipient mice each being injected. **(B)** Percentage of GFP⁺CD45.2 cells at time of injection and in peripheral blood after indicated number of weeks. Error bars indicate standard deviations. $n = 7$ for each cohort. $*p < 0.05$. **(C)** Percentage of GFP⁺ cells in bone marrow of recipient mice at day of sacrifice 30−34 weeks after injection. Error bars indicate standard deviations. $n = 14$. **(D)** Spleen weights of sacrificed mice are indicated. Error bars represent standard deviations

livers (Figure 4D; Supplementary Figure E3D). These data indicate that CITED2-GFP PU.1$^{KD/KD}$ donor c-Kit^{pos}-HSPCs contribute more rapidly to engraftment than control GFP PU.1$^{KD/KD}$ cells; however, CITED2 overexpression does not enhance the initiation of leukemia within this time frame.

shPU.1/CITED2-induced gene expression patterns correlate with gene expression profiles observed in AML

To investigate the transcriptional changes caused by shPU.1/CITED2 gene deregulation, an Ilumina Bead-Chip array was performed with CD34$^+$ CB cells transduced with the corresponding lentiviral vectors (Figure 5A; Supplementary Figure E4A, B, online only, available at www.exphem.org). PU.1 downregulation and CITED2 overexpression of sorted cells were verified by Q-PCR (Supplementary Figure E4C). We found that downregulation of PU.1, overexpression of CITED2, or their combination respectively led to 176, 205, or 148 differentially expressed genes (>2-fold up- or downregulated in both replicates, Figure 5B), as compared with control-transduced cells. Notably, the 148 probe sets that were found differently expressed in shPU.1/CITED2 cells were partly overlapping with deregulated genes found in shPU.1- or CITED2-only cells (32/148 overlap with shPU.1; 35/148 overlap with CITED2), but also contained a unique set of genes (94/148) (Figure 5B; Supplementary Table E1, online only, available at www.exphem.org). In general, gene expression changes were surprisingly modest, and unexpectedly, both pathway and GSEA analysis did not reveal significant signatures linked to stem cell maintenance or cell proliferation. Despite these findings, we decided to explore whether gene expression changes induced by combined shPU.1/CITED2 deregulation overlap with changes observed in CD34$^+$ AML cells in comparison to normal CD34$^+$ cells. We therefore downloaded gene expression data from 198 AML patients and 18 normal CD34$^+$ subsets from the Blood-Spot database (http://servers.binf.ku.dk/bloodspot/). One hundred twelve of 148 probe sets from our study could be linked to a gene, of which 67 were present in the BloodSpot database. Of these 67 genes, 34 were upregulated and 33 were downregulated in shPU.1/CITED2-transduced cells from our study. Notably, we observed that the majority of genes that were found upregulated in our shPU.1/CITED2 cells are also upregulated in AML patients (29/34), whereas 14 of 33 downregulated genes in shPU.1/CITED2 cells are also found downregulated in AML patients when compared with normal CD34$^+$ cells (Figure 5C). Spearman's ranked correlation analysis revealed that the gene expression changes we observed in shPU.1/CITED2 cells significantly correlated with the changes observed

in AML (Figure 5D), with the highest correlation observed for upregulated genes. The overlap of gene expression patterns of shPU.1/CITED2 cells and AML patients was not specific for a certain subtype of AML but could be found across various AML patients, suggesting that the modest transcriptional changes caused by combined PU.1/CITED2 deregulation could be generally supportive for AML development when combined with variable additional hits.

Discussion

In the present study, we found that combined upregulation of CITED2 and downregulation of PU.1 increase HSPC maintenance through two alternative approaches. Simultaneous overexpression of CITED2 and knockdown of PU.1 in CD34$^+$ cord blood cells using lentiviral vectors resulted in enhanced replating capacity in CFC assays and increased LTC-IC frequency. Similar results were obtained when CITED2 was upregulated in preleukemic murine PU.1$^{KD/KD}$ c-Kit^{pos}-HSPCs.

AML is characterized by a stepwise accumulation of genetic and epigenetic alterations that first result in the generation of a clonal and/or preleukemic state before eventually leading to fully transformed leukemic cells. Altered regulation of self-renewal, maintenance, and proliferation without a block in differentiation have been described as an early event in malignant transformation [2,3,45−47]. Recently, several studies have reported that clonal hematopoiesis with driver mutations can be detected in a large cohort of elderly patients whereby clonal cells outcompete the remaining cells. However, only a limited number of these patients develop AML, in particular when co-mutations occur, thereby triggering alternative pathways and making the cells vulnerable to AML transformation [3,4,48]. Apparently, cord blood shPU.1/CITED2 HSCs mimic the initial step in clonal evolution, reflected by increased replating capacity and increased LTC-IC frequency, but not a block in differentiation.

Because CITED2 expression is found to be upregulated in AML and has been reported to be essential for leukemic cell survival [33,37], we wondered if CITED2 overexpression in definite preleukemic cells contributes to their transformation. Knockdown of PU.1 in murine HSCs results in AML development after a preleukemic phase of several months [30,31], and therefore, untransformed PU.1$^{KD/KD}$ cells resemble such a condition. The results of the present study indicate that overexpression of CITED2 in untransformed PU.1$^{KD/KD}$ c-Kit^{pos} HSPCs is not sufficient for immediate leukemia onset but, however, expands the pool of preleukemic PU.1$^{KD/KD}$ HSPCs. An interesting question that could be addressed in future studies is whether upregulation of CITED2 in PU.1$^{KD/KD}$ cells prior to (serial) transplantation experiments would have an
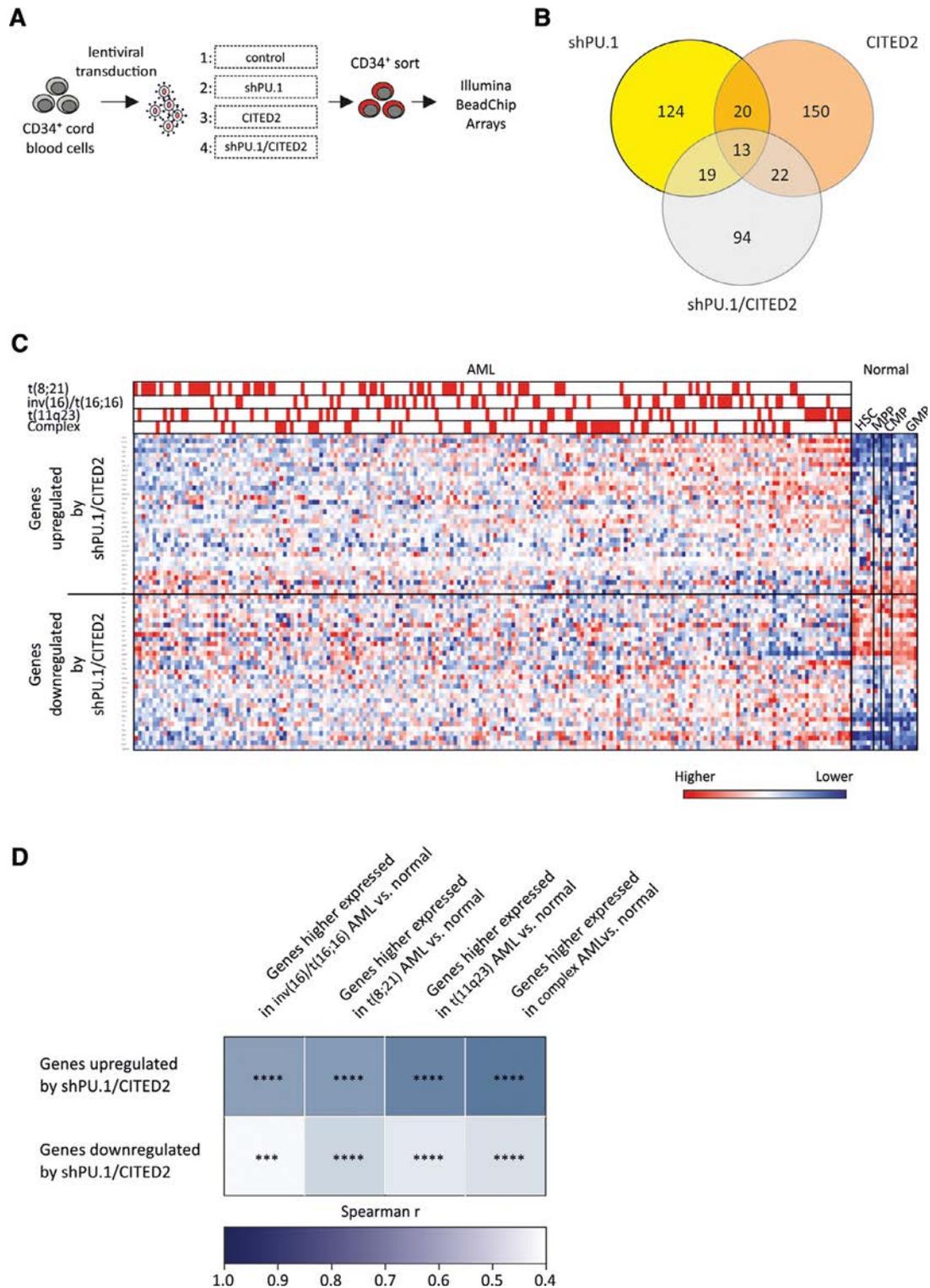
Figure 5. Combining PU.1 downregulation with CITED2 upregulation induces a gene expression pattern also observed in AML. **(A)** Schematic overview of experimental design. Isolated CD34+ cord blood cells were transduced with the indicated lentiviral constructs, and transduced CD34+ cells were sorted for Illumina BeadChip Arrays. **(B)** Venn diagram indicates that the number of genes changed >2-fold in duplicate arrays, compared with control transduced cells **(C)** Gene expression comparison of non-APL AMLs versus normal CD34+ cells. Each column is an AML sample, with the *red squares* at the top indicating the subtype. **(D)** Spearman's rank correlation between genes that are differentially expressed in shPU.1/CITED2 cells and genes differentially expressed in non-APL AMLs. ***p < 0.005. ****p < 0.001.

impact on AML development or LSC maintenance when combined with additional alterations. Other mutations have been identified that accelerate the process of leukemic transformation when combined with PU.1 downregulation. For instance, mice carrying a mutation in K-Ras rapidly progress from a myeloproliferative neoplasm to an aggressive AML when a deubiquitylase that regulates PU.1 stability is deleted [23]. In addition, in mice with a homozygous deletion of *Msh2*, a gene involved in DNA mismatch repair, slight reductions in PU.1 levels were reported to promote AML progression [32]. Similarly, reduction of PU.1 levels in p53$^{-/-}$ mice resulted in AML development, which is not observed when only p53 is deleted [49]. Mechanistically, the importance of CITED2 in maintaining both HSCs and LSCs has been linked to cell apoptosis in a p53-dependent manner [35]. We previously reported that loss of CITED2 triggers leukemic cell death through stabilization of p53 [44], an observation also made for other types of cancer [50]. It is likely that the reverse might occur in the context of CITED2 overexpression, making HSCs less sensitive to stress response pathways and facilitating the process of stem cell maintenance.

PU.1$^{KD/KD}$ HSPCs exhibited an up to 80% reduction of PU.1 levels, whereas our lentiviral-mediated PU.1 knockdown in cord blood cells caused a reduction in PU.1 levels in the range 20% to 50% (Supplementary Figure E4C). Strikingly, despite the variability of PU.1 downregulation, both genetic models resulted in similar phenotypes. These data indicate that even a modest reduction in PU.1 levels, which is also observed in AML cells [18,19,21,22,24], can lead to increased HSPC maintenance in combination with elevated CITED2 levels. We therefore also aimed to gain more insight into the transcriptional changes observed in shPU.1/CITED2 cells. Gene expression analysis revealed that there are a number of genes differentially expressed in shPU.1/CITED2 CD34$^+$ cord blood cells, which are not deregulated when only PU.1 or CITED2 levels are altered, indicating that a unique transcriptional program is altered by combined shPU.1/CITED2 alteration. Furthermore, we found that gene expression changes in shPU.1/CITED2 CD34$^+$ cells mimic a pattern found in patients with various AML subtypes, indicating that our genetic model of combined PU.1/CITED2 deregulation resembles a state that is in general supportive of AML development.

Conclusions

Our genetic models with combined CITED2/PU.1 deregulation mimic the initial step in clonal leukemia evolution and can be useful in performing further studies to reveal the molecular mechanism underlying AML development.

Conflict of interest disclosure

The authors declare no competing financial interests.

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Supplementary Material and Methods

Lentiviral transduction

Human CITED2 cDNA was obtained through Addgene (plasmid 21487) and cloned into the multiple cloning site of either pRRL-SFFV-IRES-tNGFR or pRRL-SFFV-IRES-EGFP [33]. Vectors containing shRNA against SPI1 (PU.1) were obtained from GE Healthcare Dharmacon (#V3SVHS06_8494848) and the hairpin containing region was cloned into the pGIPZ-SFFV-EGFP-shRNAmir backbone using the MluI and NotI restriction enzyme sites. Constructs for combined CITED2 overexpression and PU.1 downregulation where obtained by cloning the shRNAmir cassette from the pGPIZ-SFFV-EGFP-shRNAmir vector into the pRRL-SFFV-IRES-mCherry backbone. Sequences and plasmids are available upon request. Lentiviral particles were produced as described before.[43] After 24 hours, medium was changed to HPGM and after 12 hours, supernatant containing lentiviral particles was harvested, concentrated using CentriPrep Ultracel YM-50 Filter Units (Merck Millipore) and stored at −80°C. Cells were transduced with lentiviral particles in the presence of 4 μg/ml polybrene in 2 consecutive rounds of 12 hours. During transduction, human CD34$^+$ cells were kept in HPGM supplemented with hSCF (Novoprotein), hFLT3 ligand (Celldex) and Npllate (Amgen) (100 ng/ml each). Murine bone marrow cells were kept in StemSpan SFEM (Stemcell Technologies) supplemented with 100 ng/ml mSCF (PepProtech), 100ng/ml hFLT3 ligand (Celldex), 100ng/ml Nplate (Amgen) and 20 ng/ml mIL-3 (PepProtech). Cells were sorted 3 days after transduction on a MoFLo XDP or Astrios (DakoCytomation, Carpinteria, CA, USA) and applied to subsequent assays.

RNA isolation and Q-PCR

Total RNA was isolated using the RNeasy Micro Kit (QIAGEN) following manufacturer's instructions and reverse transcriped using the iScript cDNA synthesis kit (Bio-Rad). Real-Time PCR was performed on a CFX Connect System (Bio-Rad) using the SsoAdvanced SYBR Green Supermix (Bio-Rad). Data were quantified using CFX Manager software (Bio-Rad) and normalized to values of the housekeeping gene RPS11, RPL27, HPRT or B2M. Primer sequences are listed in Supplementary Table E2.

FACS analysis

Cells were sorted on a MoFLo XDP or Astrios (DakoCytomation, Carpinteria, CA, USA). All FACS analyses were performed on an LSRII (Becton Dickinson) flowcytometer and data was analyzed using FlowJo software. Murine lineage negative cells were selected using the Alexa Fluor 700 anti-mouse lineage cocktail (Biolegend, Uithoorn, The Netherlands, #133313). Antibodies used for flow cytometry of murine cells were: Alexa Fluor 488 anti-mouse Ly-6G/Ly-6C (Gr-1) (Biolegend, #108419), PE anti-mouse CD117 (c-kit) (Biolegend, #105807), Brilliant Violet 421 anti-mouse Ly6A/E (Sca-1) (Biolegend, #108127), PE/Cy7 anti-mouse/human CD11b (Biolegend, #101215).

Supplementary Table E1. Table lists probesets that were found differentially expressed in shPU.1/CITED2- transduced CD34^{+} cord blood cells compared to control transduced cells in an Ilumina BeadChip array.

| Unique to shPU.1/CITED2		Common with CITED2		Common with shPU.1		Common in all groups	
ProbeID	Gene Symbol	ProbeID	Symbol	ProbeID	Symbol	ProbeID	Symbol
3610626	ADM2	2850041	B3Gn-T6	7330102	CSMD1	3710722	CCDC50
4390523	AGAP7	4060632	C17orf87	1660689	GRB14	2690328	LOC647357
2510368	ARID4A	3940435	EMP1	6330767	HDAC2	1400653	LOC651075
7650209	BMF	5810577	FGA	430403	HOXB6	1240139	LOC651680
3940152	C22orf40	1260341	IL13RA1	4880192	LOC100132771	110411	LOC653701
3870441	CAPN3	360672	LOC100130928	2600095	LOC165186	7650392	MIR1267
6220382	CD68	5670176	LOC642003	580575	LOC255620	4290450	PIK3R1
4040022	CD86	6450358	LOC644641	4640136	LOC648863	5260433	PMCHL1
2470196	DNAJB5	4590192	LOC645165	2690010	LOC650620	4830632	SLC7A7
940639	ERP27	2260608	LOC649422	3520240	LOC651137	5720682	TMEM176A
60307	FCF1	5670450	PURG	3120731	LOC653643	3610020	
6040598	GSDMB	1440730	SNORA29	6620725	PPAP2C	1430494	
4890279	HIST2H4A	450682	SNORA49	1340541	RGPD5	3850369	
3870102	HNMT	4180195	SP6	1400121	SLCO2B1		
2060553	HYALP1	630722	TBX19	4880519	SPO11		
5890021	IGSF11	1710273		2760242	TIAM1		
2470743	KMO	1030243		1300026			
610037	LAMA1	1990093		6770646			
3180609	LOC100008588	3460519		6960753			
6290142	LOC100008589	7560142					
2510446	LOC100130276	1070056					
2750382	LOC100133916	2650048					
6040386	LOC100134539						
5960240	LOC100134634						
1400717	LOC121456						
240204	LOC151457						
1580082	LOC158572						
5860255	LOC284296						
7150082	LOC285296						
6760093	LOC440386						
2450326	LOC642047						
1820519	LOC642838						
2850246	LOC643713						
1010543	LOC643888						
2000369	LOC644191						
5290685	LOC645183						
3870731	LOC645722						
7560484	LOC647827						
4810717	LOC650111						
5910674	LOC650698						
6370192	LOC728288						
3140358	LOC728787						
5700736	LOC729081						
3520259	LOC730202						
50332	MDFIC						
4810114	MGC12982						
70669	MIR1224						
990328	MS4A7						
3990477	MTHFR						
2480452	MYF5						
70008	NCF2						
2650113	NR1I2						
20068	OTUD1						
2340301	PION						
2070592	PPAPDC1A						
2630519	RSHL3						
1410440	SHC2						
4180647	SHPRH						

(continued)

Supplementary Table E1 (*Continued*)

Unique to shPU.1/CITED2		Common with CITED2		Common with shPU.1		Common in all groups	
ProbeID	**Gene Symbol**	**ProbeID**	**Symbol**	**ProbeID**	**Symbol**	**ProbeID**	**Symbol**
840189	SMTN						
1300349	SOCS4						
2370368	SON						
4120475	SOX15						
7570670	SPIN1						
4590767	SPRR1B						
2350424	SUV420H2						
4180521	TMEM63B						
7040348	TMLHE						
4830273	TWF1						
5570546	U2AF1L4						
1010500	UGP2						
780180	USP9X						
610438							
6510739							
4830300							
3940609							
2000709							
670725							
7380593							
4490139							
5090102							
1260470							
6480025							
830112							
3120300							
1580070							
630615							
3360170							
7610427							
5290433							
4050296							
240259							
4670491							
1990392							
7560402							

Supplementary Table E2. Table lists primer sequences that have been used for Q-PCR.

| name	forward primer 5'-3'	reverse primer 5'-3'
hHPRT	AGTTCTGTGGCCATCTGCTTAG	CGCCCAAAGGGAACTGATAGTC
hRPL27	TCCGGACGCAAAGCTGTCATCG	TCTTGCCCATGGCAGCTGTCAC
hRPS11	AAGATGGCGGACATTCAGAC	AGCTTCTCCTTGCCAGTTTC
hCITED2	CTTTGCACGCCAGGAAGGTC	CGCCGTAGTGTATGTGCTCG
hSPI1 (PU.1)	GCGACCATTACTGGGACTTC	ATGGGTACTGGAGGCACATC
mSPI1 (PU.1)	GCCTCAGTCACCAGGTTTCC	CCTTGTCCACCCACCAGATG3
mHPRT	AGTCCCAGCGTCGTGATTAG	CCAGCAGGTCAGCAAAGAAC
mB2M	TGACCGGCCTGTATGCTATC	GATCCCAGTAGACGGTCTTG
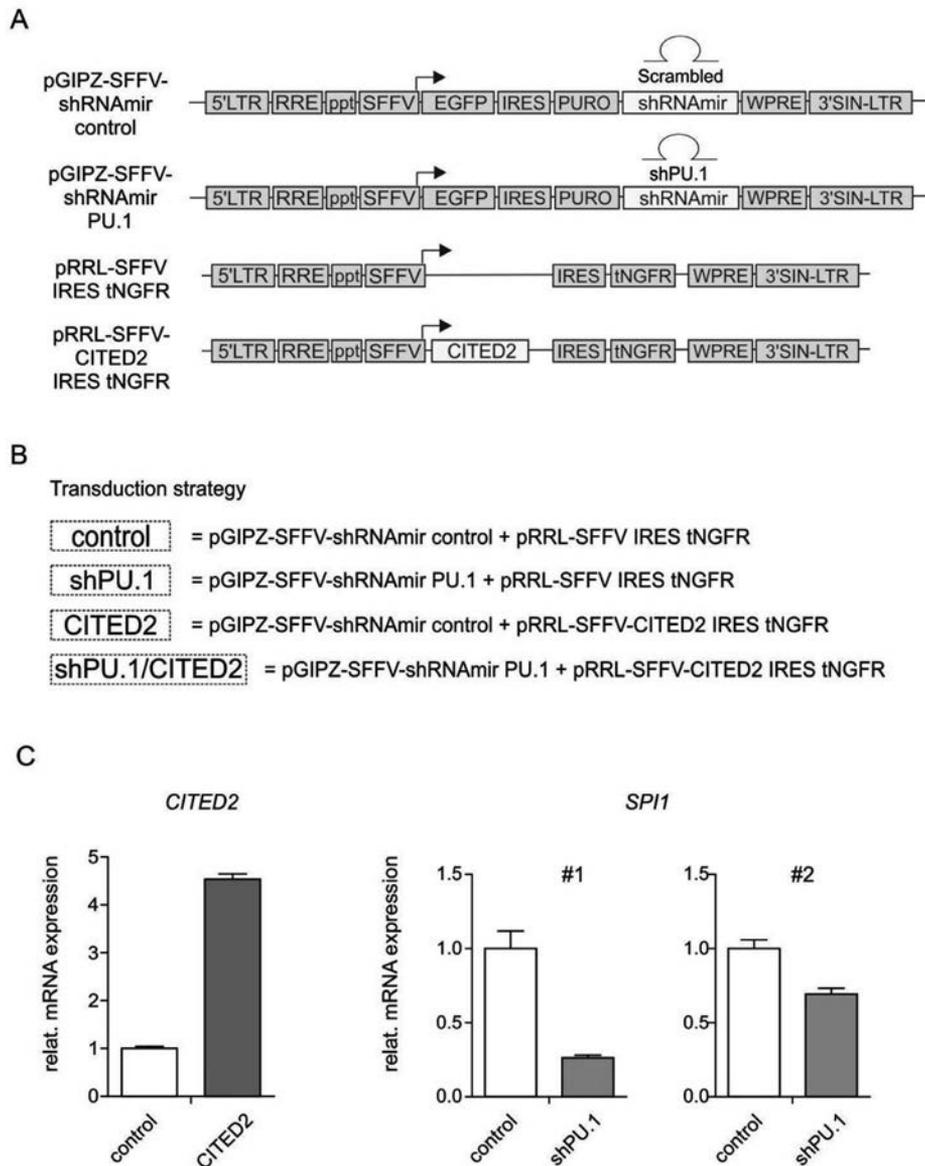
Supplementary Figure E1. (Supplement to Figure 1) **Combined PU.1 down-regulation and CITED2-upregulation in CD34⁺ cord blood cells was mediated by lentiviral constructs** **(A)** Schematic overview of the lentiviral constructs used for transduction **(B)** Double transduction strategy with indicated constructs was performed to achieve either downregulation of PU1 (shPU.1), upregulation of CITED2 (CITED2) or a combination of both (shPU.1/CTED2). **(C)** Efficiency of lentiviral constructs was tested by Q-PCR using CD34⁺ cord blood cells that had been transduced with indicated constructs and sorted for double transduced cells. The level of *SPI1* (PU.1) downregulation varied between cord blood samples (#1, #2). Error bars represent s.d. of Q-PCR triplicates.
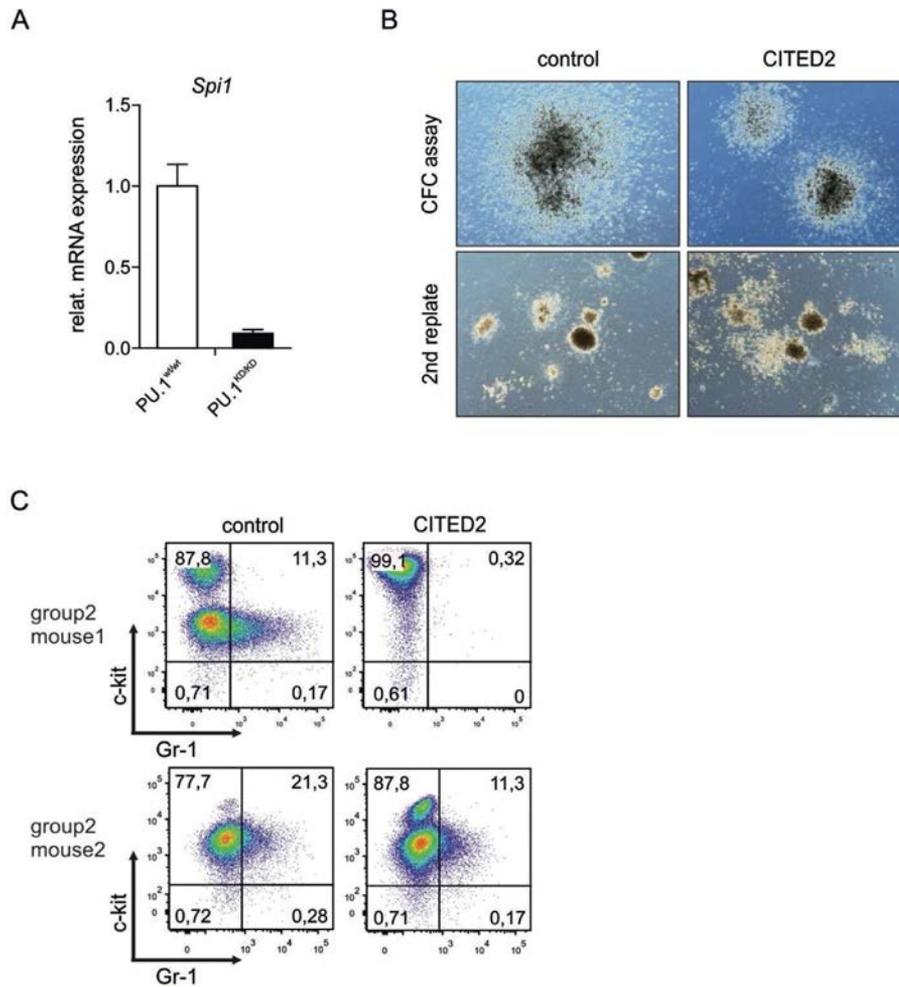
Supplementary Figure E2. (Supplement to Figure 3) **Overexpression of CITED2 in PU.1$^{KD/KD}$ cells** (A) Q-PCR for *Spi1* confirming an 80% reduction of *Spi1* (PU.1) expression in PU.1$^{KD/KD}$ BM cells compared to PU.1$^{WT/WT}$ cells. Error bars indicate s.d. of Q-PCR triplicates (B) Pictures show colonies from control- or CITED2 transduced PU.1$^{KD/KD}$ cells in primary CFC assays (upper panel) or 2nd round of replating. (C) FACS plots indicating c-Kit and Gr-1 expression of control- or CITED2 transduced PU.1$^{KD/KD}$ cells that have been harvested from CFC assay replates. Two samples of group 2, which is the group characterized by very high colony numbers in CFC assay replates, are shown.
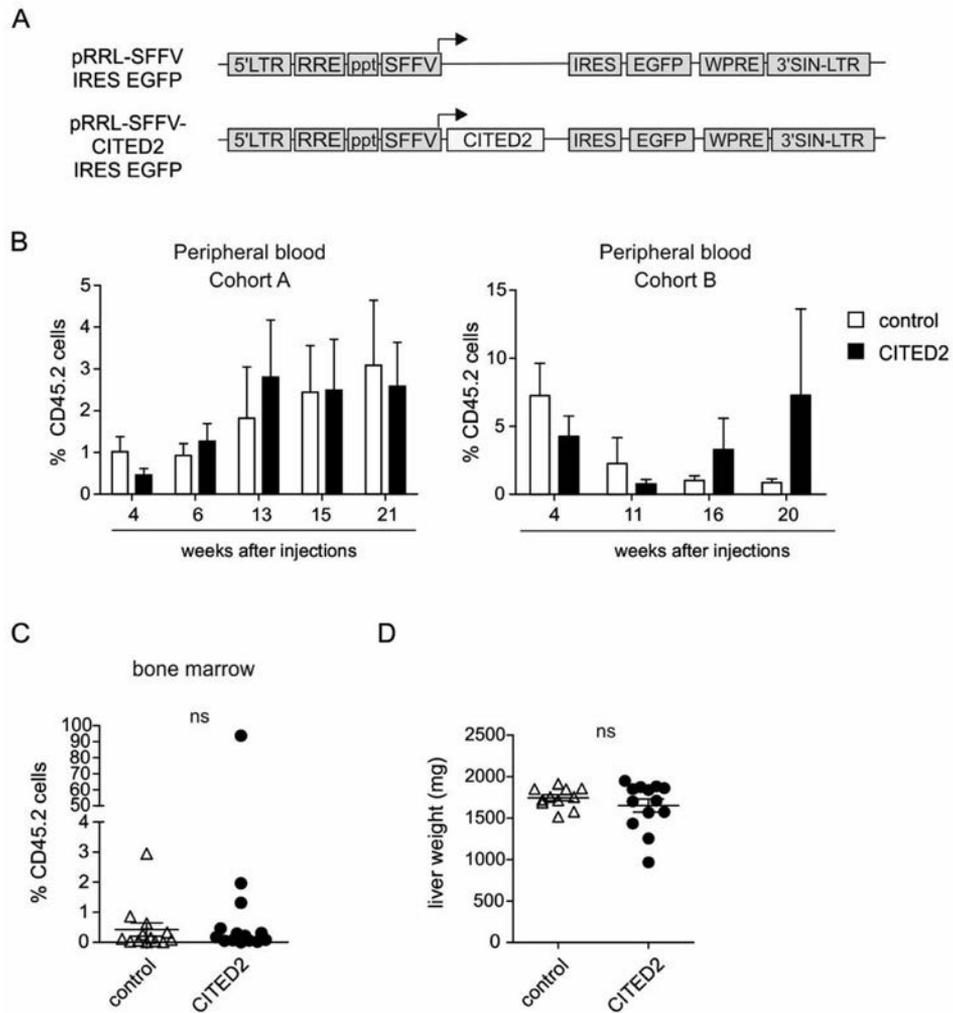
Supplementary Figure E3. (Supplement to Figure 4) **Overexpression of CITED2 in PU.1$^{KD/KD}$ HSCs is not sufficient for leukemic transformation** **(A)** Schematic overview of the lentiviral constructs used for transduction **(B)** Percentage of CD45.2 cells in peripheral blood after indicated number of weeks is shown. Error bars indicate s.d.; n=7 for each cohort; **(C)** Percentage of CD45.2 cells in bone marrow of recipient mice at day of sacrifice 30-34 weeks after injection is shown. Error bars indicate s.d.; n=14; **(D)** Liver weights of sacrificed mice is indicated; Error bars indicate s.d.; n.s: not significant
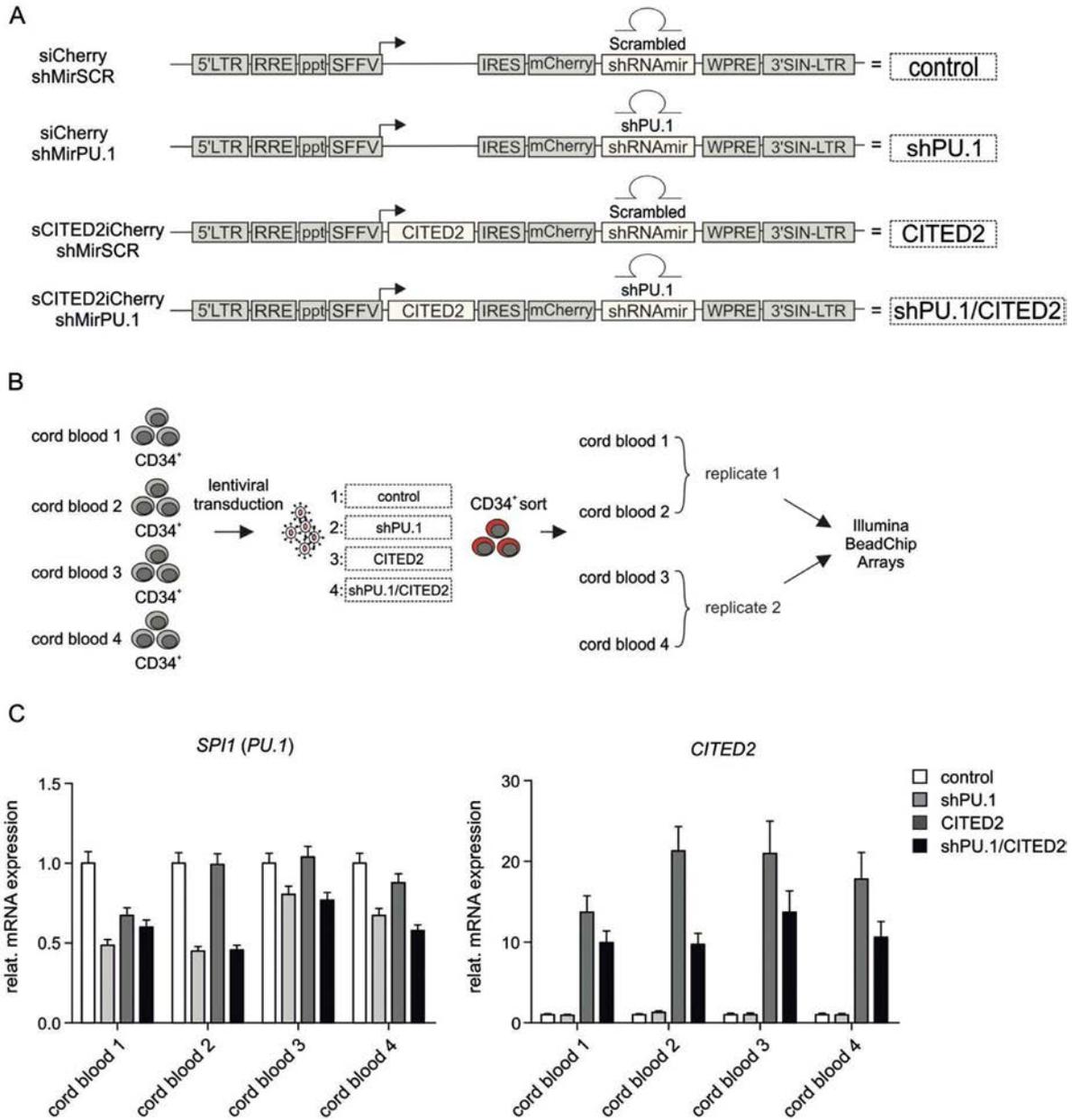
Supplementary Figure E4. (Supplement to Figure 5) **Ilumina BeadChip array was performed with control-, shPU.1-, CITED2, and shPU.1/ CITED2 CD34+ CB cells** **(A)** Schematic overview of the lentiviral constructs used for transduction **(B)** Overview of the experimental procedure: 4 different cord blood samples were transduced with the indicated constructs to achieve either downregulation of PU1 (shPU.1), upregulation of CITED2 (CITED2) or a combination of both (shPU.1/CTED2). Samples with similar levels of *SPI1* downregulation were pooled and an lumina BeadChip array was performed with 2 replicates. **(C)** Levels of *SPI1* (PU.1) downregulation and CITED2 upregulation of transduced CB cells applied to the Ilumina BeadChip array were analyzed by Q-PCR. Error bars indicate s.d. of Q-PCR triplicates.