

MLL-AF9 expression levels do not instruct lineage fate

The t(9;11) translocation results in expression of the MLL-AF9 oncofusion protein which can give rise to either acute myeloid leukemia (AML) or B-cell acute lymphoblastic leukemia (B-ALL) in pediatric patients.^{1,2} A recent study suggested that oncogene expression levels instruct lineage choice and that B-ALL patient treatment with menin inhibition may be complicated by lineage switching promoting AML relapse.³ By utilizing an exogenous lentiviral approach we were able to specifically address whether or not MLL-AF9 oncofusion protein levels causally specify lineage fate. Additionally, we show that the previously reported findings most likely are confounded by the lineage inherent epigenetic landscape determining MLL1 expression, which subsequently dictates oncogene expression levels.

Previously, various studies have addressed the question of whether cell-intrinsic or cell-extrinsic mechanisms would underly lineage choice in *MLL-AF9*-driven leukemic transformation. Clearly, cell-extrinsic factors play an important role, as cord blood (CB) CD34⁺ cells lentivirally transduced with *MLL-AF9* predominantly give rise to B-ALL when transplanted into lymphoid-biased NSG mouse models, while by transplantation into more myeloid permissive humanized mice expressing human cytokines both AML and B-ALL phenotypes were observed.⁴⁻⁷

Oncogene dosage impacts on the cell of origin from which *MLL-AF9*-induced leukemia can arise. While initial retro/lentiviral overexpression models showed that the *MLL-AF9* oncogene can efficiently transform committed granulomonocytic progenitors into leukemic GMP (L-GMP),⁸ it was later shown that when *MLL-AF9* was expressed under its own endogenous promoter only the most immature Lin⁻Sca1⁺/cKit⁺ progenitors were susceptible to *MLL-AF9*-induced transformation.⁹ Clearly, the oncogene expression levels dictate whether or not *MLL-AF9* can re-install a self-renewal and oncogenic transcriptional program in cells that have already committed to the myeloid lineage. Cell of origin dynamics have also been observed for the *MLL1* gene itself. Unlike in committed cells, *MLL1* has been found to be essential for the survival and self-renewal of hematopoietic stem cells.¹⁰ However, the comparison of *MLL/MLLr* gene expression and subsequent effects is conflicted by the fact that the binding targets have been found to be different.¹¹

Rare cases of conversion of the lymphoid and myeloid leukemic cell lineage during the course of the disease or during relapse have been reported in the literature. According to published data, lineage switching is almost exclusively observed in pediatric patients and it occurs most frequently from the lymphoid to the myeloid lineage.^{12,13} In adults, *MLL-AF9* almost exclusively results in AML and not B-ALL, and lineage switching is rarely seen. Several hypotheses

have been proposed to explain the molecular basis of the lineage switch, but the actual mechanisms remain to be discovered. Possible explanations are the emergence of new therapy-related clones or the emergence of therapy-insensitive clones of a different lineage already present at low frequencies at initial diagnosis. Another explanation might be the reprogramming of a multipotent leukemic stem cell that in response to intrinsic or environmental cues can change the lineage of its progeny.¹⁴ In *MLL-AF4* pediatric leukemia, it was shown that lymphoid to myeloid lineage switching is driven by disrupted epigenetic regulation.¹⁵ Although these complete lineage switches might occur infrequently, it is relevant to evaluate whether the leukemic blasts originate from the same leukemic clone or whether new clones emerge as a consequence of the treatment.

We first addressed this question in an *MLL-AF4* patient with a mixed leukemia phenotype presenting with both CD19⁺ lymphoid clones as well as CD33⁺ myeloid clones (Figure 1A). Both clones were sorted and cultured under myeloid-restricted MS5 co-culture conditions, or under lymphoid-permissive MS5 co-culture conditions under which both myeloid and lymphoid cells can propagate. These data demonstrated that CD19⁺ clones retain myeloid potential whereas CD33⁺ clones failed to produce lymphoid output (Figure 1A). Morphologically (Figure 1A) as well as transcriptionally (Figure 1B-E) CD19⁺-sorted cells cultured under myeloid conditions indeed switched to a myeloid phenotype. When analyzing expression of typical *MLLr* target genes such as *HOXA9*, *FLT3* and *PBX* we noted that expression levels appeared to be somewhat higher in lymphoid clones (Figure 1E), potentially indicating that *MLL-AF4* is higher expressed in those cells. This patient sample was derived from the OncoLifeS data-biobank for Oncology (Dutch Trial Register: NL7839) which entails written informed consent and approval of the study protocol by the local Institutional Review Board (UMCG METc2010/109) in accordance with the Declaration of Helsinki.

Similarly to the *MLL-AF4* patient sample, in cord blood (CB) lentiviral transduction models we could show that cells that were initially transformed along the B-lymphoid lineage retained myeloid potential when cultured under myeloid-restricted culture conditions (*Online Supplementary Figure S1A, B*). When CD19⁺ lymphoid-transformed cells were cultured under lymphoid permissive conditions for 1-3 weeks, cells retained a lymphoid phenotype, but when cells were grown under myeloid restricted conditions, cells gradually lost CD19 expression and became positive for CD33, CD11b, CD14 and CD15 (*Online Supplementary Figure S1A, B*). The growth rate of cells under both culture conditions remained similar (*Online Supplementary Figure*

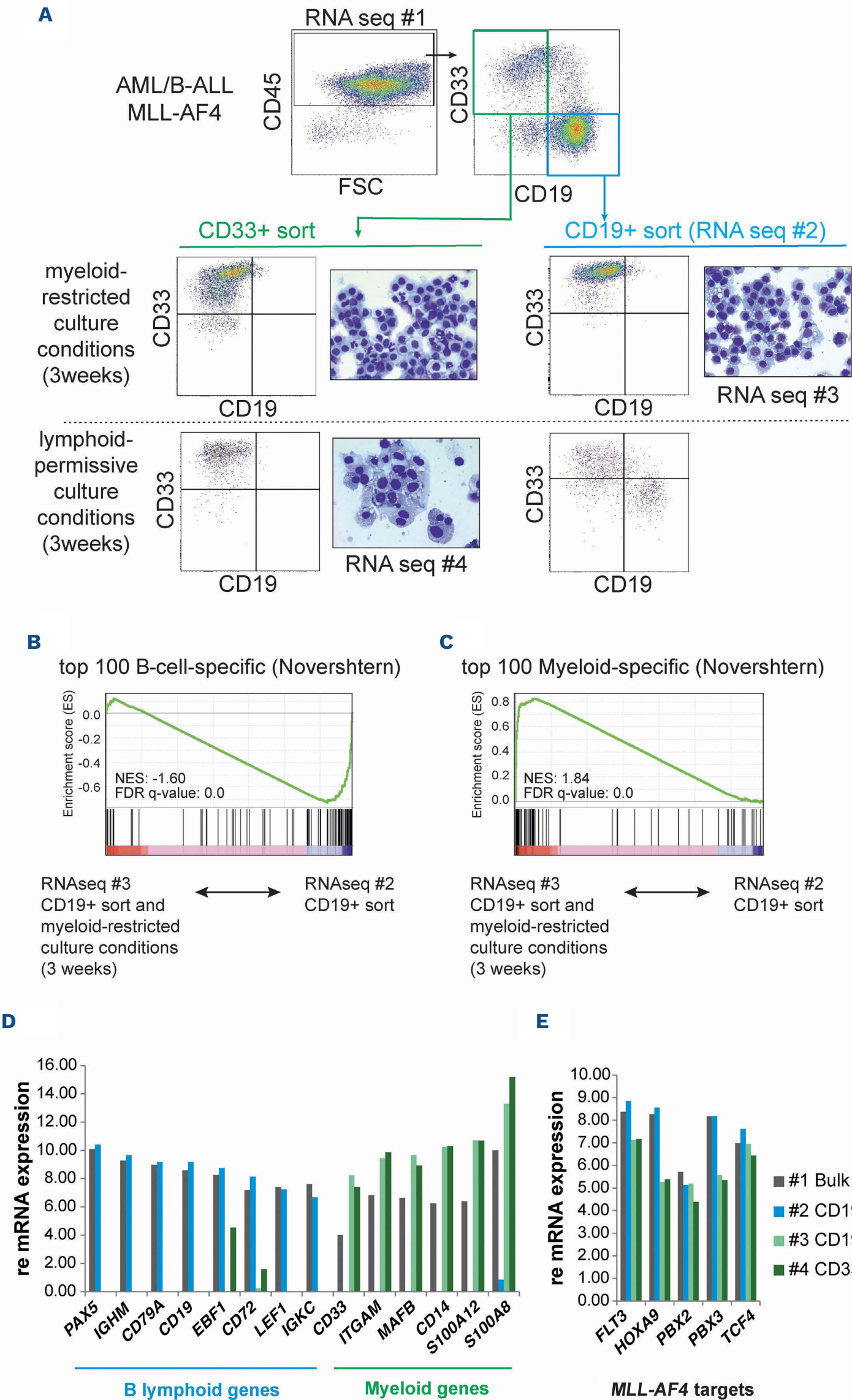


Figure 1. Lymphoid clones maintain both lymphoid and myeloid potential in contrast to myeloid clones which are committed to their lineage. (A) Pediatric *MLL-AF4* mixed phenotype leukemia primary cells were sorted for CD19⁺ (lymphoid clone) and CD33⁺ (myeloid clone) and cultured under myeloid-restricted and lymphoid-permissive MS5 co-culture conditions (see legend of the *Online Supplementary Figure S1*). The lymphoid clone maintained both lymphoid and myeloid potential whereas the myeloid clone showed commitment to the myeloid lineage as determined by CD33/CD19 immunophenotype and May-Grünwald Giemsa staining; magnification 63x. RNA was isolated at the indicated time points. (B, C) RNA sequencing (RNA seq) was performed (using the lexogen Quantseq 3' prep kit and Illumina NextSeq500) followed by gene set enrichment analyses comparing CD19⁺ cells under myeloid-restricted conditions (#3) to CD19⁺ after sorting (#2). (D, E) Examples of B-lymphoid and myeloid lineage genes (D), and *MLL-AF4* target genes (E) from RNA seq on bulk (#1), CD19 correct (#2), CD19⁺ cells under myeloid restricted conditions (#3) and CD33⁺ cells under lymphoid permissive conditions (#4). NES: normalized enrichment score; FDR: false discovery rate.

S1C). Ligation-mediated polymerase chain reaction (LM-PCR) analysis indicated that the integration site in the initial CD19⁺ lymphoid transformed cells was retained in the myeloid cells, indicating that the lineage shift did not occur because of clonal drift (*Online Supplementary Figure S1D*). A recent report suggested that MLL oncoprotein levels

would influence leukemia lineage identities.³ Whether or not higher MLL-AF9 levels are indeed causally related to transformation along the lymphoid lineage remained to be proven functionally. The use of lentiviral models where expression of the oncogene is driven by an artificial promoter that is independent of lineage type provides a unique

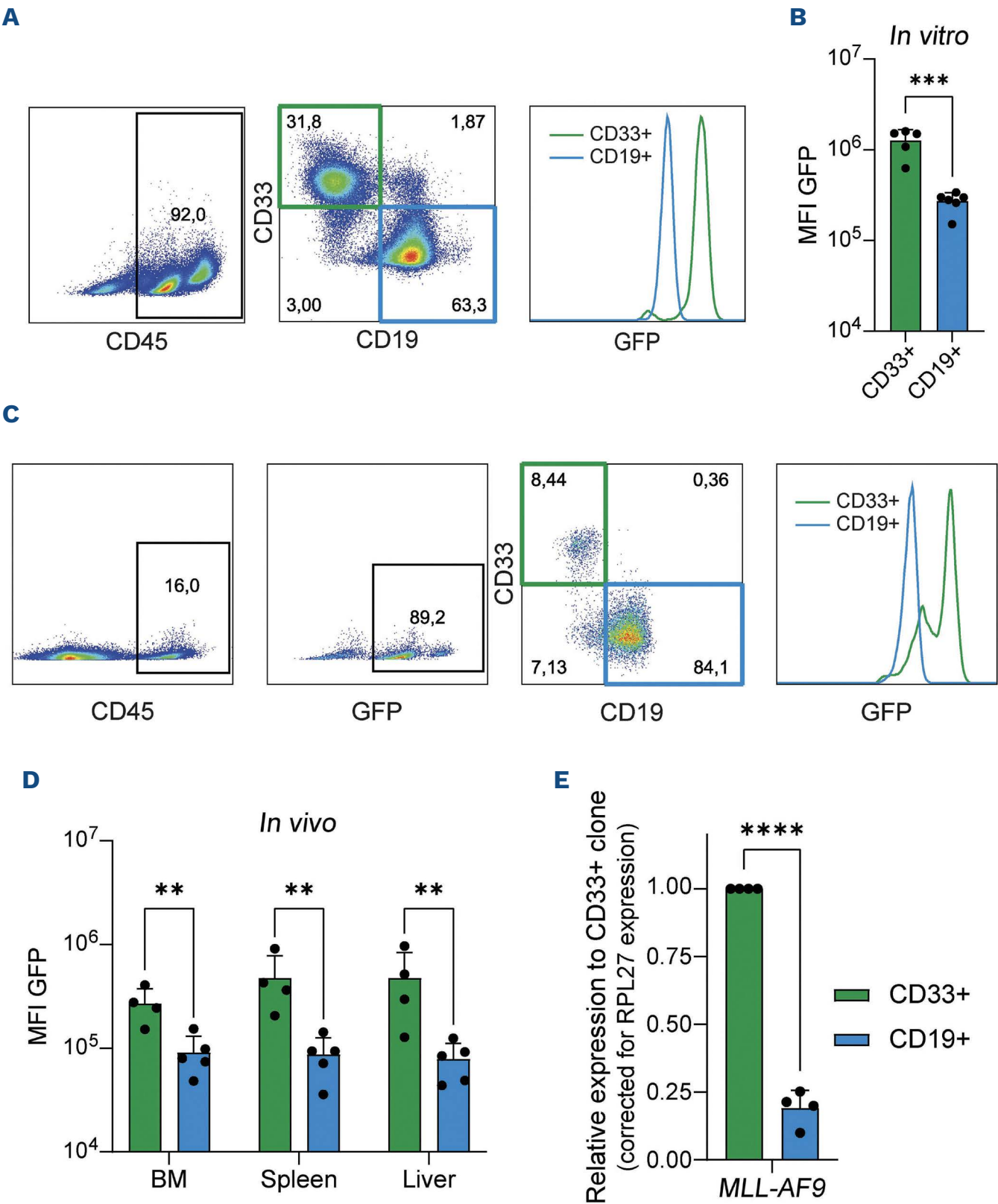


Figure 2. Lentiviral cord blood CD34⁺ transduction model showed higher *MLL-AF9* expression levels in acute myeloid leukemia clones compared to B-cell acute lymphoblastic leukemia clones. (A) Representative gating strategy of 5 independent *in vitro* cord blood (CB) CD34⁺ MLL-AF9 model experiments cultured in lymphoid permissive conditions for 10 weeks (see legends of the *Online Supplementary Figures S1* and *S2* for vector and culture condition information), CD33⁺ (acute myeloid leukemia [AML]) and CD19⁺ (B-cell acute lymphoblastic leukemia [B-ALL]) clones are identified and the EGFP signal histogram is depicted. (B) Mean fluorescent intensity (MFI) of EGFP signal in AML and B-ALL. (C) Representative gating strategy of 5 MISTRG mice showing human engraftment (CD45⁺) of CB CD34⁺ cells transduced with *MLL-AF9* and transforming into AML and B-ALL, both with distinct EGFP signal histograms. (D) MFI of EGFP signal in bone marrow (BM), spleen and liver for AML and B-ALL clones. (E) *MLL-AF9* mRNA expression measured by quantitative polymerase chain reaction relative to RPL27 from *in vitro* and *in vivo* experiments sorted as depicted in the *Online Supplementary Figure S2A, B*. *t* test (B and E), two-way analysis of variance (ANOVA) (D); ***P*<0.01, ****P*<0.001; *****P*<0.0001.

internal ribosomal entry site (IRES) followed by the *EGFP* reporter gene. In these assays, we usually observe the outgrowth of distinct clones marked with different levels of *EGFP* corresponding to different MLL-AF9 transcript levels as determined by quantitative PCR (qPCR) (see below). Under *in vitro* lymphoid-permissive MS5 co-cultures that allow outgrowth of B- lymphoid and myeloid cells we ob-

A

GENCODE V49lift37
chr11: 118,200,000 | 118,250,000 | 118,300,000 | 118,350,000 | 118,400,000 |

CD3E ENSG00000285827 CD3G ATP5MG UBE4A KMT2A TMEM25 IFT46 ARCNI1 TTC36

~ 130 K

B

Chromatin Accessibility

Distance to TSS KMT2A

HSC
CMP
GMP
MEP
MPP
CLP
B
CD4
CD8
ery
NK

C

Murine Mll1/Kmt2a (H3K27ac)

CpG: 75 CpG: 77 CpG: 119

LT-HSC
ST-HSC
MPP
MEP
CMP
GMP
Mono
Macrophage
CLP
B
NK

D

umap_2

umap_1

HSC/early
Lymphoid
Myeloid
Erythroid
Dendritic
Stromal

Expression Level (logReadCount)

KMT2A

P = 4.02e-7

Myeloid cells
Lymphoid cells

Figure 3. *MLL1* epigenetic regulation and expression show lineage dependent pattern in healthy hematopoietic cells and their progenitors. (A) UCSC visualization of chromosome 11, GRCh37, showing CD3 and *MLL1* loci located within a region of 130 Kb. (B) Assay for transposase-accessible chromatin using sequencing data revealing increased chromatin accessibility in human lymphoid cell lineages at the *MLL1* locus (GSE96772). (C) Myeloid cells and progenitors (common myeloid progenitor [CMP], granulomonocytic progenitors [GMP], monocytes and macrophages) show lower H3K27ac deposition at the *MLL1* locus compared to lymphoid (common lymphoid progenitor (CLP), B and natural killer [NK] cell) and early stem cells (hematopoietic stem cells [HSC] and multipotent progenitor [MPP]) derived from chromatin immunoprecipitation sequencing (ChIP-seq) analysis of murine (C57BL/6) hematopoiesis (GSE59636). (D) Single-cell RNA sequencing of human healthy bone marrow samples (own unpublished data), for mapping see *Online Supplementary Figure S3*). *KMT2A* expression is enriched in the lymphoid compared to myeloid lineages tested by Kruskal-Wallis with *post hoc* Benjamini-Hochberg correction.

served that *MLL-AF9*-expressing cells could give rise to both AML and B-ALL clones marked by CD33 and CD19, respectively (Figure 2A). Measuring the mean fluorescent intensity (MFI) of the EGFP signal of the separate populations revealed that B-ALL clones consistently had lower MFI levels compared to the AML clones (Figure 2A, B). *In vivo* data upon transplantation in MISTRG mice showed similar results in bone marrow, spleen and liver engrafted cells (Figure 2C, D). Sorting of the separate clones from *in vitro* and *in vivo* experiments and determination of the *MLL-AF9* oncogene mRNA levels confirmed that EGFP MFI was a good indicator of the oncogene transcript levels (*Online Supplementary Figure S2A, B*; Figure 2E). When the same analysis was repeated for the empty vector (EV)-mCherry MFI, which was co-transduced as an internal control, no difference was observed between the B-ALL and AML clones (*Online Supplementary Figure S2C-E*). Collectively, our results indicate that higher levels of *MLL-AF9* fusion oncoprotein are not instructive for B-ALL development. If anything, we consistently observe that *MLL-AF9* expression levels were lower in lymphoid clones when driven by a similar artificial promoter.

We wondered where differences in *MLLr* expression in lymphoid *versus* myeloid leukemias might arise from. Obviously, expression of the *MLL* fusion proteins is driven by the endogenous enhancers and promoters at chromosome 11 and we noted that the *MLL1* locus lies in close vicinity of the lymphoid-specific *CD3E*, *CD3D* and *CD3G* genes (Figure 3A). We therefore wondered whether this locus would be more accessible in lymphoid cells compared to myeloid cells, thereby explaining higher expression levels. Indeed, we found increased chromatin accessibility of the *MLL1* locus in lymphoid cells, along with higher accessibility around the CD3 loci (Figure 3B; *Online Supplementary Figure S3A*). In addition, lymphoid cells had higher H3K27 acetylation (H3K27ac) depositions around the promoter regions of the *MLL1* and *CD3D* genes as detected by chromatin immunoprecipitation sequencing (ChIP-seq) of murine hematopoietic cells and progenitors (Figure 3C; *Online Supplementary Figure S3B*). These epigenetic landscapes aligned with higher expression levels of *MLL1* in lymphoid compartments, both at the single cell level as well as in bulk RNAseq data gene expression (Figure 3D; *Online Supplementary Figure 3C, D*). Taken together, based on our data and those of others, we conclude that *MLL* fusion oncogenes can give rise to myeloid and lymphoid clones and that lymphoid clones can undergo a lineage switch towards a myeloid cell fate dependent on extrinsic cues. When expression is driven by

an artificial promoter we consistently observe that myeloid clones express higher levels of the oncogene compared to lymphoid clones, both *in vitro* as well as *in vivo* in humanized MISTRG mouse models. When expressed under their physiological promoter and enhancer elements at the *MLL1* locus at chromosome 11 we observed increased chromatin accessibility and H3K27ac most likely explaining the higher expression levels, but this does not necessarily indicate that high *MLL* fusion protein expression is a requirement for transformation along the lymphoid lineage.

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Disclosures

No conflicts of interest to disclose.

Contributions

EK and JJS conceived and designed the study. EK, MC, AB-V, BdB, DPM, GH and JJS performed experiments, analyzed, and interpreted data, performed statistical analysis, and drafted the article. EK and JJS wrote the manuscript which was reviewed by all authors. All authors gave their final approval of the submitted manuscript.

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Data-sharing statement

All data is available in the manuscript and is also available upon request.

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