MYELOID NEOPLASIA

Increased DNA methylation of Dnmt3b targets impairs leukemogenesis

Isabell Schulze,[1] Christian Rohde,[1] Marina Scheller-Wendorff,[1] Nicole Bäumer,[2] Annika Krause,[2] Friederike Herbst,[3] Pia Riemke,[4] Katja Hebestreit,[5] Petra Tschanter,[1] Qiong Lin,[6] Heinz Linhart,[3] Lucy A. Godley,[7] Hanno Glimm,[3] Martin Dugas,[5] Wolfgang Wagner,[6] Wolfgang E. Berdel,[2] Frank Rosenbauer,[4] and Carsten Müller-Tidow[1]

[1]Department of Hematology and Oncology, University of Halle, Halle, Germany; [2]Department of Medicine A, Hematology and Oncology, University of Münster, Münster, Germany; [3]National Center for Tumor Diseases, German Cancer Research Center Heidelberg, Heidelberg, Germany; [4]Institute of Molecular Tumor Biology and [5]Institute of Medical Informatics, University of Münster, Münster, Germany; [6]Helmholtz-Institute for Biomedical Engineering, Stem Cell Biology and Cellular Engineering, Rheinisch-Westfälische Technische Hochschule Aachen, Aachen, Germany; and [7]Department of Medicine and Comprehensive Cancer Center, The University of Chicago, Chicago, IL

Key Points

- Increased gene body methylation inhibits leukemia, and oncogenes require varying levels of DNA methylation for efficient leukemogenesis.
- Dnmt3b-induced DNA methylation in mice targets stem cell–associated genes with prognostic association in acute myeloid leukemia patients.

The de novo DNA methyltransferases Dnmt3a and Dnmt3b are of crucial importance in hematopoietic stem cells. Dnmt3b has recently been shown to play a role in genic methylation. To investigate how Dnmt3b-mediated DNA methylation affects leukemogenesis, we analyzed leukemia development under conditions of high and physiological methylation levels in a tetracycline-inducible knock-in mouse model. High expression of *Dnmt3b* slowed leukemia development in serial transplantations and impaired leukemia stem cell (LSC) function. Forced *Dnmt3b* expression induced widespread DNA hypermethylation in *Myc-Bcl2*–induced leukemias, preferentially at gene bodies. *MLL-AF9*–induced leukemogenesis showed much less pronounced DNA hypermethylation upon *Dnmt3b* expression. Nonetheless, leukemogenesis was delayed in both models with a shared core set of DNA hypermethylated regions and suppression of stem cell–related genes. Acute myeloid leukemia patients with high expression of Dnmt3b target genes showed inferior survival. Together, these findings indicate a critical role for Dnmt3b-mediated DNA methylation in leukemia development and maintenance of LSC function. (*Blood*. 2016;127(12):1575-1586)

Introduction

DNA methylation of cytosine guanine dinucleotides (CpGs) is a key epigenetic modification that affects tissue- and time-dependent gene transcription and genome integrity.[1] In mammals, CpG methylation is catalyzed by 3 DNA methyltransferases (DNMTs).[2,3] The maintenance DNA methyltransferase Dnmt1 replicates preexisting methylation patterns,[4] whereas Dnmt3a and Dnmt3b primarily act as de novo methyltransferases.[5] Both enzyme classes cooperate to establish and maintain cellular methylation patterns.[6,7] *Dnmt* deficiency induces embryonic lethality in case of *Dnmt1* and *Dnmt3b* and death at roughly 3 weeks of age in *Dnmt3a*-knockout mice.[5,8] Nonetheless, self-renewal potential is maintained in embryonic stem cells without Dnmt3a and Dnmt3b.[6] DNA methylation influences hematopoietic differentiation and lineage decisions.[9,10] In normal hematopoiesis, Dnmt1 is required for hematopoietic stem cell (HSC) self-renewal, differentiation, and niche retention.[11] In HSC, loss of both *Dnmt3a* and *Dnmt3b* diminishes self-renewal potential, whereas the differentiation capacity into all lineages is maintained.[12] Conditional deletion of *Dnmt3a* in the hematopoietic system severely impairs HSC differentiation, while simultaneously expanding HSC numbers in the bone marrow.[13] Combined loss of *Dnmt3a* and *Dnmt3b* in HSC creates a synergistic effect resulting in a more severe differentiation block than in *Dnmt3a*-deficient cells alone. Nonetheless, residual Dnmt3b activity enabled differentiation of adult HSCs, especially upon loss of *Dnmt3a*.[14]

Aberrant DNA methylation constitutes a hallmark of cancer,[15-17] and deregulated DNA methylation has been shown in a variety of hematologic malignancies.[18] Recent findings in acute myeloid leukemia (AML) patient samples indicate that DNA methylation exhibits specific patterns in AML subtypes. These methylation profiles may reflect biological differences with therapeutic implications.[19,20] Aberrant methylation patterns in cancer are characterized by global genome-wide hypomethylation,[21] and simultaneously occurring regional hypermethylation of CpG islands.[22-24] Both, hypo- and hypermethylation may facilitate tumorigenesis.[10,25-28] Accordingly, the role of de novo methyltransferases in cancer has remained unclear. Forced expression of *Dnmt3b* in tumor-prone APC_{Min} mice promoted development of gastrointestinal tumors by de novo methylation and transcriptional silencing of tumor suppressor genes.[29] Conditional *Dnmt3b* knockout inhibited early intestinal tumor formation.[30] Also,

Submitted July 19, 2015; accepted December 15, 2015. Prepublished online as *Blood* First Edition paper, January 4, 2016; DOI 10.1182/blood-2015-07-655928.

The data reported in this article have been deposited in the Gene Expression Omnibus database (accession numbers GSE71039 and GSE71040).

The online version of this article contains a data supplement.

There is an Inside *Blood* Commentary on this article in this issue.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

2 independent studies identified Dnmt3b as a tumor suppressor in *Eμ-Myc*–driven lymphomas.[31,32] Clinical data reflected these divergent roles of DNMTs in cancer. *DNMT3A* mutations have been identified in >20% of patients with AML[33,34] and ~10% of those with myelodysplastic syndromes.[35] The identified mutations are invariably heterozygous and predicted to disrupt the catalytic enzyme activity.[36] Of note, high expression levels of *DNMT3B* in leukemic bulk cells are associated with poor outcome in AML.[37,38] These data suggest an important but tightly controlled role for DNA methylation in cancer initiation and progression. However, the precise function of increased DNA methylation is not fully understood.

Here, we show that forced expression of *Dnmt3b* severely impaired leukemia development. *Dnmt3b* expression induced DNA hypermethylation with subsequent downregulation of genes highly expressed in stem cells.

Materials and methods

Plasmids, retroviral supernatant production, and cell transduction

Murine stem cell virus (MSCV) retroviral construct MLL-AF9-IRES-GFP has been described.[39] MSCV retroviral construct Myc-IRES-Bcl2-IRES-mCherry was generated by inserting an additional IRES and mCherry into the MSCV construct Myc-IRES-Bcl2.[40] Retroviral supernatants were obtained by transfection of Plat-E cells. For transduction, bone marrow cells were MACS-lineage depleted (Miltenyi) and stimulated for 72 hours in Iscove modified Dulbecco medium (Invitrogen) containing 20% fetal calf serum (PAA), mouse interleukin (IL) 3 (10 ng/mL; Peprotech), human IL-6 (5 ng/mL; Peprotech), and mouse stem cell factor (50 ng/mL; Peprotech). Lineage-negative cells were infected using Retronectin (Takara) and sorted for GFP or mCherry expression after 3 days of spin infection.

Mice, genotyping, and transgene induction

Inducible Dnmt3b-knock-in mice were described previously.[29] Inducible Dnmt3b-knock-in mice were purchased from the Jackson Laboratories, and C57Bl/6N mice from Janvier. All mice were kept in pathogen-free animal facilities at the University Hospital Münster. For genotyping, genomic tail-tip DNA was polymerase chain reaction (PCR) amplified using standard PCR. For transgene induction, mice were fed 0.5 mg/mL doxycycline in the drinking water. Drinking water was changed twice a week.

Transplantation experiments

All animal experiments were approved by the local authorities according to the German Federal Animal Protection Act. For bone marrow transplantation, 8 weeks-old C57Bl/6N recipient mice were sublethally irradiated with 7 Gy. Retrovirally transduced bone marrow cells were IV injected into the tail vein with 2×10^5 unfractionated nontransduced bone marrow cells. Expression of Dnmt3b was induced at time of transplantation, and recipient mice were kept on doxycycline until the end of the experiment. For serial transplantations, spleen cells from leukemic mice were sorted for GFP and c-Kit expression (*MLL-AF9*) or mCherry expression (*Myc-Bcl2*) and retransplanted into sublethally irradiated recipients. For competitive bone marrow transplantation, recipient mice were sublethally irradiated with 2 doses of 4.75 Gy. CD45.2$^+$ bone marrow cells (10^5 or 10^6) were injected IV into the tail vein with 10^5 CD45.1$^+$ competitor cells.

Microarray analysis

MLL-AF9 leukemic spleen cells were sorted for c-Kit expression, and gene expression was analyzed using the mouse Gene 2.0 ST Array (Affymetrix) according to the manufacturer's instructions. Arrays were scanned at 1.56-μm resolution using the Affymetrix GeneChip Scanner 3000. Raw gene expression data were imported to the Affymetrix expression console and normalized using robust multiarray average (RMA). Differential gene expression was calculated using the R/Bioconductor package RankProd.[41] Microarray data were deposited in the Gene Expression Omnibus database (accession number GSE71040).

Reduced representation bisulfite sequencing (RRBS)

A total of 0.3 to 1 μg of DNA was used for RRBS library preparation using published protocols with minor modifications.[42] Briefly, genomic DNA was digested with *Msp*I (NEB), end repaired, A-tailed, and ligated to Illumina TruSeq adapters (Illumina). Fragments in the range of 50- to 220-bp size were gel purified (NuSieve 3:1 agarose; Lonza). Libraries were bisulfite converted and sequenced on a HiScanSQ instrument (Illumina). See supplemental Methods for further details (available on the *Blood* Web site). RRBS data were deposited in the Gene Expression Omnibus database (accession number GSE71039).

Statistical analysis

All data are shown as mean ± standard deviation (SD) if not indicated otherwise. Statistical analyses were done in SPSS 22 (IBM). Student *t* tests were used to determine statistical significance. Survival was analyzed using Kaplan-Meier curves and log-rank test. A P value ≤.05 was considered significant.

Results

High levels of *Dnmt3b* expression prolong leukemia latency

Several studies described higher expression of *Dnmt3b* in AML samples when compared with healthy controls,[43,44] whereas others did not show a significant difference.[45,46] Compared with HSCs, *DNMT3B* expression was decreased in primary AML blasts (supplemental Figure 1A). To functionally investigate the role of Dnmt3b in leukemogenesis, we generated murine leukemias in a tetracycline-inducible *Dnmt3b*-knock-in mouse model by retroviral expression of an Myc-Bcl2-mCherry construct in lineage-negative bone marrow cells. The *Myc-Bcl2* combination induces a biphenotypic leukemia with myeloid and B-lymphoid blasts in mice.[40] Cells from *Dnmt3b*-wild-type and heterozygous *Dnmt3b*-knock-in cells (supplemental Figure 1B) were transduced with similar efficiency (supplemental Figure 1C). Cells were transplanted into sublethally irradiated C57Bl/6N recipient mice (Figure 1A). *Dnmt3b* expression was detected in *Myc-Bcl2 Dnmt3b*-knock-in cells (Figure 1B). All recipients of *Dnmt3b*-wild-type Myc-Bcl2-mCherry cells rapidly died of lethal leukemia characterized by splenomegaly (Figure 1C) and increased numbers of leukemic cells in bone marrow (Figure 1D) and spleen (data not shown). Recipients of *Dnmt3b*-knock-in *Myc-Bcl2* cells showed less pronounced splenomegaly (Figure 1C) and only slightly increased white blood cell counts in peripheral blood (Figure 1D). Upon cotransplantation of leukemic mCherry$^+$ and nonleukemic mCherry$^-$ cells, *Dnmt3b*-knock-in were inferior in replacing nonleukemic bone marrow cells indicating an intrinsic defect in leukemia-initiating cells (LICs) of *Dnmt3b*-knock-in mice (Figure 1E). Recipients of *Dnmt3b*-knock-in *Myc-Bcl2* cells developed leukemia with substantially prolonged latency with mean latency times of 43.4 ± 3.2 days posttransplantation (wild-type cells) and 107.3 ± 21.0 days (*Dnmt3b*-knock-in cells) ($P < .001$; Figure 1F). Transformation of *Myc-Bcl2*–transduced cells was confirmed by secondary transplantation of mCherry-sorted spleen cells (Figure 1G). *Dnmt3b*-knock-in leukemias were characterized by high expression of myeloid surface markers, whereas the bilinear phenotype of *Myc-Bcl2* leukemias[40] was only observed in recipients of wild-type cells (supplemental Figure 1D). These data indicate that deviation from physiological methylation precluded development of lymphoid neoplasia.

The increased latency of *Myc-Bcl2 Dnmt3b*-knock-in leukemias and reduced numbers of leukemic cells in competitive transplantation

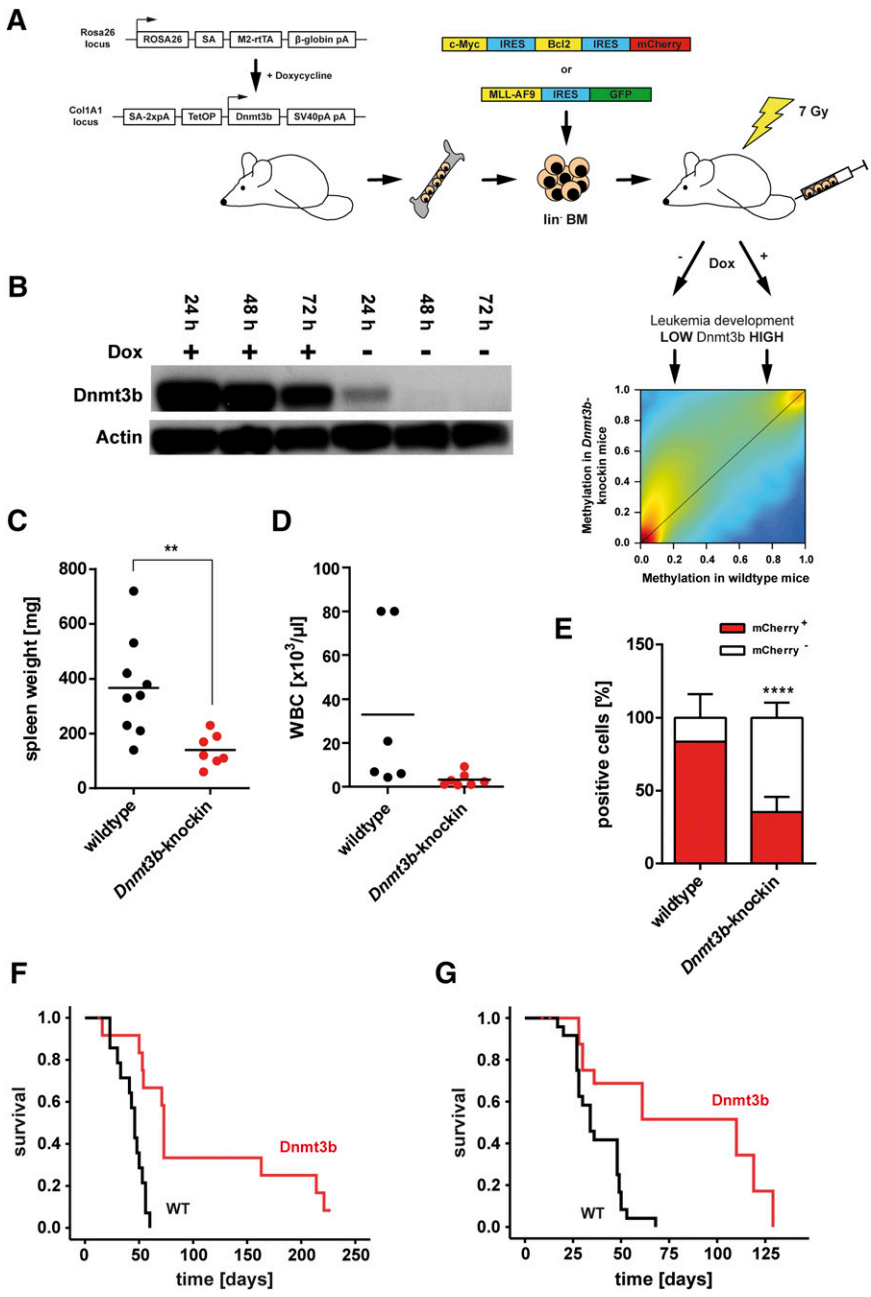

Figure 1. *Dnmt3b* expression prolongs leukemia latency. (A) Bone marrow from tetracycline-inducible *Dnmt3b* overexpressing mice was retrovirally transduced with MSCV-Myc-IRES-Bcl2-IRES-mCherry or MSCV-MLL-AF9-IRES-GFP oncogene vectors. Transduced cells were sorted for mCherry or GFP, respectively, and transplanted into sublethally irradiated recipient mice. Leukemia development was analyzed under conditions of high vs low DNA methylation. (B) Doxycycline-regulated *Dnmt3b* expression in sorted *Myc-Bcl2* leukemic spleen cells from primary recipient mice of *Dnmt3b*-knock-in cells. Spleen cells were isolated from diseased mice and cultured in the presence of 1 μg/mL doxycycline, and protein expression was analyzed after different time points of addition and subsequent withdrawal of doxycycline by western blot. Spleen weight (C) and white blood cell (WBC) count (D) in primary recipients of *Myc-Bcl2* leukemic *Dnmt3b*-knock-in cells at end of experiment. Each dot represents 1 leukemic mouse. $^{**}P < .01$. (E) Percentage of leukemic (mCherry$^+$) and nonleukemic (mCherry$^-$) cells in bone marrow of primary recipient mice at end of experiment. Mean ± SD are shown (n = 5-9 mice). $^{****}P < .0001$. (F) Survival of primary recipients of *Myc-Bcl2* leukemic wild-type and *Dnmt3b*-knock-in cells (n = 15 mice for each group). *Dnmt3b* expression was induced at time of transplantation. $P < .001$. (G) Survival of secondary recipients of *Myc-Bcl2* leukemias (n = 15 mice for each group). $P < .01$.

assays suggested that constitutive methylation is required for leukemia stem cell (LSC) function. In murine *MLL-AF9* leukemia, c-Kit$^+$ cells are highly enriched for LICs and LSCs.[39,47] We analyzed effects of enhanced DNA methylation on LSC function in *MLL-AF9* leukemia (supplemental Figure 2A). Transplantation of *MLL-AF9*–expressing *Dnmt3b*-knock-in cells led to leukemia development with prolonged latency (mean survival: *MLL-AF9* wild-type, 85.08 ± 22.44 days; *MLL-AF9 Dnmt3b*-knock-in, 153.55 ± 30.88 days) (supplemental Figure 2B). Spleen weights did not differ between recipients of wild-type and *Dnmt3b*-knock-in cells (supplemental Figure 2C). We sorted primary leukemias for high expression of c-Kit (gated for top 20%) (supplemental Figure 2D) and performed serial transplantations. Superior colony formation potential of c-Kit^{high} cells (compared with Kit^{low} cells) was confirmed by colony-forming unit assays (supplemental Figure 2E). Upon transplantation of sorted c-Kit$^+$ cells, leukemia latency was significantly prolonged in secondary (Figure 2A) and tertiary recipients (Figure 2B). Similar to *Dnmt3b*-expressing *Myc-Bcl2* leukemias, diseased recipients of *Dnmt3b*-knock-in *MLL-AF9* LSCs showed reduced numbers of white blood cells in peripheral blood at time of analysis (Figure 2C). *MLL-AF9*–induced leukemias were exclusively of myeloid lineage (Figure 2D). Of note, when secondary recipients died of leukemia, recipients of *MLL-AF9 Dnmt3b*-knock-in cells showed increased numbers of c-Kit$^+$ cells (Figure 2D). The c-Kit$^+$ *Dnmt3b*-knock-in LSCs did not show altered cell surface expression of other HSC markers (Figure 2E), which indicates that high *Dnmt3b* expression did not affect the cellular composition of c-Kit$^+$ *MLL-AF9* LSCs. The colony formation potential of c-Kit$^+$ *Dnmt3b*-knock-in LSCs was reduced when compared with recipients of *MLL-AF9* wild-type cells (Figure 2F). Colony numbers of total spleen cells were similar (Figure 2G), pointing toward impaired leukemic potential of c-Kit$^+$ leukemia cells with high *Dnmt3b* expression.

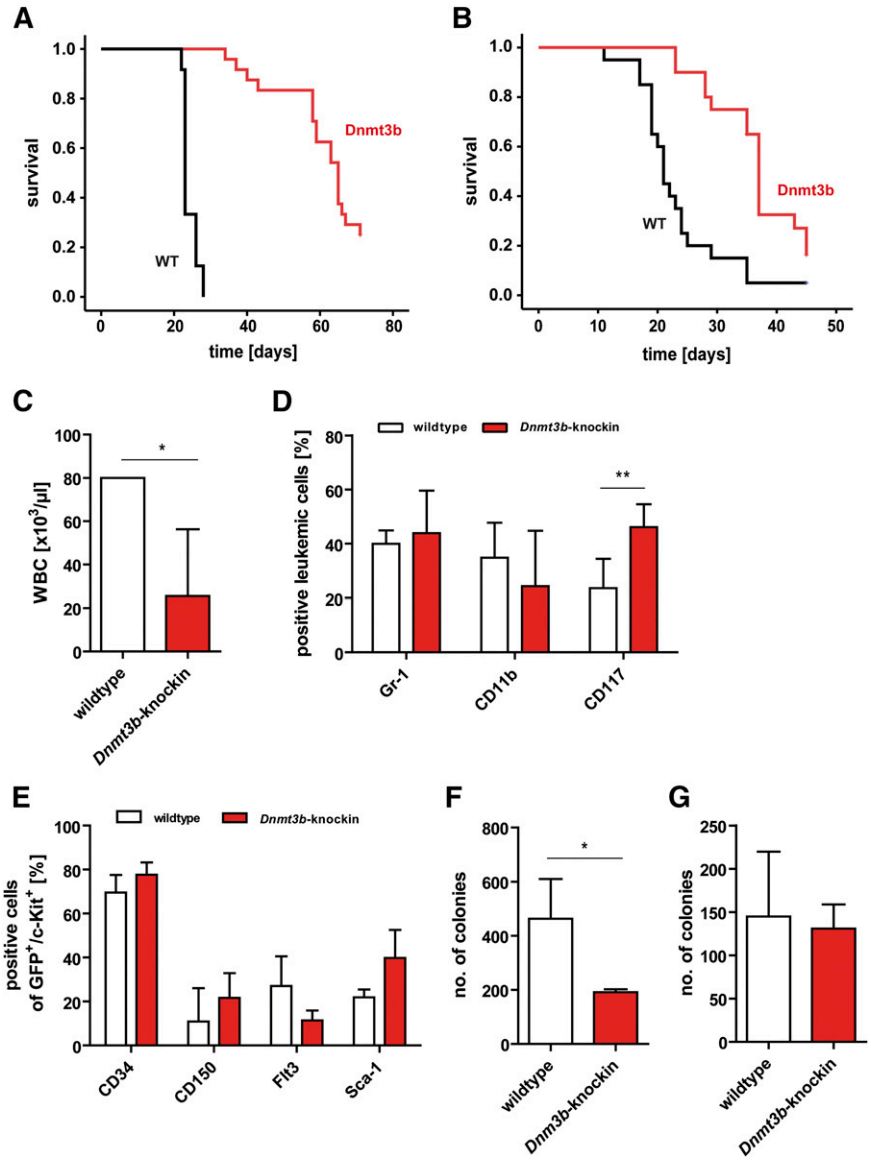

Figure 2. Effects of *Dnmt3b*-knock-in in *MLL-AF9* leukemia model. (A-B) Survival of secondary ($P < .001$) (A) and tertiary ($P < .001$) (B) recipients of *MLL-AF9*–transduced wild-type and *Dnmt3b*-knock-in c-Kit$^+$ LSCs. *Dnmt3b* expression was induced in all *Dnmt3b*-knock-in mice (n = 18-24 per group). (C) White blood cell counts of secondary recipients of wild-type and *Dnmt3b*-knock-in *MLL-AF9*–transduced cells at end of experiment; n = 3 for wild-type, n = 6 for *Dnmt3b*-knock-in. (D) Percentage of Gr-1$^+$, CD11b$^+$, and c-Kit$^+$ cells in bone marrow of secondary recipients of wild-type (n = 11) and *Dnmt3b*-knock-in (n = 4) *MLL-AF9* LSCs as determined by fluorescence-activated cell sorter analysis. (E) Expression of HSC markers CD34, CD150, Flt3, and Sca-1 on GFP$^+$/c-Kit$^+$ *MLL-AF9* LSCs from bone marrow of recipients of wild-type and *Dnmt3b*-knock-in leukemias (n = 3 for each genotype). (F-G) *Dnmt3b* expression suppresses colony growth of c-Kit$^+$ *MLL-AF9* LSCs (top 20% of c-Kit–expressing cells were sorted for wild-type and knock-in mice). C-Kit$^+$ cells (F) and total spleen cells (G) from secondary recipients of wild-type and *Dnmt3b*-knock-in *MLL-AF9* LSCs were plated in triplicates in Methocult (n = 3-11 mice). Values in panels C-G are mean ± SD. *$P < .05$; **$P < .01$

Limited effects of Dnmt3b in hematopoiesis

We analyzed whether forced *Dnmt3b* expression affected hematopoietic differentiation under nonleukemic conditions. *Dnmt3b* expression was induced in vivo for 4 to 44 weeks before analysis. *Dnmt3b*-knock-in mice showed a 1.5-fold increase in Lin$^-$Sca-1$^+$ c-Kit$^+$ (LSK) cell numbers after 4 weeks of induction ($P < .05$), mainly because of an increase in the multipotent progenitor cell compartment (LMPP) ($P < .01$; Figure 3A). The dominance of the LMPP pool in *Dnmt3b*-knock-in mice was not matched by a similar contribution of these LMPPs to the downstream progenitor populations of granulocyte-macrophage progenitors (supplemental Figure 3A) and megakaryocyte-erythroid progenitors (supplemental Figure 3B), neither were cell numbers of neutrophils altered in bone marrow of *Dnmt3b*-knock-in mice (supplemental Figure 3C). Instead, *Dnmt3b*-knock-in mice showed ~1.7-fold reduced white blood cell counts in peripheral blood (Figure 3B) and ~2.3-fold reduced lymphocyte counts in peripheral blood (Figure 3C). Fluorescence-activated cell sorter analysis of erythroid differentiation revealed slightly increased numbers of erythroblastic cells originating from *Dnmt3b*-knock-in HSCs ($P < .05$) (supplemental Figure 3D), whereas other blood parameters did not differ (supplemental Figure 3E-G). Expansion of *Dnmt3b*-knock-in LMPPs in vivo could not be attributed to altered apoptosis rates (supplemental Figure 4A). *Dnmt3b*-knock-in mice and wild-type mice showed similar survival times (Figure 3D). Both wild-type and *Dnmt3b*-knock-in animals showed comparable spleen weights (supplemental Figure 4B), and total bone marrow cells gave rise to equal colony numbers (supplemental Figure 4C). Competitive transplantation of 10^5 or 10^6 CD45.2$^+$ donor-derived bone marrow cells with 10^5 CD45.1$^+$ competitor cells did not yield differences between wild-type and *Dnmt3b*-knock-in donor-derived cells (Figure 3E), nor was multilineage engraftment altered by high *Dnmt3b* expression (supplemental Figure 4D). But *Dnmt3b*-knock-in bone marrow cells showed reduced growth in in vitro culture (Figure 3F). High *Dnmt3b* expression has been shown in mouse wild-type HSCs and different multipotent progenitor cell stages by RNA sequencing.[48] In our model, *Dnmt3b* was also highly expressed in LSK, myeloid, lymphoid, and erythroid cells upon induction. Although Dnmt3a proteins were slightly elevated in 2 out of 4 mice after *Dnmt3b*

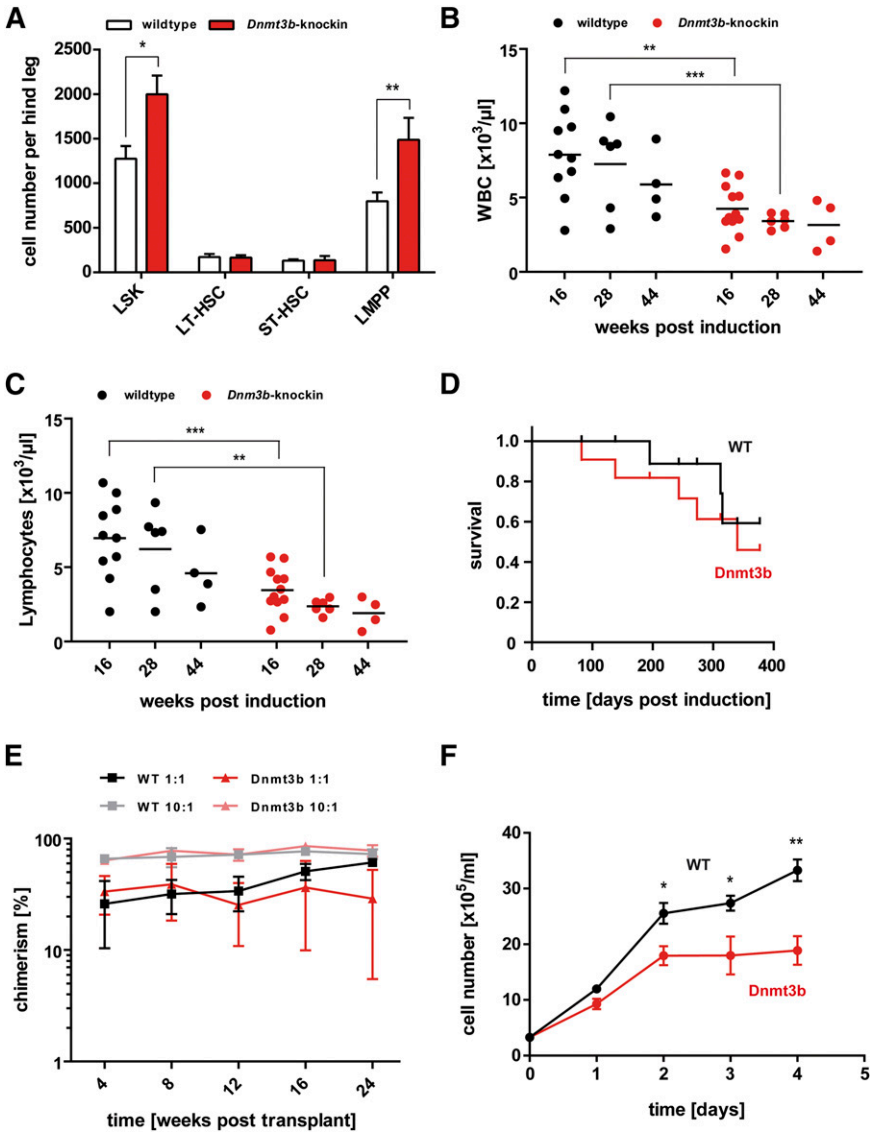

Figure 3. *Dnmt3b* expression increases the number of multipotent progenitor cells in nonleukemic mice. (A) Induction of *Dnmt3b* expression in nonleukemic *Dnmt3b*-knock-in mice increased the numbers of multipotent progenitor cells after 4 weeks (n = 3 per group). LT-HSC gated for LSK $CD34^{-}Flt3^{-}$; ST-HSC gated for LSK $CD34^{+}Flt3^{-}$; LMPP gated for LSK $CD34^{+}Flt3^{+}$. (B) White blood cell counts of wild-type (black circles) and *Dnmt3b*-knock-in (red circles) mice after 16, 28, and 44 weeks of in vivo induction of *Dnmt3b* expression. (C) Total cell counts for lymphocytes in peripheral blood of wild-type and *Dnmt3b*-knock-in mice at the indicated time points after induction of *Dnmt3b*. Each dot represents 1 individual mouse. (D) Survival of wild-type and *Dnmt3b*-knock-in mice after induction of *Dnmt3b* expression for up to 1 year (n = 6 for wild-type [WT] and n = 8 for *Dnmt3b*-knock-in mice). (E) Percentage of $CD45.2^{+}$ donor-derived peripheral blood cells in primary recipients for up to 24 weeks posttransplantation. Transplanted cell doses are 10^6 and 10^5 cells mixed with 10^5 competitor cells (n = 4 mice each). (F) *Dnmt3b*-knock-in total bone marrow cells were impaired in growth upon culture in the presence of IL-3, IL-6, and murine stem cell factor; n = 3 per group. Data in panels A-C and E-F are mean ± SD values. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$.

induction, neither Dnmt3a nor Dnmt1 showed consistent deregulation upon overexpression of *Dnmt3b* (supplemental Figure 4F).

Dnmt3b expression induces widespread DNA methylation changes

We used RRBS to map genome-wide DNA methylation in *Dnmt3b*-knock-in leukemias. For each genotype, RRBS data from 2 mice with *Myc-Bcl2* leukemias and from *MLL-AF9* LSCs (GFP^{+}/c-Kit^{+}) from 3 mice were analyzed. Raw methylation data from all covered CpG sites were used to generate an unsupervised hierarchical clustering (supplemental Figure 5A). Overall, *Dnmt3b*-knock-in *Myc-Bcl2* leukemias exhibited a larger fraction of intermediately methylated (20% to 80%) and highly methylated CpGs (80% to 100%) than wild-type *Myc-Bcl2* leukemias (supplemental Figure 5B). A similar pattern was observed in *MLL-AF9* leukemias, yet it was far less pronounced (supplemental Figure 5B-C). Smoothed methylation values confirmed genome-wide hypermethylation in both *Myc-Bcl2* and *MLL-AF9* *Dnmt3b*-knock-in leukemias (Figure 4A-B). Of note, Dnmt3b-induced hypermethylation in *MLL-AF9* leukemias was less pronounced than in *Myc-Bcl2* leukemias (Figure 4B). Differential methylation analysis in *Dnmt3b*-knock-in samples yielded 2092 differentially methylated regions (DMRs) in *Myc-Bcl2* leukemias (supplemental Figure 5D) and 105 DMRs in *MLL-AF9* LSCs (supplemental Figure 5E). Gene promoters and exons were underrepresented in hypermethylated DMRs, but transcribed regions were overrepresented in hypermethylated DMRs (supplemental Figure 5F).[49]

Analysis of single CpGs revealed 89 533 and 16 572 differentially methylated CpGs (DMCs) in *Myc-Bcl2* and *MLL-AF9* murine leukemias, respectively (supplemental Table 1). The vast majority of DMCs were hypermethylated upon *Dnmt3b* expression (supplemental Table 1). Aberrant DNA methylation was observed across all chromosomal regions, independently of the oncogene (Figure 4C). However, CpG-rich and CpG-poor regions were affected differently. DMCs more frequently mapped to CpG islands in *Myc-Bcl2* leukemias (45% vs 26%). In contrast, 60% of DMCs in *MLL-AF9* leukemias were found neither in CpG islands nor CpG shores (supplemental Figure 6A), thereby reflecting the high abundance of DMCs in regions beyond CpG shores in *MLL-AF9* leukemias. Because Dnmt3b has recently been identified as the main de novo methyltransferase of gene bodies,[50] we analyzed gene body methylation in murine leukemias. Gene bodies

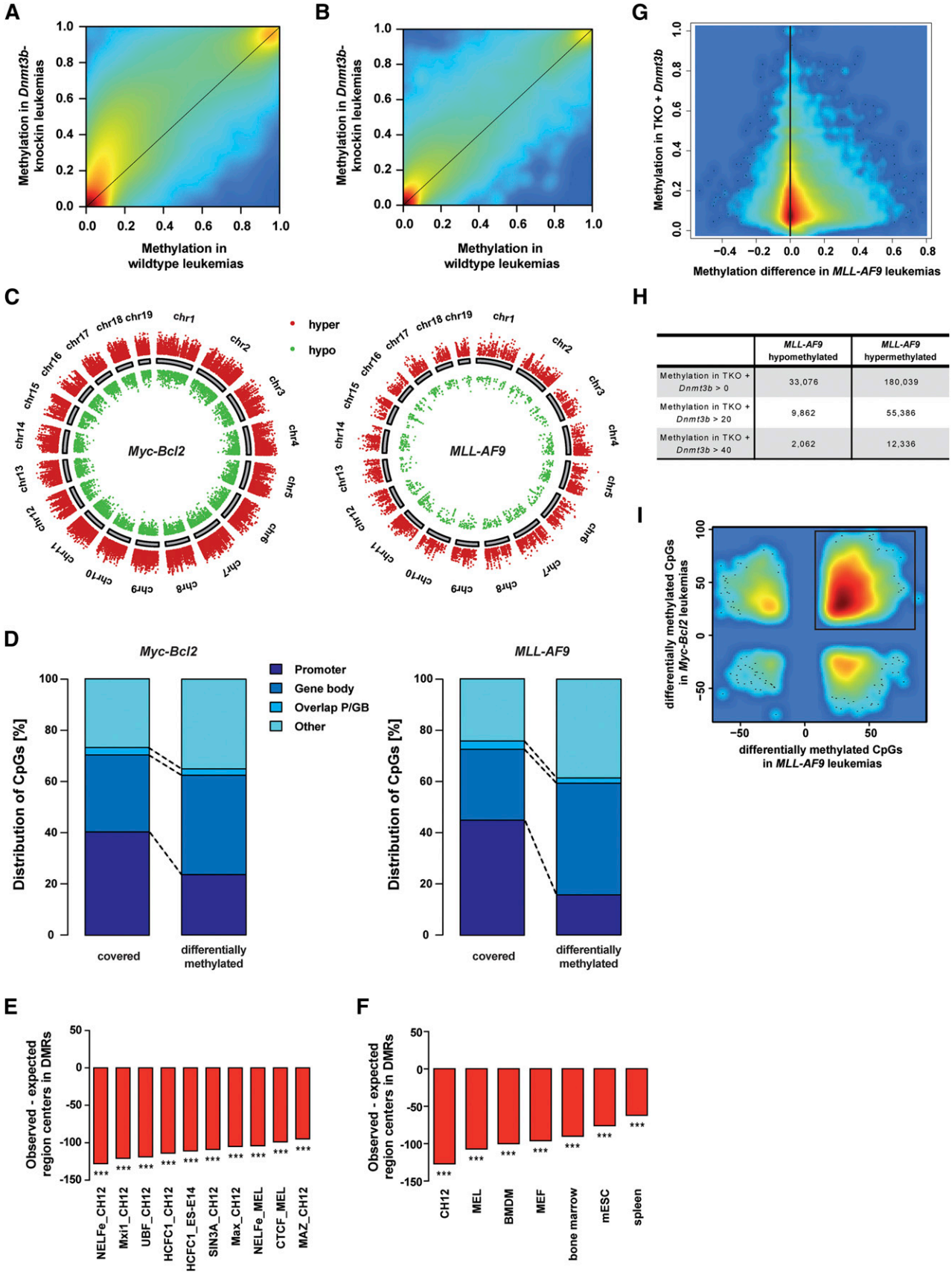

Figure 4. Dnmt3b induces DNA methylation changes detected by RRBS. (A-B) Smoothened scatter plots of methylation values for *Dnmt3b*-knock-in vs wild-type control samples in *Myc-Bcl2* (A) and *MLL-AF9* (B) leukemias, respectively. Colors represent the density of points ranging from red (high density) to blue (low density). (C) Chromosomal distribution of DMCs in *Myc-Bcl2* (left) and *MLL-AF9* (right) leukemias. Every dot represents 1 DMC. Red indicates DNA hypermethylated DMCs in

were overrepresented in hyper- and hypomethylated CpG sites in *Myc-Bcl2* and *MLL-AF9* leukemias (Figure 4D; supplemental Figure 6B-E). We also analyzed the overlap of hypermethylated regions in *Dnmt3b*-knock-in leukemias with published mouse ENCODE[51] chromatin immunoprecipitation–sequencing data. Known transcription factor binding sites were heavily underrepresented in DMRs (Figure 4E). This also held true for the transcription factors MAX and the MAX interacting protein MXI1, which have been linked to human cancers.[52] Most commonly affected by DMRs were binding sites of transcription factors that have previously been associated with leukemia (supplemental Table 4). The chromatin organizing factor CTCF was recently found to be mutated in AML patient samples[53] and in pre-LSCs.[54] Other transcription factors (eg, HCFC1 and P300) have been described to play a role in AML development and maintenance,[55] especially in *MLL*-rearranged leukemias.[56-58] In addition, sites of H3K4 trimethylation (supplemental Figure 6F) and polymerase II binding sites (Figure 4F) also showed reduced representation in DMRs. CpG sites that showed altered methylation levels in *Dnmt3b*-knock-in leukemias were compared with CpG sites that gained methylation in murine *Dnmt*-triple-knockout embryonic stem (ES) cells after reintroduction of *Dnmt3b*.[50] The number of CpG sites with methylation gain in murine ES cells and hypermethylation in *Dnmt3b*-expressing *Myc-Bcl2* and *MLL-AF9* leukemias was higher than the number of those CpG sites affected by hypomethylation in *Dnmt3b*-expressing leukemias (Figure 4G-H; supplemental Figure 7A-B). Interestingly, this phenomenon was also shown for Dnmt3a target sites (supplemental Figure 7C-F). In addition, the majority of DMCs identified in both *Myc-Bcl2* and *MLL-AF9 Dnmt3b*-knock-in samples showed DNA hypermethylation (Figure 4I), which elucidated a shared set of commonly hypermethylated CpG sites.

Dnmt3b-induced DNA methylation alters LSC gene expression profile

We generated genome-wide messenger RNA expression profiles of GFP^+/c-Kit^+ wild-type and *Dnmt3b*-knock-in *MLL-AF9* LSCs from the exact same donor mice that were used for DNA methylation profiling. We identified 74 differentially expressed genes in c-Kit^+ *Dnmt3b*-knock-in LSCs (Figure 5A). The group of genes downregulated in *Dnmt3b*-knock-in LSCs was enriched for genes highly expressed in normal HSCs, including the tyrosine kinase receptor *Flt3* and the early hematopoietic marker *CD34*. Conversely, *Dnmt3b*-knock-in LSCs showed higher expression of HSC differentiation factors. Ingenuity pathway analysis revealed significant enrichment for genes downregulated in *Dnmt3*-knock-in LSCs in the categories of cancer (25 genes), cellular growth and proliferation (18 genes), and hematologic system development and function (12 genes) ($P < .05$). Upstream regulators of differentially expressed genes in *Dnmt3b*-knock-in leukemias included transcription factors (eg, Myc and Srf), as well as the epigenetic factors Kdm6b and Asxl1. Gene set enrichment analysis revealed a strong enrichment of "stemness" genes and of genes expressed in early hematopoietic progenitors and LICs in wild-type LSCs (Figure 5B, left). Vice versa, *Dnmt3b*-knock-in hypermethylated LSCs were enriched for genes expressed in mature hematopoietic cells and genes downregulated in normal LSCs and LICs (Figure 5B, right). The concomitant downregulation of HSC and LSC genes and upregulation of differentiation genes may contribute to increased leukemia latencies upon high *Dnmt3b* expression.

We next investigated a potential connection between DNA hypermethylation, deregulated gene expression, and altered functional properties of *Dnmt3b*-knock-in LSCs. We focused on genes with extensive hypermethylation in both *MLL-AF9* and *Myc-Bcl2* leukemias and reduced expression levels in *Dnmt3b*-knock-in *MLL-AF9* LSCs. We identified 41 candidate genes, which showed at least 20% hypermethylation in murine *Dnmt3b*-knock-in leukemias and an expression log ratio of −0.5 in *Dnmt3b*-knock-in *MLL-AF9* LSCs. Expression changes for 19 of these genes were confirmed by real-time PCR, exemplarily depicted for 6 candidates (Figure 5C). Comparison of the obtained candidate genes with published expression data sets using the Leukemia Gene Atlas[59] and Gene Expression Commons[60] revealed high expression of 12 out of 19 genes in HSCs and decreasing expression levels in more differentiated cells (Figure 5D-E; supplemental Figure 8). The histone protein (H1F0) (Figure 5D) and the G-protein coupled receptor 56 (GPR56) (Figure 5E) exhibited the strongest expression differences. These data suggest that Dnmt3b-induced hypermethylation may have altered the gene expression profile of LSCs. *Dnmt3b*-knock-in *MLL-AF9* LSCs may have lost the characteristic stem cell expression profile and possibly even their functional stem cell properties. Of note, differential gene expression did not correlate with DNA methylation changes in gene bodies (Spearman's $\rho = -0.073$, $P < .001$) (supplemental Figure 9A) or promoter regions (Spearman's $\rho = -0.021$, $P = .280$) (supplemental Figure 9B). Other studies have similarly observed no correlation between gene expression and promoter methylation in human disease.[33,61] High gene body methylation has been associated with gene activation rather than transcriptional silencing.[62,63] These data support the view that Dnmt3b affects nonpromoter methylation and that Dnmt3b-mediated enhanced gene body methylation is, at least in AML, not necessarily associated with activation of gene expression.

Deregulated Dnmt3b targets have prognostic impact in AML patients

Candidate genes that showed hypermethylation and reduced gene expression in *Dnmt3b*-knock-in leukemias were analyzed for a potential association with prognosis in AML patients.[59] Overall, 5 of 19 candidate genes were associated with better overall survival when expressed at low levels in AML patients (Figure 6A-B; supplemental Figure 10A-C).[64] The methylation status of candidate genes with putative functional importance was determined in 194 AML patient

Figure 4 (continued) *Dnmt3b*-knock-in samples, green indicates hypomethylated DMCs. (D) Region presence of covered and DMC sites across different genomic regions. Promoter regions were defined as 1000 bp upstream of transcriptional start site (TSS) to 500 bp downstream of TSS; gene bodies were defined as 500 bp downstream of TSS to end of gene. GB, gene bodies; P, promoter. (E-F) Presence of transcription factor binding sites (E), and RNA polymerase II binding sites (F) among DMRs. The number of centers of a particular region of interest that could be expected in DMRs under the assumption of a uniform distribution in RRBS-covered regions (expected region centers) was subtracted from the number of region centers actually found in DMRs (observed region centers). The bars visualize the differences of observed and expected region centers in hypermethylated DMRs. ***$P < .001$. (G) Smoothed scatter plot shows CpG sites that gained DNA methylation in previously published *Dnmt* triple-knockout mice (TKO) with reintroduced *Dnmt3b*[50] and the corresponding DNA methylation difference in *MLL-AF9* leukemias (*Dnmt3b*-knock-in − wild-type). Methylation in TKO + *Dnmt3b* indicates CpGs that gain DNA methylation after eradication of existing DNA methylation patterns in TKO cells following reintroduction of *Dnmt3b*. (H) Number of CpG sites that show methylation gain in TKO murine embryonic stem cells >0, >20, or >40, and hypo- or hypermethylation in *Dnmt3b*-knock-in *MLL-AF9* leukemias. (I) Smoothed scatter plot showing all CpGs that are differentially methylated in *Myc-Bcl2* and *MLL-AF9* leukemias, respectively. The majority of DMCs showed hypermethylation in both leukemias.

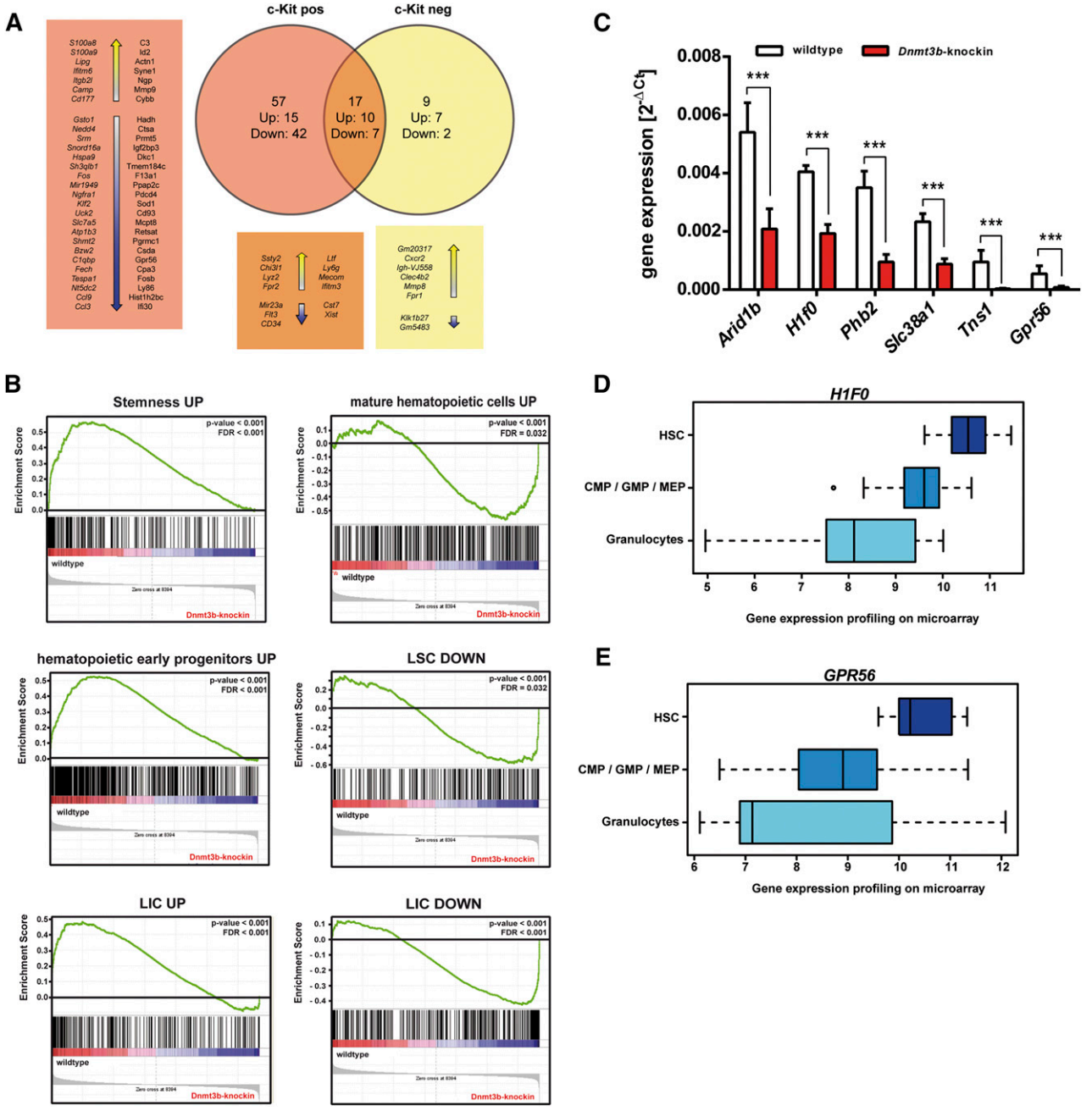

Figure 5. Dnmt3b alters the expression profile of LSCs and leads to downregulation of stem cell genes. (A) Genome-wide mRNA expression profiles of c-Kit$^+$ *MLL-AF9* LSCs and c-Kit$^-$ *MLL-AF9* leukemic bulk cells were generated using Affymetrix Mouse Gene ST 2.0 arrays. Numbers of up- and downregulated genes in c-Kit$^+$ and c-Kit$^-$ bone marrow cells from primary recipients of wild-type and *Dnmt3b*-knock-in *MLL-AF9* leukemic cells were compared. (B) Gene set enrichment analysis of expression profiles of c-Kit$^+$ wild-type and *Dnmt3b*-knock-in *MLL-AF9* LSCs. Stemness genes and genes expressed in LICs were found in wild-type LSCs (left), whereas the expression profile of *Dnmt3b*-knock-in LSCs was enriched for genes expressed in mature hematopoietic cells or genes that are downregulated in LSCs and LICs (right). (C) Differentially expressed genes were filtered for those with reduced expression levels with DNA hypermethylation in both *MLL-AF9* and *Myc-Bcl2* leukemias at the same time. Relative expression levels of representative candidate genes were measured by real-time PCR analysis. Mean ± SD values are from 3 replicates normalized to expression levels of *GAPDH*. (D-E) Gene expression profiles of representative candidate genes in HSCs, granulocyte-macrophage progenitors, and mature granulocytes. Microarray data sets were analyzed using the Leukemia Gene Atlas (http://www. leukemia-gene-atlas.org). ***$P < .001$

samples available from the TCGA database (http://cancergenome.nih.gov/). All of the candidate genes identified in the *Dnmt3b*-knock-in mouse model showed low methylation levels across the analyzed patient cohort (Figure 6C). For 34 of 41 candidates, gene expression negatively correlated with DNA methylation levels (Figure 6D). These data indicate that the identified target genes that are deregulated by Dnmt3b-mediated DNA methylation in mice may also be regulated by DNA methylation in human AML.

Discussion

DNA methylation patterns are altered during leukemogenesis. But the mechanistic relevance of altered DNA methylation for leukemogenesis has remained unclear. In the current study, we demonstrate that increased activity of de novo DNA methyltransferase Dnmt3b inhibits leukemogenesis. *Myc-Bcl2*–induced leukemias with high levels of

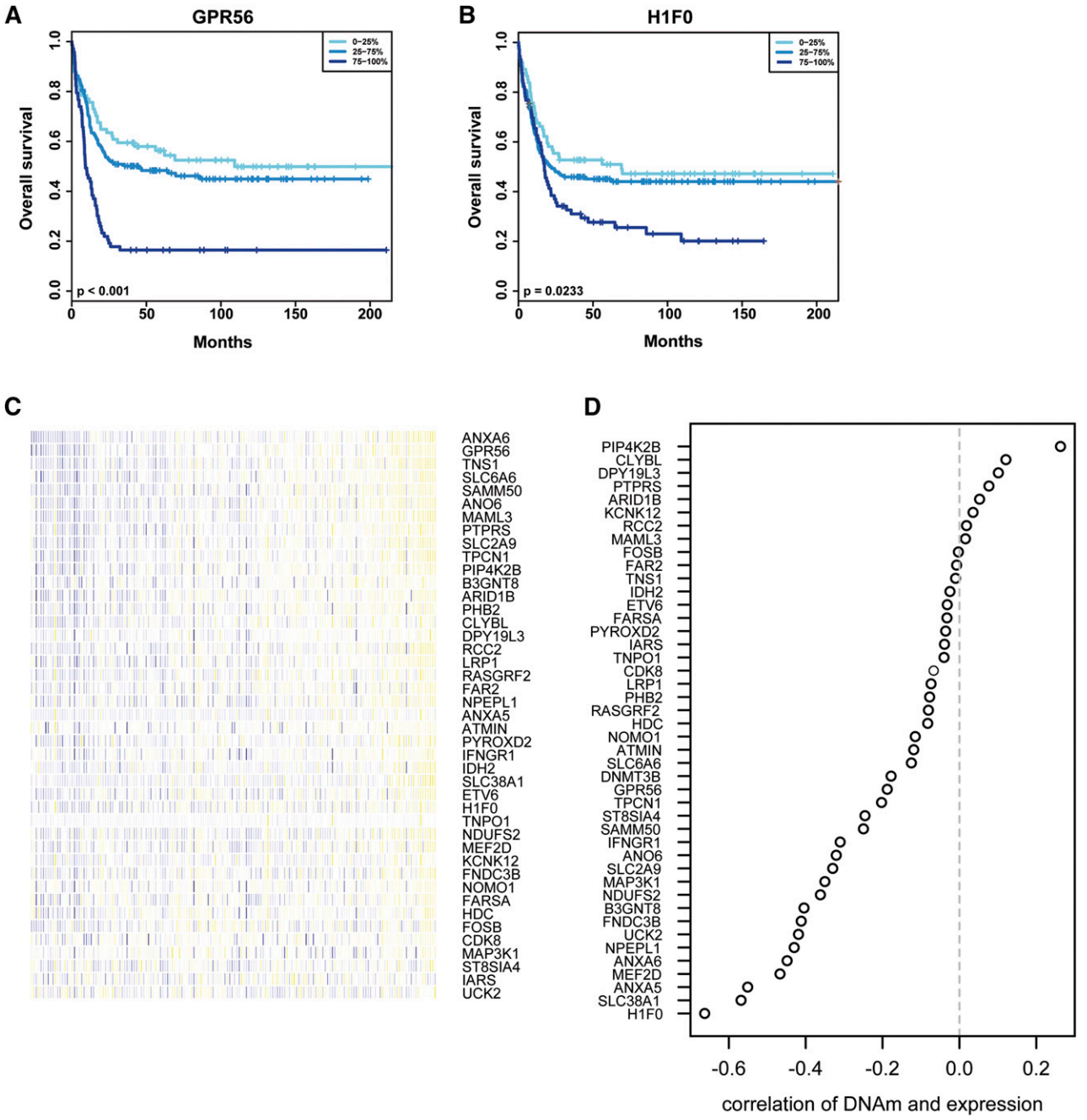

Figure 6. Analysis of Dnmt3b-regulated genes in human AML data sets. (A-B) Candidate genes were analyzed for their prognostic impact in human leukemia using published patient data and the Leukemia Gene Atlas. Gene expression data were grouped into quartiles, and survival outcome was analyzed in a patient data set published in 2009 by Verhaak et al.[64] Shown is overall survival in AML patients for low (0% to 25%), intermediate (25% to 75%), and high (75% to 100%) expression levels of the candidate genes *GPR56* ($P < .001$) (A) and *H1F0* ($P = .023$) (B). (C) DNA methylation (DNAm) levels of candidate genes were analyzed in 194 AML patients using Illumina bead array data available from the TCGA database. Each column represents 1 patient, and methylation levels are color coded with blue representing low methylation and yellow representing high methylation. (D) Correlation between DNA methylation levels and gene expression for the identified target genes from AML patients in the TCGA database. Depicted is the correlation coefficient for all candidate genes. Overall, 34 genes showed significant negative correlation between methylation and expression.

DNA methylation were affected as well as *MLL-AF9*–induced leukemias with much lower levels of altered DNA methylation.

DNA methylation plays an important role in HSC self-renewal as well as in differentiation and lineage commitment.[10,13,14] Multiple tumor suppressor genes have been identified to be epigenetically silenced presumably by DNA methylation in AML.[65-67] Oncogenes such as *AML1-ETO* were described to directly recruit DNMT activity to their target genes.[68] On the other hand, PML-RARα by itself did induce a differentiation block in promyelocytes without causing increased DNA methylation.[49] The most common mutations in the DNA methylation machinery affect *DNMT3A* and appear to induce DNA hypomethylation. Loss of *Dnmt3a* either alone or in combination with *Dnmt3b* induces stem cell expansion as an important prerequisite for leukemogenesis.[14] *DNMT3A* mutations are also the most common finding in clonal hematopoiesis in older adults.[69] These seemingly contradictory findings prompted us to evaluate the functional consequences of high de novo Dnmt activity in leukemogenesis. We chose Dnmt3b, because its expression is highest in HSCs.[12] High *DNMT3B* expression in leukemic bulk cells was associated with poor prognosis.[37]

The inducible *Dnmt3b* model has previously been used to analyze colon adenoma formation. Forced *Dnmt3b* expression in colon was not toxic but increased the number of colon adenomas.[29] In our analyses, we did not observe toxic effects in healthy hematopoiesis in vivo or in vitro.

Dnmt3b was previously identified as a haploinsufficient tumor suppressor in *Eμ-myc* mice.[32] Loss of *Dnmt3b* led to the upregulation of the tumor modifier Ment and accelerated mouse lymphomagenesis. In our analyses, leukemias with high Dnmt3b levels showed increased disease latency in serial transplantations. Accordingly, Dnmt3b might exhibit tumor suppressor functions in myeloid disease.

DNA methylation is essential for lymphoid development.[10] The *Myc-Bcl2* leukemia model can induce lymphoid as well as myeloid leukemias.[40] Upon forced *Dnmt3b* expression, we observed a shift toward myeloid leukemia with stronger suppression of lymphoid leukemias. These findings may reemphasize the notion that the myeloid lineage as the phylogenetically older system is the default pathway. DNA methylation changes might preferentially induce myeloid leukemia. Lymphoid leukemogenesis was also inhibited by global DNA hypomethylation (eg, by loss of *Dnmt1* [hypomorph]), whereas myeloid leukemias could still be formed.[10] In line with our new findings, these data suggest that lymphoid leukemias depend on a tightly controlled DNA methylation machinery whereas myeloid leukemias are more permissive for global changes in DNA methylation levels.

Leukemogenesis depends on LSC activity. In *MLL-AF9* leukemia, the population of c-Kit$^+$ cells is highly enriched for LICs.[39,47] Forced *Dnmt3b* expression in the present study increased numbers of c-Kit$^+$ leukemic cells but reduced colony formation potential. In accordance, transplantation of c-Kit^{high} cells resulted in delayed leukemia development in recipients of *Dnmt3b*-knock-in cells. Still, secondary and tertiary recipients of *Dnmt3b*-expressing c-Kit$^+$ LSCs developed a leukemia-like disease, indicating that LSCs were still present in the population of c-Kit–expressing cells, yet they induced disease with increased latency. Colony formation potential was not impaired in leukemic cells from spleen. Accordingly, DNA methylation might be most relevant at the stem cell level.

Increased numbers of c-Kit$^+$ LSCs were found in recipients of *Dnmt3b*-knock-in cells, although leukemia development was delayed. In nonleukemic *Dnmt3b*-knock-in mice, *Dnmt3b* overexpression led to a block at the multipotent progenitor (LMPP) stage. Multilineage repopulation potential of *Dnmt3b*-knock-in cells remained unaltered in competitive transplantation assays. In line with previous findings, higher Dnmt3b levels were not sufficient to ultimately drive differentiation of healthy HSCs. But altered Dnmt3b levels had a crucial effect on LSCs in AML. Nonetheless, an involvement of Dnmt3a cannot be completely excluded, because high levels of Dnmt3b might stabilize protein complexes formed by Dnmt3b, Dnmt3a, and Dnmt3L.[70,71]

Studies concerning DNA methylation patterns in human AML have found that a small common set of genes exhibited consistent aberrant methylation across several hundred cases.[19,20] *Dnmt3b*-knock-in leukemias showed increased levels of DNA methylation, as analyzed by RRBS. *Dnmt3b*-knock-in *Myc-Bcl2* leukemia samples showed a much higher number of hypermethylated CpGs than the respective *MLL-AF9* leukemic samples. This is in line with the finding that *MLL*-rearranged leukemias in AML patients are characterized by DNA hypomethylation when compared with *IDH*-mutated leukemias.[20] The extent of hypermethylation induced by forced *Dnmt3b* expression possibly depends on the basal DNA methylation level that is induced by the specific oncogene. Less pronounced DNA hypermethylation in *MLL-AF9* leukemias further suggests that these may be more susceptible to methylation-associated delay in leukemogenesis. Detailed analysis of *MLL-AF9* patient samples with high and low *DNMT3B* expression would improve our understanding of the role DNMT3B-mediated DNA methylation in human leukemogenesis. Of note, the majority of DMCs in *Dnmt3b*-knock-in leukemias mapped to gene bodies. This preferential targeting of Dnmt3b-mediated DNA methylation to nonpromoter CpGs has been described (eg, in ES cells),[50,72] but the role of gene body methylation in leukemia has not been investigated so far. An inverse relationship between transcription factor binding and DNA methylation was found in several studies involving diverse human cell and tissue types[73] and acute promyelocytic leukemia.[49] Protection of actively transcribed genes from de novo methylation has been attributed to loss of nucleosomes at sites of transcription.[70]

Apart from unique DNA methylation patterns in different leukemia subtypes, both, *Myc-Bcl2* and *MLL-AF9* leukemias shared a significant set of commonly hypermethylated CpGs. These CpGs were possibly instrumental for the consistently observed effect of delayed leukemogenesis. Although overexpression of *Dnmt3b* induced thousands of DNA methylation alterations, only few changes in gene expression were observed. We and others have shown that promoter and gene body methylation does not necessarily correlate with altered gene expression.[33,61] Low numbers of differentially expressed genes in *Dnmt3b*-knock-in leukemias could also be because of heavy underrepresentation of transcription factor binding sites in DMRs. In addition, predicted upstream regulators can affect the expression of target genes independently of DNA methylation levels. An essential role in initiation and maintenance of T-ALL was recently demonstrated for the H3K27-demethylase Kdm6b.[74]

Hypermethylated LSCs showed reduced expression of genes related to cancer and proliferation, which is in accordance with increased disease latency of *Dnmt3b*-knock-in leukemias. Furthermore, hypermethylated LSCs were characterized by loss of a stemness-associated gene expression signature. Of note, high expression of LSC- and HSC-related genes in leukemic blast cells was associated with worse survival in AML patients.[75,76] In our analyses, genes with DNA hypermethylation and reduced gene expression marked several candidate genes that were associated with poor outcome upon high expression in AML patients. Thus, suppression of selected stem cell genes by DNMT3B could be a protective mechanism that prevents LSC functions. The correlation between overall *DNMT3B* expression in leukemic bulk cells with poor outcome might reflect the stem cell phenotype of the leukemia. Studies in AML patients have only addressed the effect of *DNMT3B* expression in unsorted leukemic bulk cells from various leukemia subtypes on patient survival.[37,38] Here, we show for the first time the role of high *Dnmt3b* expression in murine LSCs and a potential relevance of DNMT3B-target genes in AML patients. Additional studies in patients with different AML subtypes would help to further clarify the function of DNMT3B in LSCs.

Taken together, increased activity of the de novo DNA methyltransferase Dnmt3b impairs leukemia development. Protection from DNA methylation and/or specific loss of DNA methylation at regions that are critical for LSC formation may constitute an important step in leukemogenesis.

Acknowledgments

This work was supported by grants from Deutsche Forschungsgemeinschaft (MU 1328/15-1 and MU 1328/9-2). W.E.B.'s laboratory is supported by Deutsche Forschungsgemeinschaft (EXC1003, Cells in Motion).

Authorship

Contribution: I.S. and C.M.-T. designed the research and wrote the manuscript; I.S., M.S.-W., N.B., A.K., P.R., H.G., F.H., and

P.T. performed experiments; C.R. performed all bioinformatics analyses on single CpG level; K.H. performed BiSeq analysis; Q.L. and W.W. performed bioinformatics analyses of TCGA data; H.L. and L.A.G. provided mice; and all authors contributed to data interpretation and the manuscript writing process.

Conflict of interest disclosure: The authors declare no competing financial interests.

Correspondence: Carsten Müller-Tidow, Department of Hematology and Oncology, University of Halle, Ernst-Grube-Strasse 40, 06108 Halle, Germany; e-mail: carsten.mueller-tidow@uk-halle.de.

References

1. Attwood JT, Yung RL, Richardson BC. DNA methylation and the regulation of gene transcription. *Cell Mol Life Sci.* 2002;59(2): 241-257.
2. Okano M, Xie S, Li E. Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. *Nat Genet.* 1998;19(3):219-220.
3. Bestor TH. The DNA methyltransferases of mammals. *Hum Mol Genet.* 2000;9(16): 2395-2402.
4. Lei H, Oh SP, Okano M, et al. De novo DNA cytosine methyltransferase activities in mouse embryonic stem cells. *Development.* 1996; 122(10):3195-3205.
5. Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell.* 1999;99(3):247-257.
6. Chen T, Ueda Y, Dodge JE, Wang Z, Li E. Establishment and maintenance of genomic methylation patterns in mouse embryonic stem cells by Dnmt3a and Dnmt3b. *Mol Cell Biol.* 2003; 23(16):5594-5605.
7. Liang G, Chan MF, Tomigahara Y, et al. Cooperativity between DNA methyltransferases in the maintenance methylation of repetitive elements. *Mol Cell Biol.* 2002;22(2):480-491.
8. Li E, Bestor TH, Jaenisch R. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell.* 1992;69(6):915-926.
9. Meissner A, Mikkelsen TS, Gu H, et al. Genome-scale DNA methylation maps of pluripotent and differentiated cells. *Nature.* 2008;454(7205): 766-770.
10. Bröske AM, Vockentanz L, Kharazi S, et al. DNA methylation protects hematopoietic stem cell multipotency from myeloerythroid restriction. *Nat Genet.* 2009;41(11):1207-1215.
11. Trowbridge JJ, Snow JW, Kim J, Orkin SH. DNA methyltransferase 1 is essential for and uniquely regulates hematopoietic stem and progenitor cells. *Cell Stem Cell.* 2009;5(4):442-449.
12. Tadokoro Y, Ema H, Okano M, Li E, Nakauchi H. De novo DNA methyltransferase is essential for self-renewal, but not for differentiation, in hematopoietic stem cells. *J Exp Med.* 2007; 204(4):715-722.
13. Challen GA, Sun D, Jeong M, et al. Dnmt3a is essential for hematopoietic stem cell differentiation. *Nat Genet.* 2011;44(1):23-31.
14. Challen GA, Sun D, Mayle A, et al. Dnmt3a and Dnmt3b have overlapping and distinct functions in hematopoietic stem cells. *Cell Stem Cell.* 2014; 15(3):350-364.
15. Baylin SB, Jones PA. A decade of exploring the cancer epigenome - biological and translational implications. *Nat Rev Cancer.* 2011;11(10): 726-734.
16. Sharma SV, Lee DY, Li B, et al. A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. *Cell.* 2010;141(1):69-80.
17. Esteller M. Epigenetics in cancer. *N Engl J Med.* 2008;358(11):1148-1159.
18. Rice KL, Hormaeche I, Licht JD. Epigenetic regulation of normal and malignant hematopoiesis. *Oncogene.* 2007;26(47): 6697-6714.
19. Figueroa ME, Lugthart S, Li Y, et al. DNA methylation signatures identify biologically distinct subtypes in acute myeloid leukemia. *Cancer Cell.* 2010;17(1):13-27.
20. Akalin A, Garrett-Bakelman FE, Kormaksson M, et al. Base-pair resolution DNA methylation sequencing reveals profoundly divergent epigenetic landscapes in acute myeloid leukemia. *PLoS Genet.* 2012;8(6):e1002781.
21. Feinberg AP, Vogelstein B. Hypomethylation of ras oncogenes in primary human cancers. *Biochem Biophys Res Commun.* 1983;111(1):47-54.
22. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med.* 2003;349(21):2042-2054.
23. Feinberg AP, Tycko B. The history of cancer epigenetics. *Nat Rev Cancer.* 2004;4(2):143-153.
24. Jones PA, Baylin SB. The epigenomics of cancer. *Cell.* 2007;128(4):683-692.
25. Laird PW, Jackson-Grusby L, Fazeli A, et al. Suppression of intestinal neoplasia by DNA hypomethylation. *Cell.* 1995;81(2):197-205.
26. Trowbridge JJ, Sinha AU, Zhu N, Li M, Armstrong SA, Orkin SH. Haploinsufficiency of Dnmt1 impairs leukemia stem cell function through derepression of bivalent chromatin domains. *Genes Dev.* 2012;26(4):344-349.
27. Eden A, Gaudet F, Waghmare A, Jaenisch R. Chromosomal instability and tumors promoted by DNA hypomethylation. *Science.* 2003;300(5618): 455.
28. Gaudet F, Hodgson JG, Eden A, et al. Induction of tumors in mice by genomic hypomethylation. *Science.* 2003;300(5618):489-492.
29. Linhart HG, Lin H, Yamada Y, et al. Dnmt3b promotes tumorigenesis in vivo by gene-specific de novo methylation and transcriptional silencing. *Genes Dev.* 2007;21(23):3110-3122.
30. Lin H, Yamada Y, Nguyen S, et al. Suppression of intestinal neoplasia by deletion of Dnmt3b. *Mol Cell Biol.* 2006;26(8):2976-2983.
31. Hlady RA, Novakova S, Opavska J, et al. Loss of Dnmt3b function upregulates the tumor modifier Ment and accelerates mouse lymphomagenesis. *J Clin Invest.* 2012;122(1):163-177.
32. Vasanthakumar A, Lepore JB, Zegarek MH, et al. Dnmt3b is a haploinsufficient tumor suppressor gene in myc-induced lymphomagenesis. *Blood.* 2013;121(11):2059-2063.
33. Ley TJ, Ding L, Walter MJ, et al. DNMT3A mutations in acute myeloid leukemia. *N Engl J Med.* 2010;363(25):2424-2433.
34. Yan XJ, Xu J, Gu ZH, et al. Exome sequencing identifies somatic mutations of DNA methyltransferase gene DNMT3A in acute monocytic leukemia. *Nat Genet.* 2011;43(4): 309-315.
35. Walter MJ, Ding L, Shen D, et al. Recurrent DNMT3A mutations in patients with myelodysplastic syndromes. *Leukemia.* 2011; 25(7):1153-1158.
36. Patel JP, Gönen M, Figueroa ME, et al. Prognostic relevance of integrated genetic profiling in acute myeloid leukemia. *N Engl J Med.* 2012;366(12):1079-1089.
37. Niederwieser C, Kohlschmidt J, Volinia S, et al. Prognostic and biologic significance of DNMT3B expression in older patients with cytogenetically normal primary acute myeloid leukemia. *Leukemia.* 2015;29(3): 567-575.
38. Hayette S, Thomas X, Jallades L, et al. High DNA methyltransferase DNMT3B levels: a poor prognostic marker in acute myeloid leukemia. *PLoS One.* 2012;7(12):e51527.
39. Somervaille TCP, Cleary ML. Identification and characterization of leukemia stem cells in murine MLL-AF9 acute myeloid leukemia. *Cancer Cell.* 2006;10(4):257-268.
40. Luo H, Li Q, O'Neal J, Kreisel F, Le Beau MM, Tomasson MH. c-Myc rapidly induces acute myeloid leukemia in mice without evidence of lymphoma-associated antiapoptotic mutations. *Blood.* 2005;106(7):2452-2461.
41. Hong F, Breitling R, McEntee CW, Wittner BS, Nemhauser JL, Chory J. RankProd: a bioconductor package for detecting differentially expressed genes in meta-analysis. *Bioinformatics.* 2006;22(22):2825-2827.
42. Smith ZD, Gu H, Bock C, Gnirke A, Meissner A. High-throughput bisulfite sequencing in mammalian genomes. *Methods.* 2009;48(3): 226-232.
43. Haferlach T, Kohlmann A, Wieczorek L, et al. Clinical utility of microarray-based gene expression profiling in the diagnosis and subclassification of leukemia: report from the International Microarray Innovations in Leukemia Study Group. *J Clin Oncol.* 2010;28(15): 2529-2537.
44. Stegmaier K, Ross KN, Colavito SA, O'Malley S, Stockwell BR, Golub TR. Gene expression-based high-throughput screening(GE-HTS) and application to leukemia differentiation. *Nat Genet.* 2004;36(3):257-263.
45. Andersson A, Ritz C, Lindgren D, et al. Microarray-based classification of a consecutive series of 121 childhood acute leukemias: prediction of leukemic and genetic subtype as well as of minimal residual disease status. *Leukemia.* 2007;21(6):1198-1203.
46. Valk PJ, Verhaak RG, Beijen MA, et al. Prognostically useful gene-expression profiles in acute myeloid leukemia. *N Engl J Med.* 2004; 350(16):1617-1628.
47. Krivtsov AV, Twomey D, Feng Z, et al. Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9. *Nature.* 2006;442(7104):818-822.
48. Cabezas-Wallscheid N, Klimmeck D, Hansson J, et al. Identification of regulatory networks in HSCs and their immediate progeny via integrated proteome, transcriptome, and DNA methylome analysis. *Cell Stem Cell.* 2014;15(4):507-522.
49. Schoofs T, Rohde C, Hebestreit K, et al. DNA methylation changes are a late event in acute promyelocytic leukemia and coincide with loss of transcription factor binding. *Blood.* 2013;121(1): 178-187.
50. Baubec T, Colombo DF, Wirbelauer C, et al. Genomic profiling of DNA methyltransferases reveals a role for DNMT3B in genic methylation. *Nature.* 2015;520(7546):243-247.
51. Rosenbloom KR, Dreszer TR, Pheasant M, et al. ENCODE whole-genome data in the

UCSC Genome Browser. *Nucleic Acids Res.* 2010;38(Database issue):D620-D625.

52. Baudino TA, Cleveland JL. The Max network gone mad. *Mol Cell Biol.* 2001;21(3):691-702.
53. Dolnik A, Engelmann JC, Scharfenberger-Schmeer M, et al. Commonly altered genomic regions in acute myeloid leukemia are enriched for somatic mutations involved in chromatin remodeling and splicing. *Blood.* 2012;120(18): e83-e92.
54. Corces-Zimmerman MR, Majeti R. Pre-leukemic evolution of hematopoietic stem cells: the importance of early mutations in leukemogenesis. *Leukemia.* 2014;28(12):2276-2282.
55. Giotopoulos G, Chan WI, Horton SJ, et al. The epigenetic regulators CBP and p300 facilitate leukemogenesis and represent therapeutic targets in acute myeloid leukemia [published online ahead of print April 20, 2015]. *Oncogene.* doi:10.1038/onc.2015.92.
56. Benedikt A, Baltruschat S, Scholz B, et al. The leukemogenic AF4-MLL fusion protein causes P-TEFb kinase activation and altered epigenetic signatures. *Leukemia.* 2011;25(1):135-144.
57. Zhou P, Wang Z, Yuan X, et al. Mixed lineage leukemia 5 (MLL5) protein regulates cell cycle progression and E2F1-responsive gene expression via association with host cell factor-1 (HCF-1). *J Biol Chem.* 2013;288(24): 17532-17543.
58. Pattabiraman DR, McGirr C, Shakhbazov K, et al. Interaction of c-Myb with p300 is required for the induction of acute myeloid leukemia (AML) by human AML oncogenes. *Blood.* 2014;123(17): 2682-2690.
59. Hebestreit K, Gröttrup S, Emden D, et al. Leukemia gene atlas–a public platform for integrative exploration of genome-wide molecular data. *PLoS One.* 2012;7(6):e39148.
60. Seita J, Sahoo D, Rossi DJ, et al. Gene Expression Commons: an open platform for absolute gene expression profiling. *PLoS One.* 2012;7(7):e40321.
61. Lister R, Pelizzola M, Dowen RH, et al. Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature.* 2009;462(7271):315-322.
62. Ball MP, Li JB, Gao Y, et al. Targeted and genome-scale strategies reveal gene-body methylation signatures in human cells. *Nat Biotechnol.* 2009;27(4):361-368.
63. Laurent LC, Ulitsky I, Slavin I, et al. Dynamic changes in the copy number of pluripotency and cell proliferation genes in human ESCs and iPSCs during reprogramming and time in culture. *Cell Stem Cell.* 2011;8(1):106-118.
64. Verhaak RG, Wouters BJ, Erpelinck CA, et al. Prediction of molecular subtypes in acute myeloid leukemia based on gene expression profiling. *Haematologica.* 2009;94(1):131-134.
65. Agrawal-Singh S, Isken F, Agelopoulos K, et al. Genome-wide analysis of histone H3 acetylation patterns in AML identifies PRDX2 as an epigenetically silenced tumor suppressor gene. *Blood.* 2012;119(10):2346-2357.
66. Agrawal S, Hofmann WK, Tidow N, et al. The C/EBPdelta tumor suppressor is silenced by hypermethylation in acute myeloid leukemia. *Blood.* 2007;109(9):3895-3905.
67. Christiansen DH, Andersen MK, Pedersen-Bjergaard J. Methylation of p15INK4B is common, is associated with deletion of genes on chromosome arm 7q and predicts a poor prognosis in therapy-related myelodysplasia and acute myeloid leukemia. *Leukemia.* 2003;17(9): 1813-1819.
68. Liu S, Shen T, Huynh L, et al. Interplay of RUNX1/MTG8 and DNA methyltransferase 1 in acute myeloid leukemia. *Cancer Res.* 2005;65(4): 1277-1284.
69. Genovese G, Kähler AK, Handsaker RE, et al. Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. *N Engl J Med.* 2014;371(26):2477-2487.
70. Ooi SK, Qiu C, Bernstein E, et al. DNMT3L connects unmethylated lysine 4 of histone H3 to de novo methylation of DNA. *Nature.* 2007;448 (7154):714-717.
71. Li JY, Pu MT, Hirasawa R, et al. Synergistic function of DNA methyltransferases Dnmt3a and Dnmt3b in the methylation of Oct4 and nanog. *Mol Cell Biol.* 2007;27(24):8748-8759.
72. Jin B, Ernst J, Tiedemann RL, et al. Linking DNA methyltransferases to epigenetic marks and nucleosome structure genome-wide in human tumor cells. *Cell Reports.* 2012;2(5):1411-1424.
73. Thurman RE, Rynes E, Humbert R, et al. The accessible chromatin landscape of the human genome. *Nature.* 2012;489(7414):75-82.
74. Ntziachristos P, Tsirigos A, Welstead GG, et al. Contrasting roles of histone 3 lysine 27 demethylases in acute lymphoblastic leukaemia. *Nature.* 2014;514(7523):513-517.
75. Gentles AJ, Plevritis SK, Majeti R, Alizadeh AA. Association of a leukemic stem cell gene expression signature with clinical outcomes in acute myeloid leukemia. *JAMA.* 2010;304(24):2706-2715.
76. Eppert K, Takenaka K, Lechman ER, et al. Stem cell gene expression programs influence clinical outcome in human leukemia. *Nat Med.* 2011; 17(9):1086-1093.