Impact of *MLL5* expression on decitabine efficacy and DNA methylation in acute myeloid leukemia

Haiyang Yun,[1] Frederik Damm,[2] Damian Yap,[3,4] Adrian Schwarzer,[5] Anuhar Chaturvedi,[1] Nidhi Jyotsana,[1] Michael Lübbert,[6] Lars Bullinger,[7] Konstanze Döhner,[7] Robert Geffers,[8] Samuel Aparicio,[3,4] R. Keith Humphries,[9,10] Arnold Ganser,[1] and Michael Heuser[1]

[1]Department of Hematology, Hemostasis, Oncology and Stem cell Transplantation, Hannover Medical School, Germany; [2]Department of Hematology, Oncology, and Tumor Immunology, Charité, Berlin, Germany; [3]Department of Molecular Oncology, British Columbia Cancer Agency, Vancouver, BC, Canada; [4]Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, BC, Canada; [5]Institute of Experimental Hematology, Hannover Medical School, Germany; [6]Division of Hematology and Oncology, University of Freiburg Medical Center, Germany; [7]Department of Internal Medicine III, University Hospital of Ulm, Germany; [8]Department of Cell Biology and Immunology, Helmholtz Centre for Infection Research, Braunschweig, Germany; [9]Terry Fox Laboratory, British Columbia Cancer Agency, Vancouver, BC, Canada; and [10]Department of Medicine, University of British Columbia, Vancouver, BC, Canada

ABSTRACT

Hypomethylating agents are widely used in patients with myelodysplastic syndromes and unfit patients with acute myeloid leukemia. However, it is not well understood why only some patients respond to hypomethylating agents. We found previously that the effect of decitabine on hematopoietic stem cell viability differed between Mll5 wild-type and null cells. We, therefore, investigated the role of *MLL5* expression levels on outcome of acute myeloid leukemia patients who were treated with decitabine. *MLL5* above the median expression level predicted longer overall survival independent of *DNMT3A* mutation status in bivariate analysis (median overall survival for high *vs.* low MLL5 expression 292 *vs.* 167 days; *P*=0.026). In patients who received three or more courses decitabine, high *MLL5* expression and wild-type *DNMT3A* independently predicted improved overall survival (median overall survival for high *vs.* low *MLL5* expression 468 *vs.* 243 days; *P*=0.012). In transformed murine cells, loss of Mll5 was associated with resistance to low-dose decitabine, less global DNA methylation in promoter regions, and reduced DNA demethylation upon decitabine treatment. Together, these data support our clinical observation of improved outcome in decitabine-treated patients who express *MLL5* at high levels, and suggest a mechanistic role of MLL5 in the regulation of DNA methylation.

Introduction

Acute myeloid leukemia (AML) has been well characterized as a heterogeneous disorder of normal hematopoiesis caused by genetic impairment and epigenetic deregulation.[1,2] Standard treatment of AML including induction chemotherapy and bone marrow transplantation has achieved limited success due to biological heterogeneity and disease evolution of patients.[3] Decitabine (DAC), a DNA hypomethylating agent (HMA) showing therapeutic efficacy against leukemic cells, was recently incorporated into standard treatment mainly for intermediate- or high-risk myelodysplastic syndromes (MDS),[4] and was also suggested for treatment of elderly AML patients ineligible for intensive chemotherapy.[5] However, molecular markers predictive for decitabine treatment response remain largely unknown and are worth exploring in the disease context.

Human trithorax-group (Trx-G) gene *MLL5* was initially identified from molecular mapping of a frequently deleted segment of chromosome 7q22 in patients with myeloid disorders.[6] Unlike the well-documented histone lysine methyltransferase (HKMT) activity of other Trx-G members, the role of MLL5 as a novel HKMT has long been under debate due to sequence divergence to its homologs.[7,8] *In vitro* gain- and loss-of-function studies of MLL5 have fully established its role in cell cycle regulation.[8-12] We and others have characterized loss-of-Mll5 mouse models.[7,13,14] Mll5 absence was not lethal, but led to a spectrum of defects including mild growth retardation, male infertility and defective hematopoiesis. Moreover, hematopoietic stem cells (HSC) deficient in Mll5 had increased sensitivity to decitabine-induced differentiation,[13] highlighting a potential role of Mll5 in control of decitabine sensitivity and DNA methylation.

Recently we reported the favorable prognostic importance of *MLL5* expression levels in patients with core binding factor AML (CBF-AML) and cytogenetically normal AML (CN-AML).[15] To find out whether *MLL5* expression levels affect decitabine response and DNA methylation in leukemia, we initiated the present study with decitabine-treated AML patients as well as transformed loss-of-Mll5 mouse bone marrow cells. Our study addresses the impact of MLL5 expression on outcome of decitabine-treated patients and establishes a link between *MLL5* activity and DNA methylation levels.

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**These authors contributed equally to this work. †These authors contributed equally to this work.*
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Correspondence: heuser.michael@mh-hannover.de

Methods

Patients with decitabine administration

This study included 57 patients (aged >60 years) with *de novo* or secondary AML (following MDS or treatment-related AML) who were treated in trial 00331 (registration n. DRKS00000069) with 135 mg/m^2 total decitabine infused intravenously over 72 h every six weeks[16] or who received 20 mg/m^2 per day (Days 1-5) every four weeks. Patients were included in the present study if RNA was available and if the sample contained at least 30% blasts (median 55%). Fifty samples (88%) were from bone marrow, 7 (12%) were from peripheral blood. Written informed consent was obtained according to the Declaration of Helsinki, and the study was approved by the local review boards of the participating centers.

Quantification of MLL5 *transcript levels*

MLL5 expression levels were quantified using the TaqMan Gene Expression Assay (Assay ID: Hs00218773_m1, Applied Biosystems, Darmstadt, Germany) and the ABL FusionQuant Standard Kit as an endogenous control (Ipsogen, Marseille, France). A detailed description of the procedures can be found in the *Online Supplementary Appendix.*

Retroviral infection of murine bone marrow cells

Primary mouse bone marrow cells were harvested from 129S6/SvEv mice with wild-type or homozygous loss of Mll5 and stimulated as previously described.[13] MSCV-HOXA9-PGKneomycin and MSCV-MEIS1-IRESYFP vectors were used for generating recombinant ecotropic retrovirus-producing GP+E86 cells. Pre-stimulated bone marrow cells were co-cultured with irradiated GP+E86 cells for 48 h and were selected by Geneticin (Gibco, Darmstadt, Germany) or were sorted for yellow fluorescent protein (YFP) expression.

Decitabine treatment in vitro

Decitabine (5-Aza-2′-deoxycytidine; Sigma-Aldrich, Seelze, Germany) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich). Cells were treated with DMSO or decitabine every 24 h for three times and at 72 h were harvested for analysis. As solvent control, DMSO was added to a final concentration of 0.1%.

Methylated DNA immunoprecipitation coupled with microarray (MeDIP-chip)

Methylated DNA immunoprecipitation coupled with microarray (MeDIP) was performed with genomic DNA from cells untreated or treated with 20 nM decitabine for three days using the MagMeDIP kit (Diagenode, Liège, Belgium). MeDIP-enriched DNA from 3 independently treated samples were amplified with GenomePlex Complete Whole Genome Amplification (WGA) Kit (Sigma-Aldrich) and pooled for the hybridization on Agilent custom mouse promoter microarrays. The primary data are available at Gene Expression Omnibus (GEO) with accession number GSE52199. Details about MeDIP-chip preparation and data processing can be found in the *Online Supplementary Appendix.*

Correlation of DNA methylation and gene expression levels in AML patients according to MLL5 *expression*

Details about the clinical profiles, assay description and data processing were previously described.[17-19] Details of the bioinformatic analysis can be found in the *Online Supplementary Methods.*

Further details of methods are described in the *Online Supplementary Appendix.*

Statistical analysis

All statistical analyses were performed with the GraphPad Prism 5 Software (Statcon, Witzenhausen, Germany) or the software package SPSS v. 20.0 (SPSS, Chicago, USA). Two-tailed $P<0.05$ was considered significant. Details of the statistical analysis can be found in the *Online Supplementary Appendix.*

Results

Prognostic impact of MLL5 *expression levels in AML patients treated with decitabine*

MLL5 expression was quantified in 57 elderly patients with newly diagnosed AML who received decitabine as first-line treatment. Relative *MLL5* transcript levels ranged from 1.56 to 61.77 expression values of *MLL5/ABL*. Patients were dichotomized into high *MLL5* expressing patients and low *MLL5* expressing patients at the median level of *MLL5* expression (9.21 expression values of *MLL5/ABL*). There were no significant differences between patients with high and low *MLL5* expression levels with respect to age, sex, FAB subtype, blast count in peripheral blood or bone marrow, type of AML, additional all-trans retinoic acid (ATRA) treatment, hemoglobin, platelet count, lactate dehydrogenase (LDH), Eastern Cooperative Oncology Group (ECOG) performance status, cytogenetic risk group, *NPM1, FLT3*, or *DNMT3A* mutation status, or best response (*Online Supplementary Table S1*). In agreement with our previous study, high *MLL5* expression predicted longer overall survival (OS) (median 292 *vs.* 167 days; P=0.026) (Figure 1A). None of the other parameters, such as age (P=0.37), antecedent MDS (P=0.97), ECOG performance status (P=0.24), WBC count (P=0.52), cytogenetic risk (P=0.49), *NPM1* mutation status (P=0.22), or presence of FLT3-ITD (P=0.85) were associated with OS. However, patients with mutated *DNMT3A* had significantly shorter OS than patients with wild-type *DNMT3A* (P=0.001) (*Online Supplementary Figure S1*). In bivariate analysis, only *MLL5* expression levels and *DNMT3A* mutation status independently predicted OS (P=0.046 and P=0.01, respectively) (Table 1).

To evaluate whether the treatment effect of decitabine was associated with *MLL5* expression, we separated the patients into a group with short decitabine exposure (1 or 2 courses) and a group with long decitabine exposure (3 or more courses). The latter group included all patients with stable disease or better response (complete response, CR; partial response, PR; or antileukemic effect, ALE, defined as a greater than 25% reduction of bone marrow blasts relative to the initial blast percentage but not enough to fulfill the criteria for a partial remission) after two courses of decitabine and thus represents all patients with potential benefit from decitabine. Median OS was similarly short in patients with high or low *MLL5* expression in the group with short decitabine exposure (median 90 *vs.* 50 days; P=0.802) (Figure 1B). In patients with long decitabine exposure, high *MLL5* expression levels predicted significantly improved OS compared to patients with low *MLL5* expression (median 468 *vs.* 243 days; P=0.012) (Figure 1C). To identify potential confounding factors, we compared patients' characteristics in the group with long decitabine exposure between high and low *MLL5* expressing patients. Variables were similarly distributed between patients with high or low *MLL5* expression levels except a trend for more frequent ATRA treatment in patients with low *MLL5* expression (*Online Supplementary Table S2*). In addition, bivariate analysis including *MLL5* expression

levels and cytogenetic risk identified high *MLL5* expression as independent favorable marker, while high cytogenetic risk was not significant (*Online Supplementary Table S3*). We did not include *DNMT3A* mutation status in this analysis as there was only one patient with *DNMT3A* mutation in this cohort. In human cell lines *MLL5* mRNA expression correlated with protein expression (*Online Supplementary Figure S2)*. These data suggested that decitabine-responsive patients in the *MLL5* high-expressing group benefit more from decitabine than patients in the *MLL5* low-expressing group and pointed to a potential biological link between MLL5 and decitabine response.

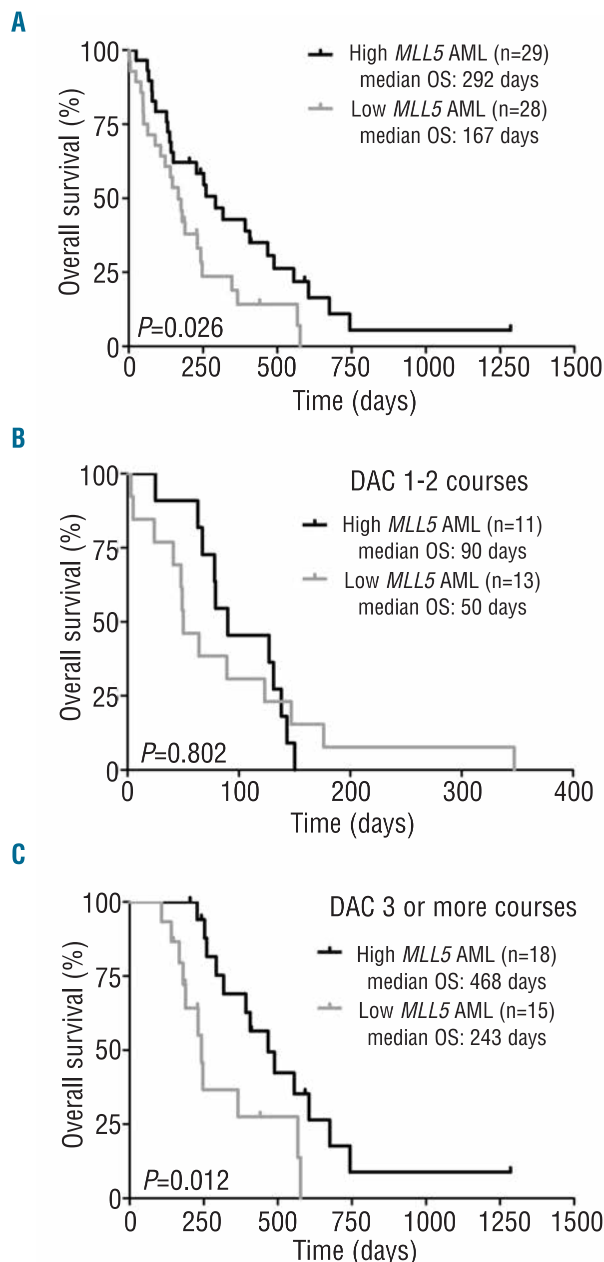


Figure 1. **Prognostic impact of *MLL5* expression in AML patients treated with decitabine (DAC). (A to C) Overall survival (OS) of all patients treated with DAC (irrespective of treatment courses) (A), OS of patients who received 1-2 courses of DAC (B), and OS of patients who received 3 or more courses of DAC (C), according to high *versus* low *MLL5* expression levels.**

To see if loss of chromosome 7 or 7q, where *MLL5* is located, influences OS of patients treated with decitabine as well as *MLL5* expression, 49 patients were selected for whom detailed cytogenetic information was available for the comparison of OS and *MLL5* mRNA expression between 37 patients with diploid chromosome 7 (chr7) and 12 patients with chr7 abnormalities including monosomy 7 (-7) or deletion of 7q (del(7q)). OS was similar between patients with diploid chr7 and patients with -7 or del(7q) (*P*=0.63) (*Online Supplementary Figure S3A*). This was also true when the analysis was restricted to patients who received at least three courses of decitabine (*P*=0.36) (*Online Supplementary Figure S3B*). *MLL5* expression levels were slightly higher in patients with diploid chr7 compared to patients with -7 or del(7q) (mean 13.2 *vs.* 8.2; *P*=0.16) (*Online Supplementary Figure S3C*).

Immortalized Mll5 wild-type bone marrow cells are more sensitive to decitabine treatment than Mll5 null cells

To further evaluate whether *MLL5* expression determines decitabine sensitivity, we generated HOXA9-transformed[20] mouse bone marrow cells with Mll5 wild-type (*Mll5*$^{+/+}$ HOXA9) or knockout (*Mll5*$^{-/-}$ HOXA9) background via retroviral gene transfer of HOXA9. Decitabine exerts antileukemic efficacy at low doses *in vitro*[21-23] and in the clinical setting.[24-26] We therefore treated the cells with decitabine for three days comparing low-dose (10nM) and high-dose (100nM) treatments. *Mll5*$^{+/+}$ cells were more sensitive to decitabine than *Mll5*$^{-/-}$ cells at low doses of decitabine, whereas viability was reduced equally effectively in *Mll5*$^{+/+}$ and *Mll5*$^{-/-}$ cells at 100 nM decitabine treatment (Figure 2A).

Expression of the myeloid differentiation marker CD11b increased more in *Mll5*$^{+/+}$ than in *Mll5*$^{-/-}$ cells upon either low-dose or high-dose decitabine exposure (Figure 2B). The colony number in CFC assays decreased by 60% upon low-dose decitabine exposure in *Mll5*$^{+/+}$ cells, but remained unchanged in *Mll5*$^{-/-}$ cells compared to solvent treated control (Figure 2C). Upon higher dose decitabine treatment total colony numbers were reduced and colonies appeared smaller in size in *Mll5*$^{+/+}$ than in *Mll5*$^{-/-}$ cells (Figure 2C and D).

Considering the role of Mll5 as a cell cycle regulator, cell cycle profiles were compared in HOXA9 cells upon decitabine exposure when Mll5 was present or absent. Decitabine increased the proportion of cells in G0/G1 phase as well as decreased the proportion of cells in both

Table 1. **Univariate and multivariate analysis for OS in all patients.**

	OS Univariate analysis			OS Multivariate analysis		
	HR	95% CI	*P*	HR	95% CI	*P*
MLL5 high *vs.* low	0.51	0.28-0.93	0.029	0.53	0.29-0.99	0.046
DNMT3A mutation status mutated *vs.* wildtype	4.46	1.65-12.09	0.003	3.80	1.38-10.49	0.01

Hazard ratios greater than 1 indicate an increased risk of an event for the first category listed. OS: overall survival; HR: hazard ratio; CI: confidence interval; ECOG: performance status of the Eastern Cooperative Oncology Group.

S and G2/M phases in a dose-dependent manner (Figure 2E). However, there was no significant difference in this effect between *Mll5*$^{+/+}$ and *Mll5*$^{-/-}$ cells. In addition, no difference in the rate of apoptosis was observed between *Mll5*$^{+/+}$ and *Mll5*$^{-/-}$ cells upon low- or high-dose decitabine treatment (Figure 2F). In summary, these data suggested that the presence of Mll5 sensitizes HOXA9-transformed cells to decitabine-induced cell differentiation and its anti-proliferative effects, especially at low doses of decitabine.

It remains an interesting question whether MLL5 may regulate *HOXA9* itself, as MLL1 does.[27] We measured *HOXA9* expression in 35 samples and correlated expression levels to *MLL5*. There was no correlation (R= -0.18) (*Online Supplementary Figure S4A*). *MLL5* and *HOXA9* expression was also evaluated in a published AML gene expression profiling data set with 241 AML patients.[19] In these patients, again no clear correlation was observed (R= 0.05) (*Online Supplementary Figure S4B*).

Leukemic Mll5 wild-type cells exhibit higher global DNA methylation levels in promoter regions compared to Mll5 null cells

In addition to the HOXA9-immortalized mouse bone marrow cells, Mll5-associated decitabine sensitivity was also investigated in a murine leukemia cell model, which was generated by co-transfer of HOXA9 and MEIS1[28] into Mll5 wild-type or knockout mouse bone marrow cells. Both cell lines showed a gradual decrease in cell proliferation upon increasing decitabine concentration (Figure 3A). However, *Mll5*$^{+/+}$ HOXA9/MEIS1 cells were significantly more sensitive to decitabine as demonstrated by the reduced cell viability upon decitabine exposure. In addition, treatment with 20 nM decitabine significantly increased Gr-1/CD11b expression in Mll5 wild-type cells compared to knockout cells (Figure 3B).

To gain insight into the methylation changes associated with decitabine sensitivity, we performed methylated DNA immunoprecipitation combined with hybridization to mouse promoter microarrays (MeDIP-chip)[29] in *Mll5*$^{+/+}$ HOXA9/MEIS1 and *Mll5*$^{-/-}$ HOXA9/MEIS1 leukemic cells, untreated or treated with 3-day exposure of 20 nM decitabine. DNA methylation at promoter regions covered by each probe was quantified by the $\log_2$ ratio of fluorescence intensity of immunoprecipitated (IP) DNA compared to input DNA. The number of methylated probes was compared between untreated Mll5 wild-type and knockout HOXA9/MEIS1 cells. The number of methylated probes in Mll5 wild-type cells was significantly higher than in Mll5 knockout cells at different methylation cut-offs (where increasing $\log_2$ ratios indicate increased methylation) (Figure 3C). Among all probes, 34% were methylated with a $\log_2$ ratio over 0.5 in *Mll5*$^{+/+}$ HOXA9/MEIS1 cells, in comparison with 25% of probes that were methylated in *Mll5*$^{-/-}$ HOXA9/MEIS1 cells (Figure 3D). In summary, promoter methylation was more frequent in Mll5 wild-type cells compared to Mll5 null cells.

Some of the most differentially methylated promoter regions were promoter regions of *Rpl37, Stx6, Surf1* and *Cebpa*, which were methylated in *Mll5*$^{+/+}$ HOXA9/MEIS1

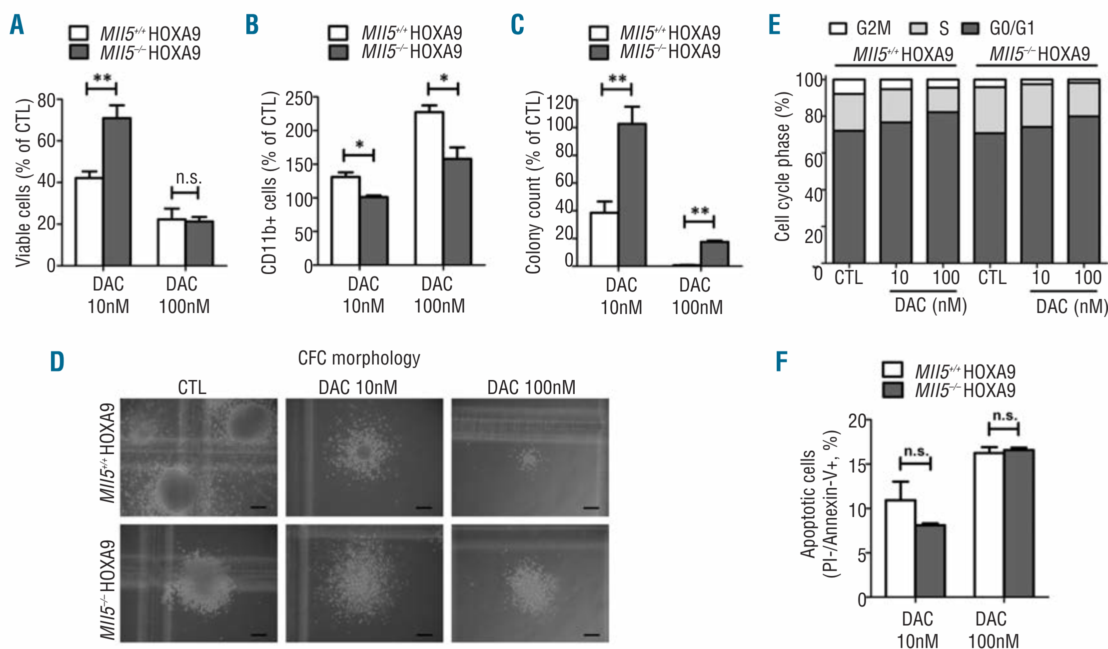


Figure 2. **Immortalized Mll5 wild-type mouse bone marrow cells are more sensitive to decitabine (DAC) treatment than Mll5 null cells. (A) Cell viability upon low- or high-dose DAC treatment relative to DMSO solvent control (CTL) treatment. 1 x 10^5 cells were plated in duplicate (mean ± SEM, n=5). (B) DAC-induced cell differentiation by immunophenotyping CD11b expression (mean ± SEM, n=3). (C) Colony yield from CFC assays performed with DAC-treated cells in comparison to CTL-treated cells (mean ± SEM, n=3). (D) Morphology of representative colonies from 3 independent CFC assays. Scale bars represent 100µm. (E) Cell cycle profiles upon CTL or DAC treatment (mean, n=3). (F) Frequency of apoptotic cells represented by PI negative and Annexin-V positive staining upon DAC treatment and normalized to CTL treatment (mean ± SEM, n=3). n.s.: not significant; *P < 0.05; **P < 0.01.**

cells but non-methylated in *Mll5*$^{-/-}$ HOXA9/MEIS1 cells, while *Lrcc45* was non-methylated in Mll5 wild-type cells but methylated in Mll5 null cells (Figure 3E). These methylation differences were confirmed by MeDIP-PCR (Figure 3F). To further confirm the differentially methylated targets revealed by MeDIP-chip, bisulfite sequencing was performed on selected genomic regions of these targets. Among them, *Rpl37, Stx6, Surf1* showed more methylated CpGs (92.5% *vs.* 75.0%, 6.5% *vs.* 2.5%, 11.3% *vs.* 5.0%, respectively), while the negative control Lrcc45 showed

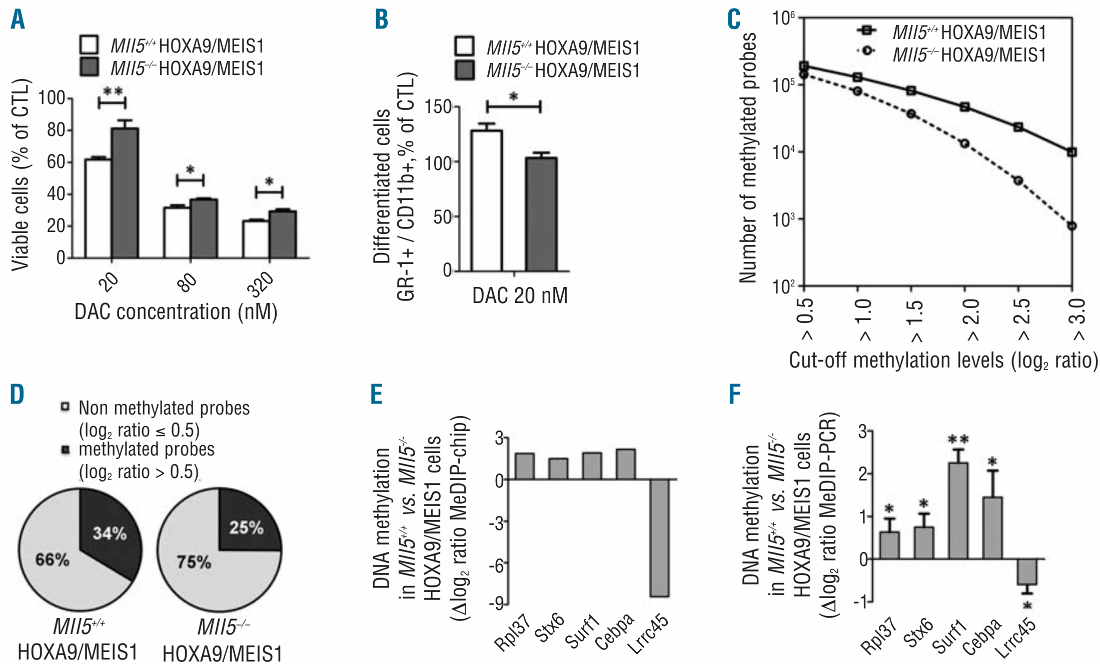
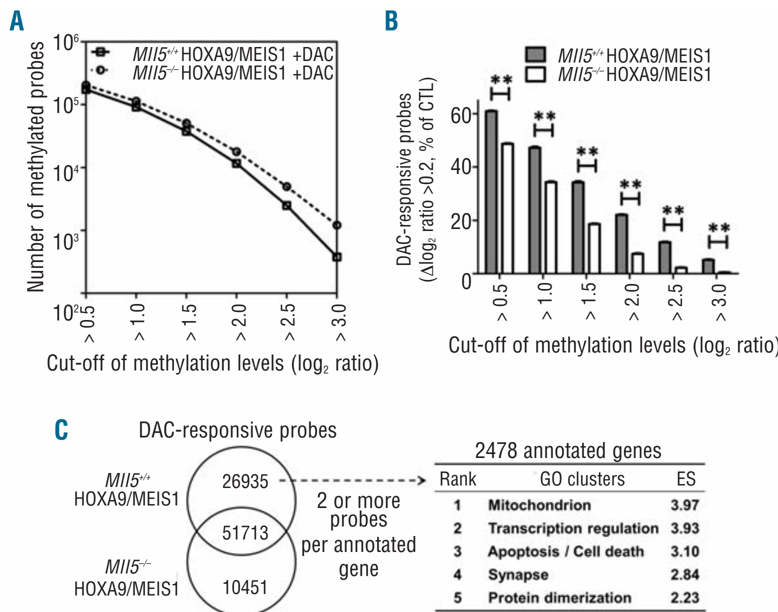


Figure 3. Leukemic Mll5 wild-type cells exhibit higher global DNA methylation levels in promoter regions compared to Mll5 null cells. (A) Cell viability upon low-, medium- or high-dose DAC treatment relative to CTL treatment. 2 x 10^4 cells were plated in duplicate (mean ± SEM, n=3). (B) DAC-induced cell differentiation by immunophenotyping Gr-1/CD11b expression (mean ± SEM, n=3). (C) Promoter DNA-methylation represented by the number of methylated array probes at different methylation-defining cut offs of MeDIP-chip assay. The log$_2$ ratio of immunoprecipitated DNA compared to input DNA at 0.5 was chosen as the threshold to discriminate methylated from non-methylated probes for downstream analyses. (D) Percentage of methylated and non-methylated probes in Mll5 wild-type and null cells. (E) Differential methylation of selected gene promoters based on MeDIP-chip log$_2$ ratio difference (Δlog$_2$ ratio). (F) Validation of differentially methylated gene promoters by MeDIP-PCR. Methylation difference was calculated as log$_2$-transformed ratio of enrichment of methylated DNA of Mll5 wild-type compared to null cells (log$_2$ ratio) (mean ± SEM, n=3).



Rank	GO clusters	ES
1	Mitochondrion	3.97
2	Transcription regulation	3.93
3	Apoptosis / Cell death	3.10
4	Synapse	2.84
5	Protein dimerization	2.23

Figure 4. Leukemic Mll5 wild-type cells are more responsive to DAC-induced promoter demethylation compared to Mll5 null cells. (A) Promoter DNA-methylation analysis of cells upon 20nM DAC exposure. (B) Percentage of DAC-responsive probes in Mll5 wildtype *versus* null cells. Methylation values of each probe were compared between untreated and 20nM DAC-treated cells. Among all the methylated probes (log$_2$ ratio > 0.5) in untreated sample, those probes with a decrease of the log$_2$ ratio of more than 0.2 (Δlog$_2$ ratio > 0.2) upon DAC treatment were defined as DAC-responsive probes. (C) Functional annotation of genes that were differentially demethylated upon DAC treatment in Mll5 wild-type cells. Mll5 wild-type specific DAC-responsive probes were matched to their closest gene if 2 or more probes were DAC responsive. **P < 0.01.

less methylated CpGs (0.2% *vs.* 8.3%) in Mll5 wild-type compared to Mll5 null cells, respectively (*Online Supplementary Figure S5*). CEBPA is a well-studied transcription factor required for granulocytic differentiation.[30] Higher levels of DNA methylation were confirmed by bisulfite sequencing in Mll5 wild-type compared to knockout cells (15.6% *vs.* 11.7%) (*Online Supplementary Figure S6A*). When treated with 20 nM decitabine, both Mll5 wild-type and null cells had less methylated CpGs at this locus compared to solvent treated cells (15.6% *vs.* 6.25, 11.7% *vs.* 5.5%, respectively) (*Online Supplementary Figure S6A*). In addition, decitabine-induced demethylation was associated with an increase in *Cebpa* mRNA expression, suggesting that *Cebpa* is regulated through DNA methylation (*Online Supplementary Figure S6B*).

Leukemic Mll5 wild-type cells are more responsive to decitabine-induced promoter demethylation compared to Mll5 null cells

To evaluate the effect of decitabine on methylation of promoter regions, we first compared the number of methylated probes in Mll5 wild-type and knockout HOXA9/MEIS1 cells upon decitabine exposure. Opposite to the untreated samples, the number of methylated probes in decitabine-treated Mll5 wild-type cells was significantly lower than in decitabine-treated Mll5 null cells at different cut offs of methylation (Figure 4A). Further analysis was performed on the probes that were methylated ($\log_2$ ratio >0.5) in the untreated samples of either Mll5 wildtype or knockout but became demethylated in the decitabine-treated samples with a decrease in the $\log_2$ ratio of more than 0.2 ($\Delta\log_2$ ratio >0.2), and these corresponding probes were defined as decitabine-responsive probes. The number of decitabine-responsive probes was significantly higher in Mll5 wild-type cells compared to Mll5 null cells independent of the $\log_2$ ratio chosen for the definition of a methylated probe (e.g. wildtype 60.8% *vs.* knockout 48.7%, for $\log_2$ ratio >0.5) (Figure 4B). Similar results were found when decitabine-induced demethylation was defined with other more stringent criteria ($\Delta\log_2$ ratio >0.4 or 0.8) (*Online Supplementary Figure S7*). We next generated a list of genes with decitabine-responsive promoter methylation (*Online Supplementary Table S4*) and performed gene ontology (GO) analysis to determine the functional relevance of the decitabine responsive gene sets. The top-ranked GO clusters for decitabine-responsive genes in Mll5 wild-type cells were "mitochondrion", "transcription regulation", and "apoptosis / cell death"

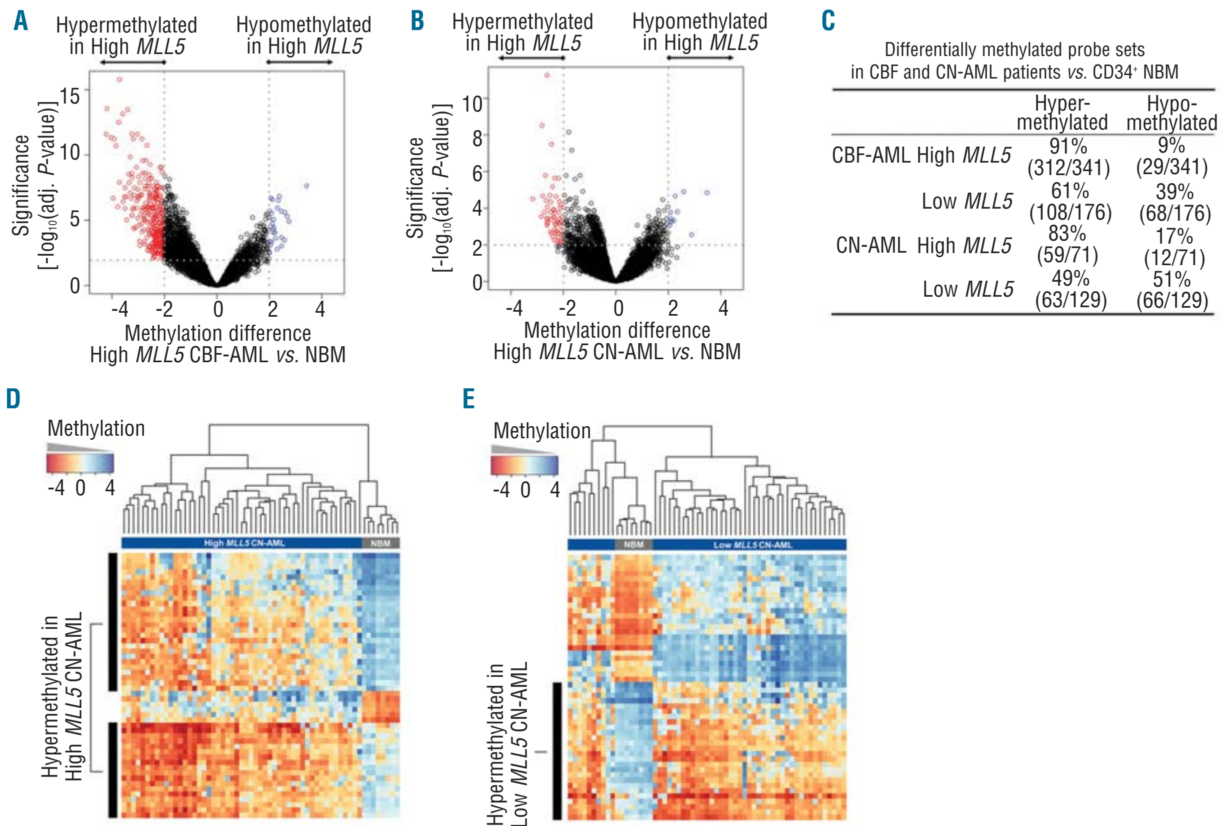


Figure 5. High *MLL5* expression is associated with increased DNA methylation in promoter regions in patients with CBF-AML and CN-AML. (A and B) Volcano plot of methylation difference of *MLL5* high-expressing CBF-AML patients (n=18) (A) and CN-AML (n=51) (B) patients *versus* CD34+ normal bone marrows (NBM) (n=8). Hyper- or hypomethylated probe sets were marked by red or blue circles, respectively. (C) Number and percentage of hyper- or hypomethylated probe sets in patients *versus* CD34+ NBM. (D and E) Heatmap with supervised hierarchical clustering showing the 50 most differentially methylated probe sets of CN-AML patients with high MLL5 (n=51) (D) and low MLL5 (n=51) (E) expression *versus* CD34+ NBM (n=8). Black bars indicate the hypermethylated probes in patients compared to CD34+ NBM.

(Figure 4C), suggesting that Mll5 plays a regulatory role in these biological processes through epigenetic mechanisms involving DNA methylation. Enriched GO clusters for probes only responsive in Mll5 null cells are listed in *Online Supplementary Figure S8A*. The number of responsive genes was much lower than in Mll5 wild-type cells (277 *vs.* 2478 genes, respectively). The enrichment score for top-listed GO clusters was quite low in Mll5 null compared to Mll5 wild-type cells (*Online Supplementary Figure S8A*). A total of 343 genes were responsive to decitabine in both Mll5 wild-type and knockout cells and enriched GO clusters were "transcription regulation", "neuron differentiation" and "NLI-interacting factor" (*Online Supplementary Figure S8B*). Taken together, promoter hypomethylation occurred more frequently upon decitabine treatment in leukemic Mll5 wild-type cells than in Mll5 null cells.

High MLL5 *expression is associated with increased DNA methylation in promoter regions in patients with CBF-AML and CN-AML*

We have previously reported the favorable prognostic impact of *MLL5* expression levels in two subgroups of AML patients with t(8;21) or inv(16) chromosomal aberrations (CBF-AML) and with normal karyotype (CN-AML).[15] We identified 54 patients with CBF-AML and 153 patients with CN-AML in a previously published cohort of 344 patients, for whom global gene expression and promoter methylation data are available.[17-19] *MLL5* expression levels of individual patients are represented by probe *223189_x_at* and *223190_s_at* from gene expression arrays (*Online Supplementary Figure S9A*). Expression values from the two probes correlated well with each other (*Online Supplementary Figure S9B and C*). Promoter methylation was compared between patients with high or low *MLL5* expression using normal $CD34^+$ bone marrow cells ($CD34^+$ NBM) as baseline (Figure 5A and B).[31] In total, 341 and 71 probe sets were differentially methylated in CBF- and CN-AML patients with high *MLL5* expression, respectively, with absolute difference in methylation more than 2 and adjusted *P* less than 0.01 (Figure 5C). Among them, 91% (312 of 341) and 83% (59 of 71) were significantly hypermethylated in *MLL5* high-expressing CBF- and CN-AML patients, respectively, whereas only 9% (29 of 341) and 17% (12 of 71) were hypomethylated. In contrast, 61% (108 of 176) and 49% (63 of 129) of the differentially methylated probe sets were significantly hypermethylated in CBF- and CN-AML patients with low *MLL5* expression, respectively, whereas 39% (68 of 176) and 51% (66 of 129) were hypomethylated (*Online Supplementary Figure S10*). Supervised hierarchical clustering of the 50 most differentially methylated probe sets in CN-AML patients with either high or low *MLL5* expression showed that the majority of probe sets were hypermethylated in MLL5 high-expressing CN-AML patients, while only 50% of the probe sets were hypermethylated in CN-AML patients with low *MLL5* expression (Figure 5D and E). Similarly, clustering analysis also revealed more hypermethylated probe sets in CBF-AML patients with high *MLL5* expression than patients with low *MLL5* expression (*Online Supplementary Figure S11*). Collectively, a positive correlation between MLL5 expression levels with global DNA methylation was observed in our study, further supporting the correlation of Mll5 expression with global promoter methylation found in murine leukemia cells.

Discussion

In the present analysis, we observed that decitabine-responsive patients with high *MLL5* expression levels had an improved overall survival compared to patients with low *MLL5* expression levels, while patients not responding to decitabine during the first two courses had a poor outcome irrespective of *MLL5* expression levels. We transformed Mll5 wild-type and null mouse bone marrow cells with the established oncogenes HOXA9 and MEIS1[28] to study the impact of Mll5 on decitabine response in leukemic cells. Loss of Mll5 was associated with resistance to low-dose decitabine, lower methylation levels of global promoters, fewer methylation changes upon decitabine treatment, and reduced methylation of global promoters was found in *MLL5* low-expressing AML patients, supporting our clinical observation and suggesting a mechanistic role of *MLL5* in mediating the effects of decitabine.

A recent study evaluated the genome-wide distribution of mouse full-length Mll5 protein in the promoter regions in C2C12 mouse myoblast cells.[32] Interestingly, Mll5 preferentially targeted the regions downstream of transcription start sites (TSSs), and its binding was exclusively enriched at CpG islands but not at the flanking regions, suggesting a link between Mll5 and the regulation of DNA methylation. Mll5 may affect DNA methylation through TET proteins, a family of hydroxymethylases that regulate 5-methyl cytosine hydroxylation and thus govern DNA methylation by converting 5-methylcytosine to 5-hydroxymethylcytosine.[33] TET2 and TET3 directly interact with O-GlcNAc transferase (OGT) and co-localize on chromatin.[34] The cell cycle regulator 'host cell factor' (HCF-1) is a specific GlcNAcylation target of TET2/3-OGT and associates with TET2/3. Interestingly, the MLL5 protein interacts with HCF-1 and largely co-localizes with HCF-1 in the nucleus.[35] Therefore, MLL5 might be involved in a complex associated with TET2/3 through interaction with HCF-1/OGT, which might influence the level of DNA methylation.

Global DNA methylation was higher in transformed Mll5 wild-type cells, while upon decitabine treatment it decreased to lower levels compared to Mll5 null cells. Our data suggested a link between higher DNA methylation levels prior to decitabine treatment and better sensitivity to HMAs. In gastric cancers, Zouridis *et al*.[36] reported the association of the CpG island methylator phenotype (CIMP) with better sensitivity to DNA methylation inhibitors. CIMP tumor type was identified in 68 of 203 primary gastric tumors and 7 of 17 gastric cancer cell lines, which displayed a unique feature of widespread hypermethylation at a global level compared to non-CIMP tumor type. Interestingly, CIMP cell lines displayed significantly better response to decitabine-induced reduction of *in vitro* proliferation and decrease of tumor growth in a murine xenograft model compared to non-CIMP cell lines. Global methylation data from decitabine-treated patients confirmed that differential demethylation occurs in decitabine responding patients, while non-responding patients have no differentially methylated regions.[37]

To date, only a few genetic markers are known that predict treatment response to hypomethylating agents. *TET2* mutations were reported as an independent predictor of a higher overall response rate to azacitidine, the ribonucleoside form of decitabine, in patients with MDS and low blast count AML.[38] In addition, *DNMT3A* mutations or

lower *DNMT3A* expression have been associated with better response to hypomethylating agents in AML patients,[39] in contrast to our findings regarding *DNMT3A* mutation status, which predicted a poor outcome of decitabine-treated patients. High *MLL5* levels predicted improved outcome with prolonged treatment rather than response to decitabine.

Some patients with low *MLL5* levels also responded to a longer exposure of decitabine, thus it is interesting to know if base-line *MLL5* promoter methylation in these patients was reversed by decitabine and hence *MLL5* expression was converted to higher levels. We correlated *MLL5* promoter DNA methylation with *MLL5* transcript expression levels in a published data set including 344 AML patients.[17-19] Only 8 patients had hypermethylated *MLL5* promoters, and they did not show decreased expression of *MLL5* compared to other patients (*Online Supplementary Figure S12*), suggesting that the DNA methylation status of the *MLL5* promoter does not correlate with *MLL5* expression.

In the current study, the transformed Mll5 wild-type cells were more sensitive to decitabine, while we previously found a preferential loss of normal HSCs in Mll5 null cells treated with decitabine.[13] The major difference between these studies is that we looked at stem cells in the first study, while the leukemic cells correspond to myeloid progenitor cells.[40] None of the AML patients went into long-term remission upon decitabine treatment, suggesting that decitabine primarily inhibited the mature AML cells but not the leukemic stem cell. Thus, *MLL5* may have different effects on decitabine response at different stages of hematopoiesis and between normal and leukemic cells. As Mll5 expression increases with maturation along the myeloid lineage,[13] AML cells with high *MLL5* expression may indicate leukemias that are arrested at a later stage of differentiation and respond better to chemotherapy or HMAs. The current study suggested a similar role of high *MLL5* expression as an independent favorable prognostic marker in multivariate analysis, as in our previous report.[15] However, we do not see a prognostic effect of *MLL5* in patients who received less than three courses of decitabine. Therefore, we believe that the prognostic effect of *MLL5* is dependent at least on some form of drug treatment.

Here we describe the role of *MLL5* expression as a biomarker for prolonged survival in AML patients treated with decitabine and for improved decitabine response in murine leukemic cells. Murine leukemic cells deficient in Mll5 had lower levels of DNA methylation and were less responsive to the biological and demethylating effects of decitabine. Human AML cells with high *MLL5* levels had higher DNA methylation levels. Overall, high *MLL5* expression predicted improved outcome in AML patients. Therefore, *MLL5* expression may be useful to predict clinical outcome of AML patients treated with hypomethylating agents.

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Authorship and Disclosures

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References

1. Lowenberg B. Acute myeloid leukemia: the challenge of capturing disease variety. Hematology Am Soc Hematol Educ Program. 2008;1:1-11.
2. Shih AH, Abdel-Wahab O, Patel JP, Levine RL. The role of mutations in epigenetic regulators in myeloid malignancies. Nat Rev Cancer. 2012;12(9):599-612.
3. Burnett A, Wetzler M, Lowenberg B. Therapeutic advances in acute myeloid leukemia. J Clin Oncol. 2011;29(5):487-94.
4. Lubbert M, Suciu S, Baila L, Ruter BH, Platzbecker U, Giagounidis A, et al. Low-dose decitabine versus best supportive care in elderly patients with intermediate- or high-risk myelodysplastic syndrome (MDS) ineligible for intensive chemotherapy: final results of the randomized phase III study of the European Organisation for Research and Treatment of Cancer Leukemia Group and the German MDS Study Group. J Clin Oncol. 2011;29(15):1987-96.
5. Kantarjian HM, Thomas XG, Dmoszynska A, Wierzbowska A, Mazur G, Mayer J, et al. Multicenter, randomized, open-label, phase III trial of decitabine versus patient choice, with physician advice, of either supportive care or low-dose cytarabine for the treatment of older patients with newly diagnosed acute myeloid leukemia. J Clin Oncol. 2012;30(21):2670-7.
6. Emerling BM, Bonifas J, Kratz CP, Donovan S, Taylor BR, Green ED, et al. MLL5, a homolog of Drosophila trithorax located within a segment of chromosome band 7q22 implicated in myeloid leukemia. Oncogene. 2002;21(31):4849-54.
7. Madan V, Madan B, Brykczynska U, Zilbermann F, Hogeveen K, Dohner K, et al. Impaired function of primitive hematopoietic cells in mice lacking the Mixed-Lineage-Leukemia homolog MLL5. Blood. 2009;113(7):1444-54.
8. Sebastian S, Sreenivas P, Sambasivan R, Cheedipudi S, Kandalla P, Pavlath GK, et al. MLL5, a trithorax homolog, indirectly regulates H3K4 methylation, represses cyclin A2 expression, and promotes myogenic differentiation. Proc Natl Acad Sci USA. 2009;106(12):4719-24.
9. Cheng F, Liu J, Zhou SH, Wang XN, Chew JF, Deng LW. RNA interference against mixed lineage leukemia 5 resulted in cell cycle arrest. Int J Biochem Cell Biol. 2008;40(11):2472-81.
10. Deng LW, Chiu I, Strominger JL. MLL 5 protein forms intranuclear foci, and overexpression inhibits cell cycle progression. Proc Natl Acad Sci USA. 2004;101(3):757-62.
11. Liu J, Wang XN, Cheng F, Liou YC, Deng LW. Phosphorylation of mixed lineage leukemia 5 by CDC2 affects its cellular distribution and is required for mitotic entry. J Biol Chem. 2010;285(27):20904-14.
12. Sambasivan R, Pavlath GK, Dhawan J. A gene-trap strategy identifies quiescence-induced genes in synchronized myoblasts. J Biosci. 2008;33(1):27-44.
13. Heuser M, Yap DB, Leung M, de Algara TR, Tafech A, McKinney S, et al. Loss of MLL5 results in pleiotropic hematopoietic defects, reduced neutrophil immune function, and extreme sensitivity to DNA demethylation. Blood. 2009;113(7):1432-43.
14. Zhang Y, Wong J, Klinger M, Tran MT, Shannon KM, Killeen N. MLL5 contributes to hematopoietic stem cell fitness and homeostasis. Blood. 2009;113(7):1455-63.
15. Damm F, Oberacker T, Thol F, Surdziel E, Wagner K, Chaturvedi A, et al. Prognostic importance of histone methyltransferase MLL5 expression in acute myeloid leukemia. J Clin Oncol. 2011;29(6):682-9.
16. Lubbert M, Ruter BH, Claus R, Schmoor C,

Schmid M, Germing U, et al. A multicenter phase II trial of decitabine as first-line treatment for older patients with acute myeloid leukemia judged unfit for induction chemotherapy. Haematologica. 2012;97(3): 393-401.

17. Valk PJ, Verhaak RG, Beijen MA, Erpelinck CA, Barjesteh van Waalwijk van Doorn-Khosrovani S, Boer JM, et al. Prognostically useful gene-expression profiles in acute myeloid leukemia. N Engl J Med. 2004;350(16):1617-28.
18. Figueroa ME, Lugthart S, Li Y, Erpelinck-Verschueren C, Deng X, Christos PJ, et al. DNA methylation signatures identify biologically distinct subtypes in acute myeloid leukemia. Cancer Cell. 2010;17(1):13-27.
19. Verhaak RG, Wouters BJ, Erpelinck CA, Abbas S, Beverloo HB, Lugthart S, et al. Prediction of molecular subtypes in acute myeloid leukemia based on gene expression profiling. Haematologica. 2009;94(1): 131-4.
20. Calvo KR, Sykes DB, Pasillas M, Kamps MP. Hoxa9 immortalizes a granulocyte-macrophage colony-stimulating factor-dependent promyelocyte capable of biphenotypic differentiation to neutrophils or macrophages, independent of enforced meis expression. Mol Cell Biol. 2000;20(9): 3274-85.
21. Hollenbach PW, Nguyen AN, Brady H, Williams M, Ning Y, Richard N, et al. A comparison of azacitidine and decitabine activities in acute myeloid leukemia cell lines. PLoS One. 2010;5(2):e9001.
22. Klco JM, Spencer DH, Lamprecht TL, Sarkaria SM, Wylie T, Magrini V, et al. Genomic impact of transient low-dose decitabine treatment on primary AML cells. Blood. 2013;121(9):1633-43.
23. Tsai HC, Li H, Van Neste L, Cai Y, Robert C, Rassool FV, et al. Transient low doses of DNA-demethylating agents exert durable antitumor effects on hematological and epithelial tumor cells. Cancer Cell. 2012;21(3):430-46.
24. Cashen AF, Shah AK, Todt L, Fisher N, DiPersio J. Pharmacokinetics of decitabine administered as a 3-h infusion to patients with acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS). Cancer Chemother Pharmacol. 2008;61(5):759-66.
25. Issa JP, Garcia-Manero G, Giles FJ, Mannari R, Thomas D, Faderl S, et al. Phase 1 study of low-dose prolonged exposure schedules of the hypomethylating agent 5-aza-2'-deoxycytidine (decitabine) in hematopoietic malignancies. Blood. 2004;103(5): 1635-40.
26. Kantarjian H, Oki Y, Garcia-Manero G, Huang X, O'Brien S, Cortes J, et al. Results of a randomized study of 3 schedules of low-dose decitabine in higher-risk myelodysplastic syndrome and chronic myelomonocytic leukemia. Blood. 2007;109(1):52-7.
27. Rozovskaia T, Feinstein E, Mor O, Foa R, Blechman J, Nakamura T, et al. Upregulation of Meis1 and HoxA9 in acute lymphocytic leukemias with the t(4 : 11) abnormality. Oncogene. 2001;20(7):874-8.
28. Kroon E, Krosl J, Thorsteinsdottir U, Baban S, Buchberg AM, Sauvageau G. Hoxa9 transforms primary bone marrow cells through specific collaboration with Meis1a but not Pbx1b. EMBO J. 1998;17 (13):3714-25.
29. Weber M, Davies JJ, Wittig D, Oakeley EJ, Haase M, Lam WL, et al. Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. Nat Genet. 2005;37(8):853-62.
30. Zhang DE, Zhang P, Wang ND, Hetherington CJ, Darlington GJ, Tenen DG. Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein alpha-deficient mice. Proc Natl Acad Sci USA. 1997;94(2):569-74.
31. Lugthart S, Figueroa ME, Bindels E, Skrabanek L, Valk PJ, Li Y, et al. Aberrant DNA hypermethylation signature in acute myeloid leukemia directed by EVI1. Blood. 2011;117(1):234-41.
32. Ali M, Rincon-Arano H, Zhao W, Rothbart SB, Tong Q, Parkhurst SM, et al. Molecular basis for chromatin binding and regulation of MLL5. Proc Natl Acad Sci USA. 2013;110 (28):11296-301.
33. Ko M, Huang Y, Jankowska AM, Pape UJ, Tahiliani M, Bandukwala HS, et al. Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2. Nature. 2010;468(7325):839-43.
34. Deplus R, Delatte B, Schwinn MK, Defrance M, Mendez J, Murphy N, et al. TET2 and TET3 regulate GlcNAcylation and H3K4 methylation through OGT and SET1/COMPASS. EMBO J. 2013;32(5): 645-55.
35. Zhou P, Wang Z, Yuan X, Zhou C, Liu L, Wan 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):17532-43.
36. Zouridis H, Deng N, Ivanova T, Zhu Y, Wong B, Huang D, et al. Methylation subtypes and large-scale epigenetic alterations in gastric cancer. Sci Transl Med. 2012;4(156):156ra40.
37. Yan P, Frankhouser D, Murphy M, Tam HH, Rodriguez B, Curfman J, et al. Genome-wide methylation profiling in decitabine-treated patients with acute myeloid leukemia. Blood. 2012;120(12): 2466-74.
38. Itzykson R, Kosmider O, Cluzeau T, Mansat-De Mas V, Dreyfus F, Beyne-Rauzy O, et al. Impact of TET2 mutations on response rate to azacitidine in myelodysplastic syndromes and low blast count acute myeloid leukemias. Leukemia. 2011;25(7):1147-52.
39. Metzeler KH, Walker A, Geyer S, Garzon R, Klisovic RB, Bloomfield CD, et al. DNMT3A mutations and response to the hypomethylating agent decitabine in acute myeloid leukemia. Leukemia. 2012;26(5): 1106-7.
40. Goardon N, Marchi E, Atzberger A, Quek L, Schuh A, Soneji S, et al. Coexistence of LMPP-like and GMP-like leukemia stem cells in acute myeloid leukemia. Cancer Cell. 2011;19(1):138-52.