Title: A Therapeutically relevant difference in leukemia and normal stem cell self-renewal

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Abstract:

Abnormal self-renewal and differentiation are defining features of acute myeloid leukemia (AML). Using models of hematopoiesis, we show that persistent self-renewal that ignores differentiation signals can be produced through two distinct pathways. One pathway prevents repression of stem cell genes, producing daughter cells that resemble parental stem cells despite differentiation inducing lineage-specifying factor or cytokines. Runx1 deficiency, a frequent abnormality in AML, demonstrates the second pathway, which allows cytokine/lineage-specifying factor mediated repression of stem cell genes but impairs subsequent activation of differentiation genes that terminate transit-amplification, producing self-renewal in daughter cells that do not resemble the parental stem cell. Use of this pathway by AML cells has therapeutic relevance: cell-fate regulation by the chromatin-modifying protein DNMT1 is differentiation-phase dependent. In the post lineage-commitment differentiation-phase at which AML cell self-renewal occurs, DNMT1 has an opposite role than in normal stem cells. Consequently, DNMT1 depletion, using a clinically applicable method, terminates AML cell self-renewal but increases normal stem cell self-renewal.

Introduction

Some cells in an acute myeloid leukemia (AML) clone self-renew in increased or unregulated fashion, driving clonal expansion. Characterization of the mechanisms by which AML cells self-renew could lead to treatment that selectively destroys AML cells. Current drug therapy mostly targets cellular components necessary for the health of both AML and normal dividing cells, causing significant toxicity often in return for only limited benefits.

In the hierarchy of hematopoietic cells, hematopoietic stem cells are cells that exhibit self-renewal and multi-lineage differentiation. Self-renewal is cell division that generates one or two daughter cells that resemble the parental stem cell. Differentiation is cell division that generates one or two daughter cells that express high levels of lineage-specifying factors, and divide symmetrically to produce daughter cells that are advanced to the next differentiation level. This process of exponential cell expansion, called transit-amplification, ends when daughter cells arrive at the terminal differentiation state. Since differentiating daughter cells eventually exit cell-cycle, self-renewal by the hematopoietic stem cell is necessary for clonal persistence.

One approach to understanding AML cell self-renewal is to relate it to pathways involved in normal stem cell function. One potential pathway is deregulation of hematopoietic stem cell self-renewal, which is usually linear rather than exponential . With this pathway, self-renewing AML cells can be expected to phenocopy normal hematopoietic stem cells, and utilize molecular mechanisms for self-renewal engaged by normal hematopoietic stem cells . Another potential pathway, proposed as a mechanism by which abnormalities of the *MLL* gene can lead to leukemia , is acquisition in non-stem cells (for example, lineage-committed progenitors) of abnormalities that aberrantly activate stem cell self-renewal pathways in the non-stem cell context. Here, a third set of pathways were examined. A defining feature of AML, which becomes quite obvious as clinically aggressive AML evolves from a preceding hematologic disorder known as myelodysplastic syndrome (MDS), is the accumulation of immature or differentiation-impaired hematopoietic cells (myeloblasts). This suggests that differentiation impairment and abnormal self-renewal could have a cause-effect relationship . We identified two distinct molecular pathways for producing self-renewal that ignores differentiation signals. Interestingly, only one of these

pathways widely operates in AML. Importantly, this pathway produces AML self-renewal in a differentiation-context distinct from that of normal stem cell self-renewal, such that common cellular components have an opposite role in cell-fate regulation in the two contexts. We demonstrate that one such component, the DNA methylating enzyme DNMT1, can be depleted using a clinically applicable method to concurrently increase normal stem cell and terminate AML cell self-renewal.

Results

Lineage-specific differentiation requires repression of stem cell genes prior to activation of terminal differentiation genes. Terminal hematopoietic differentiation depends on lineage-specifying transcription-factors such as PU.1, which is essential for producing macrophages. PU.1, like other lineage-specifying DNA binding factors, demonstrates both transcription repression and transcription activating functions, determined by interactions with either corepressors or coactivators (reviewed in). The murine PUER cell-line is derived from Pu.1 knock-out hematopoietic precursors which have been transduced with a retroviral vector to express Pu.1 fused to the estrogen-receptor. In cell-culture with mIL-3, PUER cells self-renew indefinitely. Addition of tamoxifen (OHT) to these cells (as an estrogen agonist) causes functional reintroduction of Pu.1 through translocation into the nucleus, and triggers terminal macrophage differentiation . In PUER cells induced to differentiate by OHT, serial analysis of gene-expression by real-time quantitative polymerase chain reaction (RQ-PCR) demonstrated an early repression of genes associated with stem cells (HoxB4, Bmi-1, c-Kit) that was followed by activation of genes associated with terminal differentiation (Mcsfr, Gmcsfr, F4/80) (**figure 1A**).

To determine if the initial repression of stem cell genes is necessary for lineage-specific differentiation, DNA methyltransferase 1 (Dnmt1) was knocked-down by lentiviral transduction of shRNA (PUER shDnmt1). Dnmt1 occupies a central and critical position in the chromatin modifying protein network that mediates transcription repression . In PUER shDnmt1 cells, Pu.1 mediated repression of stem cell associated genes was impaired and the cells continued to self-renew, implying that repression of stem cell genes is critical for initiation of differentiation (**figure 1B**).

To determine if the requirement for Dnmt1 activity is temporally confined to the initial phase in the differentiation-chronology, we used the United States Food and Drug Administration (FDA) approved drug decitabine to deplete Dnmt1 concurrent with Pu.1 activation or 6 hours after Pu.1. Decitabine is a cytosine analogue that is incorporated into the newly synthesized DNA strand during the S-phase of cell-cycle. Dnmt1 is depleted by covalent modification and degradation when it attempts to methylate the DNA-incorporated decitabine. Significant Dnmt1 depletion can be achieved with very small quantities of drug that do not cause measurable DNA damage, and which allow continued cell-division (**figure S1A-E**). Decitabine has no known effects other than depleting Dnmt1 and damaging DNA .

As expected, decitabine addition concurrent with Pu.1 prevented the repression of stem cell associated genes and maintained self-renewal. However, decitabine addition 6 hours later, after significant stem cell gene repression has already occurred, did not inhibit terminal differentiation (**figure 1C**).

Similar observations were seen using primary human CD34+ hematopoietic cells induced to terminal differentiation by the granulocyte differentiation promoting cytokine granulocyte colony-stimulating factor (G-CSF). Adding decitabine concurrent with G-CSF prevents stem cell gene repression and maintains self-renewal (**figure 1D, figure S1F**) (previously, we demonstrated that decitabine treatment prior to G-CSF maintains NOD-SCID repopulating activity of human CD34+ hematopoietic stem cells). However, adding decitabine 6 hours after G-CSF is too late to prevent stem cell gene repression, and differentiation gene expression and terminal differentiation can proceed (**figure 1D, figure S1F**).

Therefore, the consequences of DNMT1 depletion on cell-fate relate to the differentiation-chronology of sequential repression of key stem cell genes followed by activation of differentiation genes. DNMT1 depletion prior to or concurrent with a differentiation inducing stimulus prevents stem cell gene repression and maintains stem cell self-renewal.

A frequent molecular event in leukemogenesis, deficiency of Runx1, allows stem cell gene repression and lineage-commitment but impairs activation of key terminal differentiation genes. To determine if genetic abnormalities in leukemia increase self-renewal and impair differentiation in a

manner similar to DNMT1 depletion, Runx1 was depleted in PUER cells. Runx1 is a key transcription factor required for hematopoiesis. Runx1 cooperates with lineage-specifying hematopoietic transcription factors, including Pu.1 and CEBPα, to regulate gene-expression (reviewed in). Functional or actual deficiency of RUNX1, by mutation or chromosome translocation of the *RUNX1* gene, is one of the most frequent molecular events in hematologic malignancies (reviewed in).

Lentivirus was used to introduce shRNA for Runx1 into PUER cells (PUER shRunx1). In shRunx1 cells, Runx1 protein levels were <50% of that in empty vector transduced control cells (**figure 2A**). Unlike shDnmt1 cells, repression of stem cell genes is intact upon Pu.1 introduction in PUER shRunx1. However, Pu.1 mediated activation of genes associated with differentiation is significantly impaired (**figure 2B**). In micro-array gene expression analysis of multiple genes, genes repressed early after Pu.1 introduction into control cells were also significantly repressed in PUER shRunx1. However, genes activated by Pu.1 in control cells had significantly impaired activation in PUER shRunx1 (**figure S2A**). After Pu.1 introduction, PUER shRunx1 continued to proliferate with minimal morphologic changes despite repression of stem cell genes, exhibiting a significant growth advantage over control cells (**figure 2C**). Therefore, the growth advantage of PUER shRunx1 cells over control PUER cells only became apparent in differentiation-promoting conditions.

To extend these observations, lineage-negative hematopoietic stem cells isolated from the bone marrow of Runx1 haplo-insufficient mice (Runx1 +/-) (a gift of the Jim Downing laboratory) were cultured with self-renewal promoting cytokines (stem cell factor [SCF], thrombopoietin) or differentiation promoting cytokines (SCF, interleukin 6 and G-CSF). In self-renewal cytokines, both Runx1 +/- cells and control cells underwent minimal expansion (**figure 2D**). However, in differentiation promoting cytokines, Runx1 +/- cells continued to expand exponentially for 14 days after cell counts in wild-type cells began to decline (**figure 2D, figure S2B**). However, Runx1 abnormalities by themselves are insufficient to cause leukemia , and the Runx1 +/- cells in G-CSF eventually stop proliferating.

RQ-PCR gene expression analysis was used to analyze expression of self-renewal, lineage-commitment and terminal differentiation genes in Runx1 +/- cells. Hoxb4 promotes hematopoietic stem cell self-renewal . Cebpα promotes granulocyte lineage-commitment in response to G-CSF. Cebpε is required for terminal granulocyte differentiation (reviewed in). Lineage negative Runx1 +/- cells and

wild-type control cells were cultured in media supplemented with G-CSF. After 10 days of culture with G-CSF, Runx1 +/- cells expressed higher levels of Cebpα, but lower levels of Hoxb4 and Cebpε, than wild-type cells, demonstrating that Runx1 deficiency allowed lineage-commitment (high Cebpα and high Cebpα/Hoxb4 ratio) but impaired terminal granulocyte differentiation (low Cebpε and low Cebpε/Cebpα ratio) (**figure 2E**).

Therefore, unlike Dnmt1 deficiency, which increased self-renewal by preventing stem cell exit, Runx1 deficiency allows repression of stem cell genes and lineage-commitment, but impairs the activation of key terminal differentiation genes that terminate the cell-division (transit-amplification) of lineage-committed cells. Although this effect by itself is insufficient to produce the persistent unregulated self-renewal necessary for leukemia, possibly these abnormally proliferating linage-committed daughter cells could be the substrate for additional genetic or epigenetic abnormalities for evolution into leukemia.

Transcript and epigenetic analysis of MDS and AML cells indicates progressive impairment of forward differentiation of lineage-committed cells. If abnormal self-renewal in AML cells occurs in a post lineage-commitment differentiation phase, AML cells from patients should express low levels of HOXB4 (which promotes stem cell self-renewal), increased levels of PU.1 and CEBPα (lineage-specifying factors associated with myeloid lineage-commitment), but relatively decreased levels of CEBP ε (required for terminal granulocyte differentiation).

To compare cellular sub-sets with a similar phenotype, CD34+ cells were isolated from AML patient bone marrow (n=3) and normal donor bone marrow (n=3). AML CD34+ cells expressed higher levels of CEBPα, but lower levels of HOXB4 and CEBPε, than normal CD34+ cells. This is consistent with lineage-commitment (high CEBPα and high CEBPα/HOXB4 ratio) but impaired terminal granulocyte differentiation (low CEBPε and low CEBPε/CEBPα ratio) in the AML CD34+ cells (**figure 3A, figure S3A**). The same pattern of gene expression was noted in AML CD34+ cells (n=10) compared to normal CD34+ cells (n=11) (**figure 3B**), and AML myeloblasts (n=311) compared to normal myeloblasts (n=3) (cells morphologically at the same level of differentiation), analyzed by gene-expression microarray (**figure 3C**) (raw data extracted from GEO Datasets). HOXB4 expression data was not available in the micro-array gene expression analysis of myeloblasts.

AML arises through a multi-hit process, frequently proceeding through an intermediate stage called MDS. Evolution of MDS to AML is marked by a progressive decrease in mature blood elements concurrent with an increase in morphologically immature myeloblasts. To determine if this increase in immaturity and disease aggressiveness is from a shift towards cells with stem cell phenotype ('dedifferentiation'), or decreasing forward differentiation in lineage-committed cells, the transcript and DNA methylation profile in normal, MDS and AML bone marrow cells were compared.

Lineage-specifying factor levels were measured by RQ-PCR in bone marrow aspirate cells from patients with low-risk MDS (< 5% myeloblasts, 4 cases) and patients with high-risk MDS and AML (average myeloblast percentage 40%, 12 cases). Controls were CD34+ selected cells and whole bone marrow aspirates from normal donors. The high-risk samples, although morphologically the least mature, had the highest levels of the myeloid lineage-specifying factors CEBPα and PU.1 (**figure 3D, figure S3B**).

DNA methylation of cytosines that precede guanines (CpG), catalyzed by DNMT, is an epigenetic mark associated with transcription repression. Promoter CpG methylation patterns reflect the level of hematopoietic differentiation . An array-based DNA methylation assay, which measures DNA methylation at ~1500 CpG sites in the 5'-regulatory region of ~800 genes , was used to identify CpG which undergo significant ($p<0.001$) methylation changes between normal precursor (CD34+) and mature cells. CpG with greater methylation (switched off) in mature cells are labeled precursor CpG (108 CpG – **Table S2A**) and CpG with less methylation (switched on) in differentiated cells are labeled differentiation-CpG (162 CpG – **Table S2B**). Methylation status was examined in 32 samples of low risk MDS and 132 samples of high risk MDS and AML (patients described in), and two in-vitro models of leukemia: CD34+ cells transduced with the AML fusion protein RUNX1-ETO as a leukemia first-hit or pre-leukemia model , and the RUNX1-ETO containing Kasumi-1 cell-line as a model of advanced transformation that initiates leukemia in transplanted mice . Precursor CpG methylation increased from normal, to MDS, to AML. Therefore, the increasing morphologic immaturity was not from 'de-differentiation' or increasing stem cell phenotype. Differentiation CpG methylation also increased from MDS to AML. Taken together, this epigenetic profile suggests that increasing morphologic immaturity is

from increasing differentiation impairment that prevents forward differentiation of lineage-committed cells, rather than dedifferentiation or increase of stem cell phenotype (**figure 3E**).

Therefore, despite the diversity of genetic and epigenetic abnormalities found in hundreds of cases of MDS and AML, the transcript and epigenetic profile indicates convergence during disease evolution onto a common pathway of decreased forward differentiation of lineage-committed cells. Furthermore, the increase in differentiation CpG methylation from MDS to AML suggests epigenetic mechanisms play a role in impairing forward differentiation of lineage-committed cells.

The opposite cell fate consequences in response to DNMT1 depletion in AML and normal hematopoietic stem cells indicates AML cell self-renewal occurs in a post-lineage commitment differentiation phase. In normal hematopoietic stem cells, DNMT1 depletion prior to or concurrent with a differentiation inducing cytokine or lineage-specifying factor, but not after, increases self-renewal by preventing repression of stem cell genes. Therefore, if AML cells self-renew in a post lineage-commitment context, as suggested by the above analyses, then the functional consequences of DNMT1 depletion should differ from that in normal hematopoietic stem cells.

AML models were CD34 RUNX1-ETO and Kasumi-1 cells, and control cells were normal human CD34+ hematopoietic precursor cells isolated from cord-blood. DNMT1 depletion by decitabine allowed continued proliferation of normal cells but diminished AML cell counts (**figure 4A**). This contrasted with the effects of another clinically utilized cytosine analogue, cytosine arabinoside (Ara-C). Ara-C is the mainstay of myeloid leukemia chemotherapy. Ara-C does not deplete DNMT1 but is a potent DNA damaging agent (**figure S1B,C**). Ara-C, at the same molar concentration as the decitabine (0.5 μM), was toxic to both normal cells and AML cells (**figure 4A**).

The decitabine-induced decrease in AML cell-counts was not via apoptosis (annexin/7AAD-staining measured by flow-cytometry) (**figure 4B**). Decitabine maintained primitive morphology of the normal cells but induced morphologic differentiation of the AML cells (decreased nuclear cytoplasmic ratio, nuclear segmentation and condensation, cytoplasmic granulation and vacuolation) (**figure 4C**). Decitabine treated normal CD34+ cells produced fewer colonies in semi-solid media than untreated cells, however, the colonies formed were larger and mixed. Furthermore, decitabine treatment enabled colony

formation by normal cells even after a prolonged period of liquid culture, which severely diminished colony formation by untreated cells (**figure 4D**) (previously, we demonstrated expanded NOD/SCID engraftment potential of decitabine treated normal CD34+ hematopoietic precursors). In contrast, colony forming ability was abrogated in decitabine treated AML cells compared to untreated cells (**figure 4D, figure S4A**). Depletion of DNMT1 by transfection of siRNA into Kasumi-1 cells also induced differentiation and terminated proliferation (**figure S4B,C,D**).

DNA methylation changes induced by decitabine treatment were measured by micro-array analysis (as described for figure 3). In normal cells, but not in the AML cells, decitabine significantly decreased precursor-CpG methylation, consistent with the difference in produced functional phenotype (**figure 4E**). In both normal and AML cells, decitabine significantly decreased differentiation-CpG methylation, although this only triggered terminal differentiation in the AML cells (**figure 4E**).

The cell-fate consequences in response to DNMT1 depletion can distinguish between self-renewal in a stem cell context or aberrant self-renewal in a post lineage-commitment differentiation phase. In RUNX1-ETO containing AML cells, terminal differentiation in response to DNMT1 depletion is consistent with abnormal self-renewal post-lineage commitment.

DNMT1 depletion with decitabine induces terminal differentiation of primary leukemia cells and cell-line models containing a variety of chromosome abnormalities. The gene-expression and DNA methylation profile of hundreds of MDS and AML samples suggests that self-renewal in lineage-committed daughter cells occurs frequently in AML and is not confined to cases containing RUNX1-ETO. If so, terminal differentiation in response to DNMT1 depletion should be observed in AML cells containing different genetic abnormalities.

Human CD34+ hematopoietic precursor cells isolated from cord-blood were transduced with retrovirus to express the leukemia fusion protein MLL-AF9. The indefinitely self-renewing and leukemia-initiating cells that arose from this transduction do not express CD34 and express high levels of lineage-specifying factors, consistent with self-renewal in lineage-committed daughter cells (**figure S5A,B**). Non-DNA damaging levels of decitabine (**figure S5C**) deplete DNMT1 and induce terminal differentiation of MLL-AF9 cells, including MLL-AF9 cells co-transduced to express activated mutants of RAS and FLT3,

frequent cooperating events noted in AML (**figure 5A**). Colony formation is a surrogate for leukemia-initiating activity by MLL-AF9 cells . DNMT1 depletion abrogates colony formation by viable MLL-AF9 cells (**figure 5B**) (the cells were harvested from liquid culture on day 5 while there were still large numbers of viable cells, there were no viable cells by day 14). Identical treatment of normal hematopoietic stem cells sustains colony forming ability for an extended period (**figure 4D**).

Non-DNA damaging levels of decitabine were also used to deplete DNMT1 in the following AML variants: primary leukemia cells from 14 patients with different patho-morphologic sub-types of AML containing a variety of chromosome abnormalities, and the UT7 and K562 erythro-megakaryoblastic leukemia cell-lines which contain multiple chromosome abnormalities. Decitabine 0.5 μM on day 1, 4 and 7 induced terminal differentiation in 12/14 patient primary leukemia samples and in both erythroblastic leukemia cell-lines (**figure 5C**).

In different AML cases containing a variety of genetic abnormalities, the cell-fate consequences in response to DNMT1 depletion are consistent with the operation of a common pathway of abnormal self-renewal in a post-lineage commitment context.

A non-DNA damaging, DNMT1-depleting regimen of decitabine increases survival in a murine xeno-transplantation model of aggressive human leukemia. Since decitabine is an FDA approved drug, the potential clinical relevance of the above findings was explored in a xeno-transplantation model of aggressive human AML. Mice transplanted with $1x10^6$ MLL-AF9 cells develop lethal invasive leukemia with a median survival of 5 weeks . These mice were treated with a very low dose of decitabine to deplete DNMT1 without causing DNA damage (0.3mg/kg [~1mg/m^2]). A frequent but intermittent schedule of drug administration was used, to allow cell-division, since this should trigger terminal differentiation in leukemia cells but increase self-renewal of normal hematopoietic stem cells. This contrasts with the usual intent with chemotherapy, which is to produce cytostasis or cytotoxicity. The in-vivo DNMT1-depleting, non-DNA damaging properties of the regimen were confirmed by immuno-fluorescence staining for DNMT1 and flow-cytometric measurement of phospho-H2AX in bone marrow cells (**figure 6A,B**). This dose and schedule of decitabine significantly extended murine survival, although it did not cure the mice (**figure 6C**).

Discussion

Although AML is heterogeneous at the genetic and epigenetic level, most cases utilize a common pathway for abnormal self-renewal. Possibly, only a few recurrent abnormalities initiate abnormal self-renewal, and much of the genetic and epigenetic heterogeneity seen in AML relates to other aspects of neoplastic evolution. For example, *RUNX1* and *CEBPα* abnormalities, both of which directly influence CEBPε expression and terminal myeloid differentiation (but not necessarily lineage-commitment), are detected in a substantial portion of MDS and AML cases (reviewed in), and account for most familial MDS or AML . Presumably the utilization of this pathway for abnormal self-renewal has advantages over the others that have been proposed.

A requirement for intact transcription repression machinery in the initial phase of differentiation (**figure 6D**) explains increased self-renewal of hematopoietic stem cells and embryonic stem cells treated with inhibitors of histone deacetylase and increased self-renewal of embryonic stem cells subject to knock-down or inhibition of DNMT . Similarly, the described pathway for abnormal self-renewal in a post-lineage-commitment, post stem cell gene repression differentiation phase, explains terminal differentiation of a variety of leukemia and cancer models in response to DNMT1 depletion and histone deacetylase inhibition (reviewed in) (**figure 6D**).

Although *DNMT1* and the histone deacetylase family are not typical oncogenes or tumor suppressor genes, they have an opposite role in cell-fate regulation in the context of AML versus normal stem cell self-renewal. Clinically available drugs can be used to inhibit DNMT1 or histone deacetylase. Distinct from typical oncotherapeutic drug regimens, which intend cytostatic or cytotoxic effects, drug dose and schedule can be modified to allow cell-division, thereby terminally differentiating malignant cells and concurrently increasing self-renewal of normal stem cells. The consequent decrease in toxicity may enable chronic treatment to maintain the therapeutic effect. The mechanism of AML self-renewal is incentive for improving the dose, schedule or pharmacology of existing drugs, and developing new agents, to target these components of the transcription repression machinery.

Not all cells in an AML clone have self-renewal capacity . Two terms used in describing the heterogeneity of AML cell populations are cell of origin (CO) and leukemia-initiating cells (LIC) (cells which initiate leukemia after transplantation into murine recipients) . The CO is a cell that sustained the first-hit in a multi-hit process that leads to AML, and is probably a stem cell in most cases. LIC can be separate from the CO: the described AML pathway promotes abnormal self-renewal in lineage-committed daughter cells of the CO . These abnormally self-renewing lineage-committed daughter cells of a stem cell CO can form an expanded pool of cells available for random additional genetic or epigenetic hits to evolve into LIC. Therefore, secondary mutations implicated in LIC generation can be lineage-restricted despite evidence for a stem cell CO . Consistent with this pathway for LIC generation, AML LIC frequently have a lineage-committed phenotype (CD34+CD38+ and CLL-1 expression) .

One caveat to the proposed treatment approach is that DNMT1 depletion is expected to terminally differentiate LIC with features of lineage-commitment, but would not be expected to terminally differentiate CO or LIC which phenocopy hematopoietic stem cells. In malignancies in which terminal differentiation responses appear intact, for example chronic phase chronic myeloid leukemia (CML), self-renewal may utilize stem cell associated pathways and not the described AML pathway. However, transformation of CML into AML seems likely to utilize the described pathway, since CML blast crisis LIC demonstrate lineage-commitment .

In conclusion, persistent self-renewal that ignores environmental differentiation cues can be produced via two distinguishable pathways. One pathway generates daughter cells that resemble the parental stem cell (true self-renewal). The second pathway converts transit-amplification of lineage-committed daughter cells into abnormal self-renewal. AML cells exhibit the second pathway. Importantly, common cellular components, such as elements of the transcription repression machinery, have differentiation-phase specific function, such that they have an opposite role in cell-fate regulation in the differentiation-phase at which AML self-renewal occurs compared to their role in normal stem cells. These cellular components can be targeted by clinically available drugs, to concurrently increase normal stem cell and terminate AML cell self-renewal.

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Figure 1

Figure 1. Lineage-specific differentiation requires repression of genes associated with stem cells prior to activation of genes associated with terminal differentiation. A) OHT 100nM (which functionally introduces Pu.1) mediated differentiation of PUER involves stem cell gene (Bmi1, HoxB4, cKit) repression followed by differentiation gene (Mcsfr, Gmcsfr, F4/80) activation (gene expression measured by RQ-PCR). **B) Dnmt1 suppression impairs OHT (Pu.1) mediated stem cell gene repression and terminal differentiation.** Dnmt1 expression in PUER cells was suppressed by shRNA. Images: phase-contrast and Giemsa-stained cytospins of PUER 48h after OHT. **C) Dnmt1 depletion after the stem cell gene repression phase does not impair differentiation.** Decitabine (DAC) 0.5 μM was used to deplete Dnmt1 in PUER cells concurrent with, or 6 hours after, OHT differentiation stimulus. Gmcsfr and Mcsfr expression were not measured in DAC 6h. Images: 48h after OHT. **D) Decitabine effect on normal human CD34+ hematopoietic precursor cell-fate depends on timing of decitabine addition in relationship to a cytokine-mediated (G-CSF) differentiation stimulus.** G-CSF (100ng/ml) was added to CD34+ selected cord-blood cells cultured in media supplemented with SCF, FLT3, TPO, IL-3 and IL-6. DAC (0.5μM) was added either concurrently with G-CSF or 6 hours after G-CSF. Images: day 7 after G-CSF. Data for colony formation in semi-solid media are in figure S1.

Figure 2

Figure 2. Deficiency of Runx1 allows Pu.1 (OHT) mediated stem cell gene repression but impairs activation of terminal differentiation genes. A) Lenti-viral transduction of shRNA suppresses Runx1 protein expression in PUER cells (measured by Western blot)**. B) shRunx1 allows Pu.1 mediated stem cell gene repression but impairs differentiation gene activation.** Gene expression measured by RQ-PCR. Results of micro-array gene expression analysis are in figure S2. **C) PUER shRunx1 cells retain proliferative capacity and undifferentiated morphology despite stem cell gene repression**. Cell-counts daily by automatic cell-counter. Images: phase-contrast 24h after OHT. **D) Lineage negative hematopoietic stem cells isolated from the bone marrow of Runx1 +/- mice demonstrate a growth advantage over wild-type hematopoietic stem cells when cultured in differentiation promoting conditions** (SCF, IL-6 and G-CSF) compared to self-renewal promoting conditions (SCF, thrombopoietin). Images: colonies 14 days after plating bone marrow cells isolated from Runx1 +/- or wild-type congenic mice ($2x10^4$ cells/ml) in methyl-cellulose supplemented with self-renewal or differentiation promoting cytokines. **E) Lineage-commitment but impaired terminal differentiation of Runx1 +/- cells demonstrated by the expression of Cebpα, Hoxb4 and Cebpε.** Gene expression 10 days after culture of lineage-negative stem cells in SCF, IL-6 and G-CSF (bar-graphs). The Cebpα/Hoxb4 ratio increases with lineage commitment, and the Cebpε/Cebpα ratio increases with terminal differentiation.

Figure 3

Figure 3. The transcript and epigenetic profile of patient AML cells suggests decreased forward differentiation of lineage-committed cells. **A) CD34+ AML cells express higher levels of CEBPα, but lower levels of HOXB4 and CEBPε, than normal CD34+ hematopoietic precursors**. The CEBPα/HOXB4 and CEBPε/α ratio were calculated in each sample as indices of lineage-commitment and terminal differentiation respectively. Gene expression by RQ-PCR. Distribution of individual expression ratios represented by box-plot. Clinical characteristics of patients in figure S3. **B) Similar findings for CD34+ AML cells** (n=10) **and normal CD34+ cells** (n=11) **analyzed by gene-expression micro-array** (raw data extracted from GEO Datasets). Expression levels represented by heat-map. **C) AML myeloblasts (n=311) display lineage-commitment but impaired terminal differentiation compared to normal cells at different levels of differentiation (n=38), including normal myeloblasts (n=3)** (HOXB4 expression data not available for this Microarray platform). **D) Lineage-specifying factor levels increase from low risk MDS (<5% myeloblasts) to high risk MDS and AML (5% myeloblasts)** (measured by RQ-PCR). Samples are bone marrow from normal donors, patients with low risk MDS (<5% myeloblasts), and high risk MDS/AML (5% myeloblasts). Clinical characteristics of patients in figure S3. **E) Precursor CpG methylation increases from normal, to MDS, to AML, suggesting increasing morphologic immaturity is not from increasing stem cell phenotype**. **Differentiation CpG methylation also increased from MDS to AML, suggesting a role for DNA methylation in differentiation-impairment that prevents forward differentiation of lineage-committed cells.** CpG with greater methylation (p<0.001) (switched off) in normal mature cells are labeled precursor CpG (108 CpG – Table S2A) and CpG with less methylation (p<0.001) (switched on) in differentiated cells are labeled differentiation-CpG (162 CpG – Table S2B). nCD34 = CD34+ cells isolated from normal bone marrow (n=9), nBM = normal whole bone marrow (n=42), LR-MDS = bone marrow from low-risk MDS patients (n=32), RAEBAML = bone marrow from high-risk MDS/AML (n=132) patients . In-vitro models: nCD34 = CD34+ cells isolated from cord-blood, RUNX1-ETO = CD34+ hematopoietic precursors retrovirally transduced with RUNX1-ETO as a model of pre-leukemia, Kasumi1 =Leukemia cell line from patient with RUNX1-ETO leukemia. Box-plot boundaries = inter-quartile range, horizontal line = median, '+' = mean, whiskers = range of values, small boxes = out-lying values.

Figure 4

Figure 4: The opposite cell fate consequences in response to DNMT1 depletion in AML and normal hematopoietic stem cells indicates AML cell self-renewal occurs in a post-lineage commitment differentiation phase. Leukemia models: CD34+ cells transduced with RUNX1-ETO and Kasumi-1 cell-line.

Control: normal cord blood CD34+ cells. Treatment: Decitabine (DAC) 0.5 - 1 μM or cytosine arabinoside (AraC) 0.5 μM on day 1, 4 and 7. DAC 0.5μM depleted DNMT1 without causing measurable DNA damage (figure S1). **A) Non-DNA damaging DNMT1 depletion has opposite effects on the proliferation of normal and leukemia cells.** Cell counts by automated cell counter. **B) DAC did not increase apoptosis in normal or leukemia samples**. Apoptosis measured by flow-cytometry for annexin and 7AAD staining on Day 5. Similar results were noted in the leukemia samples. **C) DAC treated normal precursors retain primitive morphology while leukemia cells differentiate.** Giemsa stained cytospin preparations on day 9 of liquid culture. **D) DAC treatment of normal cells prolongs colony-forming ability, increases colony-size and mixed character, while abrogating colony formation by CD34 RUNX1-ETO.** Cells were plated in semisolid media without DAC after 7, 14 or 21 days in liquid culture (DAC treatment was stopped on day 7). Phase-contrast images of colonies. U=Untreated, D=DAC treated, A=Ara-C treated. **E) DAC treatment significantly decreases precursor-CpG methylation in normal but not leukemia cells.** Precursor and differentiation-CpG as defined in text and figure 3 legend.

Figure 5

Figure 5: DNMT1 depletion with decitabine (DAC) induces terminal differentiation of leukemia cells containing a variety of chromosome abnormalities. A) DAC terminally differentiates MLL-AF9 leukemia cells. MLL-AF9 (MA9) leukemia-initiating cells are produced by retroviral transduction of CD34+ human hematopoietic precursor cells. Some clones are additionally transduced to express leukemia associated mutants of FLT3 (ITD) and RAS (NRas). These cells self-renew indefinitely in-vitro and initiate invasive leukemia in transplanted mice. Cells were treated with DAC 0.5 μM added on days 1, 4 and 7. Non-DNA damaging DNMT1 depletion was confirmed by immune-fluorescence and flow-cytometry (figure S5). Cell counts by automated cell counter. Images: Giemsa-stained cytospins Day 9. **B) DAC treated MLL-AF9 cells lose clonogenicity.** Colony formation by MLL-AF9 cells is a surrogate for their ability to initiate leukemia. Viable untreated (U) and DAC treated (D) cells were plated in semi-solid medium which did not contain DAC after 6 days of liquid culture. **C) DAC terminally differentiates primary leukemia cells from patients and leukemia cell-lines**. Primary leukemia cells were aspirated from the bone marrow of patients with AML and cultured in media supplemented with cytokines (SCF, FLT-3, TPO, IL-3, IL-6) with (full-lines) or without decitabine (dashed-lines) (DAC) (0.5 μM) added on day 1, 4 and 7. These samples represent a spectrum of morphologic classification and chromosome abnormalities (figure S5). Leukemia cell lines: Erythro-megakaryoblastic leukemia (UT7, K562). Cell counts by automatic cell counter on day 8. Images: day 8. MLL-AF9 and cell-line cell cultures were maintained until day 21 to confirm that there was no rebound increase in cell numbers after discontinuation of DAC treatment on day 7.

Figure 6

Figure 6: A dose and schedule of decitabine that depletes DNMT1 but does not produce measurable DNA damage (1mg/m^2 3X/wk intra-peritoneal) increases survival in a murine xeno-transplantation

model of aggressive human leukemia. **A-C)** In-vivo depletion of DNMT1 without DNA damage was confirmed by measuring DNMT1 depletion in bone marrow cells using immune-fluorescence (red dots) (A) and DNA damage in bone marrow cells by flow-cytometric measurement of phosphorylation of H2AX (B). Grey = isotype control. Mice were transplanted with $1x10^6$ MLL-AF9 leukemia cells. Mock treatment was with intra-peritoneal PBS. 5 mice in each treatment group. This regimen increased survival in the treated mice by approximately 20% (C). **D) Two pathways for persistent self-renewal that ignores differentiation-inducing signals, and the therapeutic implications. Left panel:** In hematopoietic stem cells (HSC), decitabine (DAC) to deplete DNMT1 and antagonize transcription repression prevents a necessary first step in lineage-commitment, which is repression of stem cell gene expression. Therefore, daughter cells resemble the parental stem cell, even in differentiation promoting conditions. If DAC is added shortly after the differentiation-inducing stimulus (after the phase of stem cell associated gene repression), it does not prevent and may even increase differentiation. **Right panel:** The cell of origin (CO) is the cell that sustains the first genetic or epigenetic abnormality leading to leukemia. The CO could be a stem cell, however, abnormal self-renewal and leukemia initiating ability (LIC) may manifest in lineage-committed daughter cells, since the genetic/epigenetic abnormalities may allow stem cell gene repression but impair terminal differentiation gene activation that terminates transit-amplification. In these cells, primed to differentiate with high levels of lineage-specifying factors, repression of stem cell genes, and with aberrant repression of differentiation genes that requires the transcription repression machinery, DAC terminates self-renewal and resumes differentiation.

Supporting Online Material

Tables (attached excel file)

S1 A: Genes repressed >2-fold by micro-array gene expression analysis 6 hours after OHT (Pu.1) introduction to PUER cells

S1 B: Genes activated >2-fold by micro-array gene expression analysis 24 hours after OHT (Pu.1) introduction to PUER cells

S2A: List of Precursor CpG (CpG that are significantly hypermethylated during hematopoietic differentiation)

S2B: Differentiation CpG (CpG that are significantly hypomethylated during normal hematopoietic differentiation)

Figure S1

Figure S1. The nucleoside analogue decitabine (DAC) can deplete DNMT1 without causing measurable DNA damage. A) Increasing levels of DAC produce increasing DNMT1 depletion in normal hematopoietic precursor cells. Normal CD34+ hematopoietic precursor cells were isolated from cord-blood. DAC concentration in μM, number of DAC additions in a 1 week period in parentheses. DNMT1 was quantified by immuno-fluorescence using a Q-dot based secondary anti-body (red dots) and Image-Quant software. DAPI was used to stain nuclei (blue stain). **B & C) These levels of decitabine do not produce measurable DNA damage.** DNA damage was measured by flow-cytometric assessment for (B) phosphorylation of histone H2AX or by (C) the Fast Micromethod for DNA scission 24 hours after DAC exposure. Equimolar doses of another cytosine analogue, cytosine arabinoside (AraC) produce significant DNA damage. Grey histogram = isotype control for flow-cytometric analysis. **D) DAC, at these non-DNA damaging but DNMT1 depleting levels, produces transient cell-cycle arrest followed by rebound hyperproliferation**. Cell-cycle status was measured at the various time-points by flow-cytometric assessment of propidium iodide staining. **E) Kinetics of DNMT1 depletion and recovery in normal hematopoietic precursors exposed to a 1X addition of DAC 0.5μM.** DNMT1 was quantified with the Q-dot based assay (red dots – appear pink overlayed on blue DAPI stain). Nuclei are stained with DAPI (blue). **F) DAC can inhibit or enhance G-CSF mediated myeloid differentiation depending on the timing of DAC addition in relationship to the G-CSF stimulus**. G-CSF (100ng/ml) was added to CD34+ selected cord-blood cells cultured in media supplemented with SCF, FLT3, TPO, IL-3 and IL-6. DAC (0.5μM) was added either concurrently with G-CSF or 6 hours after G-CSF. Cells were plated into methyl-cellulose (20,000/ml methyl-cellulose) 9 days after the addition of G-CSF/DAC. Colonies were counted and photographed (phase-contrast) after 12 days of culture in methyl-cellulose. DAC added concurrent with G-CSF encourages formation of larger and mixed colonies, whereas DAC added 6 hours after G-CSF results in significantly decreased colony-forming ability and smaller colonies.

Figure S2

Figure S2: Runx1 deficiency allowed OHT (Pu.1) mediated repression gene repression but impairs Pu.1 mediated gene activation. A) Microarray gene expression analysis was used to identify genes repressed early or activated more than 2-fold after Pu.1 introduction into control vector transduced PUER cells. The expression pattern of these repressed and activated genes (table S1) was compared in PUER shRunx1 at 6 and 24h after Pu.1 introduction. **B)** Colony forming units (CFU) in Runx1 +/- and wild-type congenic murine bone marrow cells plated into methyl-cellulose ($2x10^4$ cells/ml) supplemented with cytokines to promote self-renewal versus differentiation were counted at day 10. After counting, colony cells were washed twice with PBS to remove methylcellulose and replated under identical conditions.

Figure S3

Figure S3. The samples analyzed are representative of the clinical spectrum of AML. A) WHO classification and detected chromosome abnormalities in AML CD34+ cells analyzed by RQ-PCR. M5 = Acute monoblastic leukemia; M2 = Acute myeloblastic leukemia with maturation. **B) WHO classification and detected chromosome abnormalities in MDS and AML bone marrow aspirate specimens analyzed by RQ-PCR**. RA = refractory anemia; RCMD = refractory cytopenia with multi-lineage dysplasia; RCMD-RST = refractory cytopenia with multi-lineage dysplasia, ring sideroblasts and thrombocytosis. M1 = Acute myeloblastic leukemia without maturation. M4 = acute myelomonocytic leukemia.

Figure S4

Figure S4: Suppression of DNMT1 expression in Kasumi-1 cells using decitabine or siRNA induces terminal differentiation. A) DAC significantly decreased the colony-forming ability of Kasumi-1 cells. Viable Kasumi-1 cells were plated into methyl-cellulose ($2x10^3$ cells/ml) on day 9 after treatment with decitabine 0.5μM on day 1 and 2. Colonies were counted 12 days after plating. **B). DNMT1 suppression by siRNA was confirmed by immuno-fluorescence measurement of DNMT1 levels.** Kasumi-1 cells were transfected with control siRNA to or siRNA to deplete DNMT1. **C) DNMT1 depletion by siRNA terminally differentiated Kasumi-1 cells.** Cell-counts measured by automatic counter. Images: Giemsa-stained cytospins 5 days after transfection.

Figure S5

Figure S5: DNMT1 depletion by non-DNA damaging levels of decitabine (DAC) induces terminal differentiation in leukemia cells containing a variety of chromosome abnormalities. A) MLL-AF9 cells are CD34 negative, consistent with self-renewal in a post lineage commitment context. MLL-AF9 (MA9) leukemia-initiating cells are produced by retroviral transduction of CD34+ human hematopoietic precursor cells, but self-renewing and leukemia-initiating cells are CD34 negative (measured by flow-cytometry). Grey = isotype control. **B) MA9 cells express high levels of lineage-specifying factors (CEBPα, PU.1) and intermediate levels of stem cell associated factors (HOXB4, BMI-1) compared to normal CD34+ hematopoietic precursors.** Gene-expression by RQ-PCR. **C) Decitabine (DAC) 0.5μM on day 1, 4, and 7 depletes DNMT1 without causing DNA damage.** DNMT1 levels measured by immuno-fluorescence (red-dots). DNA damage measured by flow-cytometry for phospho-H2AX. Positive control for DNA damage = cells treated with equimolar concentration of AraC. Grey histogram = isotype control. **D) Morphologic classification by WHO categories and metaphase cytogenetic analysis of primary leukemia samples treated with DAC.** M1 = Acute myeloblastic leukemia without maturation; M2 = Acute myeloblastic leukemia with maturation; M4 = acute myelomonocytic leukemia; M5 = Acute monoblastic leukemia; M6 = Acute erythroid leukemia; M7 = acute megakaryoblastic leukemia.

Materials and Methods

Normal volunteer and patient samples: Umbilical cord blood was collected during normal full-term deliveries with informed consent of the mother on a CASE IRB approved protocol. Bone marrow aspirates were collected from patients and healthy volunteers. Informed consent for sample collection was obtained according to protocols approved by the Cleveland Clinic IRB. All these samples are associated with anonymized clinical hematopathology data that indicate the morphological classification and immuno-phenotype of the disease. Patients were grouped according to the World Health Organization (WHO) classification system.

Human hematopoietic cell culture: Normal human hematopoietic cells, CD34 RUNX1-ETO cells, and MLL-AF9 cells were cultured in IMDM supplemented with 10% fetal bovine serum and 10ng/ml of the following human cytokines: stem cell factor (SCF), FLT3 ligand (FLT3), thrombopoietin (TPO), interleukin-3 (IL-3) and interleukin-6 (IL-6).

Isolation of CD34+ cells: CD34+ cells from umbilical cord blood or bone marrow aspirates were immunopositively purified using a magnetic cell sorting system a CD34 MicroBead Kit (catalog #: 130-046-702, Miltenyi Biotec) according to manufacturer instructions. The purity of the CD34+ population (ranged typically from 95 to 99%) was determined by immunolabeling with FITC-conjugated monoclonal antibodies against CD34 (Clone 581, Beckman Coulter, Miami, FL, USA) that reacted with an epitope other than the antibody used for separation.

Generation of PUER cells with stable suppression of Runx1 or Dnmt1 expression: A lentiviral vector pLenti6-DEST (Invitrogen, Carlsbad, CA) was used to construct short hairpin (sh) RNA for Runx1 and Dnmt1. The specific 21-bp target sequences for mouse shRunx1 (5′ -TGTGGATGACTGAGTACCTGA-3′) and mouse shDnmt1 (5′ -GAACGGCATCAAGGTGAAC-3′) were synthesized in sense and antisense orientation by Advanced DNA Technology (ADT), the single-strand oligos were then annealed to form double-strand oligos, and subsequently ligated with pENTRY vector (Invitrogen, Carlsbad, CA) downstream of an RNA promoter. The ligated constructs were transformed into E. Coli TOPO10. Positive clones were verified by

DNA sequencing. The verified clones were then recombined into pLenti6-DEST vector using Invitrogen's ViralPack kit, resulting in pLenti6-shRunx1 and pLenti6-Dnmt1. The pLenti6-shRunx1 and pLenti6-Dnmt1 constructs were then transfected together with envelop encoding plasmid (VSVG) into 293FT packaging cell line to produce lentivirus. The supernatant containing lentivirus was harvested at 48 hours after transfection. Titers were determined on NIH3T3 cells as transducing units using serial dilutions of vector stocks with 8 µg/ml polybrene (Sigma Chemical, St. Louis, MO).

PUER cells (kindly provided by Dr. Harinder Singh, University of Chicago) are murine hematopoietic precursor cells that have been retrovirally transduced to express Pu.1 fused to the estrogen receptor. To knock-down Runx1 and Dnmt1 in cells, murine PUER cells were grown in Iscove's modified Eagle's medium, without phenol-red, with 10% fetal bovine serum, 2.5ng/ml mouse IL-3, 1μg/ml Puromycin, 55μM beta-ME, 1% penicillin/streptomycin at 37°C in a humidified atmosphere with 5% CO2 in air. The lentivirus-containing supernatant was added to the cell culture at appropriate 4 particles/cell concentration with 8 µg/ml polybrene. Twenty-four hours after infection, 4ug/ml of blasticidin (bln) was added to the cell culture for positive clone selection. The Bln-resistant cells were analyzed for Runx1 and Dnmt1 expression by RQ-PCR and Western blot.

Addition of tamoxifen (OHT) to PUER triggers their terminal differentiation into macrophages . Differentiation status was analyzed by: (i) presence of adherent cells by light microscopy, (ii) morphological changes in Giemsa stained cytospin preparations, and (iii) cKit (eBioscience catalog number 11-1171-82) and F4/80 (eBioscience catalog number 15-4801-82) expression by flow cytometry.

Real time PCR (RQ-PCR): mRNA levels were assayed using RQ-PCR. Briefly, total cellular RNA is isolated from 5x10^5 cells using RNeasy Plus (QIAGEN), according to the manufacturer's protocol. For cDNA synthesis, after a denaturation step of 5 min at 70°C, 1 μgof RNA were reverse transcribed to single-stranded cDNA using a mix of random hexamers and oligo dT primers and M-MLV reverse transcriptase for first strand synthesis (Promega). Real-time PCR was performed with Real-time PCR Master Mix containing Sybrgreen I and hotstart Taq DNA polymerase (Takara). GAPDH was amplified as control. Primer sequences available upon request. Real-time detection of the emission intensity of SYBR Green bound to double-stranded DNA will be detected using the iCycler instrument (Bio-Rad).

Micro-array gene expression analysis: Total cellular RNA was isolated from PUER and PUER-shRunx1 cells, at base-line and 6 and 24 hours after OHT addition,using a RNeasy Kit (Cat#74104, Qiagen GmbH, Hilden, Germany) per manufacture's instruction. The purity, concentration and quality of the RNAs were determined using a NanoDrop spectrophotometer and agarose gel electropherosis. All the RNA samples had an A260/280>1.8 and A260/A230>1.8, with intact 28S and 18S ribosome RNA visible on 1% agarose gel.

20μgof RNA from each sample was submitted to NimbleGen (Roche NimbleGen Inc, Iceland) for gene-expression microarray analysis. At NimbleGen, double-stranded cDNA was synthesized from each RNA sample using Invitrogen Superscript Double-Stranded cDNA Synthesis Kit. The cDNAs were then labeled with Cy3 and used for hybridization with array. 385K 1-plex arrays were used in the experiments. Gene-expression data from the array experiments were analyzed using ArrayStar 2 software (DNAStar, Inc).

Quality controlled raw data (Affymetrix CEL files or SOFT files for GDS2118 dataset) from previously published experiments (references GDS2118, GSE123326, GSE1159, GSE9476, GSE1729, GDS1064) was downloaded from Gene Expression Omnibus (GEO) datasets (www.ncbi.nlm.nih.gov/geo). Log_2 expression values for individual probe sets utilizing the same micro-array platform were combined using RMA normalization supplemented with the median over the entire array method (BRM-ArrayTools version 3.7.1 developed by Dr. Richard Simon and BRB-ArrayTools Development Team). By comparing ratios of gene expression that were calculated within each sample (CEBPα/HOXB4 expression and CEBPε/CEBPα expression) the gene expression data was also internally controlled. Heat-maps were generated using ArrayStar® v3.0 (DNASTAR, Madison, WI).

Methylome analysis: 1 µg of DNA extracted using the Pure-Gene kit was subject to analyses by the Cancer Methylation Panel 1 (1505 selected CpG sites) in the Golden Gate Assay kit (Illumina) following the protocol provided with the kit. Briefly, bisulfite conversion of DNA samples is done using the EZ DNA methylation kit (Zymo Research). For each CpG site, there are two pairs of probes which corresponded to either the methylated or unmethylated state of the CpG site. Through allele-specific extension and ligation, PCR templates are generated and then amplified by PCR using fluorescently labeled common primers. The resulting PCR products are hybridized to a bead array at sites bearing a complementary address sequence. These hybridized targets contain a fluorescent label that denotes a methylated or unmethylated state for a given locus. Methylation status of the interrogated CpG site is then calculated as the ratio of fluorescent signal

from one allele relative to the sum of both methylated and unmethylated alleles. The image extraction and statistical analysis is done with Beadstudio Methylation Module Software (Illumina). The β-value provides a continuous measure of levels of DNA methylation at a CpG site, ranging from 0 in the case of completely unmethylated sites to 1 in completely methylated sites.

Clonogenic progenitor assays: Cells in liquid culture were treated with decitabine on day 1, 4 and 7. After 7, 14 or 21 days in liquid culture, identical numbers of cells from treated and untreated control samples (20,000/ml methyl-cellulose of normal, RUNX1-ETO CD34+ cells and MLL-AF9 cells, and 2000/ml methyl-cellulose of Kasumi-1 cells) are plated in decitabine free-semisolid media (MethoCult H4434, Stem Cell Technology) supplemented with SCF (50 ng/mL), GM-CSF (10 ng/mL), IL-3 (10 ng/mL), and erythropoietin (3 U/mL). Different colony-forming units (CFU) were identified by morphology and counted under an inverted microscope at 10 to 12 days post-plating.

Immunofluorescence with Q-Dots to measure DNMT1 levels: Cells on cytospin slides were fixed and permeabilized with 10% formalin and 0.25% triton. Non specific binding sites were blocked with 10% normal goat serum and 6% BSA. Slides were incubated overnight with mouse anti-DNMT1 antibody (Abcam catalog number ab13537) (diluted 1:500 in blocking solution), followed by a 655 nm Quantum Dots™-conjugated goat anti-mouse antibody (Invitrogen catalog number Q11022MP) (diluted 1:500). Finally, cells were stained with 3 µM DAPI for 5 min before dehydration in graded alcohols and xylene. Image files were loaded into the Image-Pro Plus environment and individual cells were segmented for quantification of fluorescence signal with the "count/size" function of Image-Pro Plus. Precise segmentation was achieved by setting a lower threshold just above background emission. A filter was set for an area of whole cells and excluded fluorescent fragments from the measurement. Image-Pro Plus generates a high-content array of measurements that include event number, area, mean of pixel intensities, and maximum and minimum pixel intensities. These scores were integrated into a mean intensity fluorescence (MIF) score. An MIF 25% of untreated control is the threshold that was used to evaluate decitabine effectiveness.

DNA damage measurement by Fast Micromethod®: The Fast Micromethod DNA single-strand break assay is applied as described in Schroder H et al. Methods in Molecular Biology 2006; 314: 287-305. The method is

based on the ability of the specific fluorochrome dye PicoGreen® to make a stable complex with dsDNA, but not ssDNA in highly alkaline conditions. Cells (3000 cells/25 μl) were incubated in lysis buffer (4.5 M urea, 0.2 M EDTA, 0.1% SDS, pH 10.0) supplemented with PicoGreen® (Invitrogen). DNA denaturation is triggered by addition of NaOH–EDTA solution (pH = 12) and recorded after 20 min by measuring the fluorescence (excitation 485 mn/emission 520 nm) of the ssDNA-PicoGreen complex. Results are expressed as strand scission factors (SSF), calculated as the $\log_{10}$ of the ratio of the percentage of ssDNA from treated and control samples, respectively, after 20 min of denaturation. For practical reasons to facilitate display of the data, the SSF are multiplied by -1.

DNA damage measurement by γH2AX staining: Phosphorylation of the histone H2A family member H2AX at Ser139 (γH2AX) was measured by flow cytometry. Cells were fixed with 2% paraformaldehyde and then permeabilized by adding an iced cold 90% methanol solution. Cells were then incubated in blocking solution (0.5% BSA) containing saturating concentration of Alexa 488-conjugated γH2AX antibody (Cell signaling technology catalog number 9719). Percentage of γH2AX positive cells is analyzed by using a Coulter Epics XL-MCL flow cytometer equipped with CXP software (Beckman-Coulter).

Cell cycle analysis: Cell cycle status in normal CD34+ cells after a single exposure to decitabine was analyzed by measuring single cell DNA quantity. Cells were harvested at different time points and incubated with 100 mg/ml propidium iodide (Sigma) and 0.2 mg/ml DNase-free RNaseA (Boehringer Mannheim). After 30 minutes, single cell DNA content was analyzed by flow-cytometry.

Apoptosis detection: Apoptosis was detected by Annexin-V and 7AAD co-staining using the APOAF commercial kit (Sigma). Cells ($5x10^5$) were washed and incubated for 30 minutes with FITC-conjugated Annexin-V at room temperature. Cells were then resuspended in 400 mL of binding buffer containing 7AAD and immediately analyzed by flow cytometry.

Flow cytometry studies: Cell analysis was performed on a Coulter Epics XL-MCL flow cytometer equipped with CXP software (Beckman-Coulter). Kasumi or CD34+ cord blood cells were stained for 20 min with a combination of fluorochrome-conjugated monoclonal antibodies for CD34, CD38, CD90, CD33 and/or CD133.

The CD90 antibody (catalog #: IM1840U) was purchased from Coulter/Immunotech. The CD133 antibody (catalog #: 130-080-801) was from MACS (Miltenyi Biotec). The antibodies against CD34, CD38, and CD33 (catalog #: 348053, 340409, and 347787 respectively) were from Becton Dickinson. For each mAb, an isotype-matched immunoglobulin was used as a control in all experiments. The percentage of CD90+, CD33+ and CD133+ positive cells was assessed in the CD34+CD38- subpopulation.

Murine studies: The MLL-AF9 xeno-transplantation experiments were approved by the Cincinnati Children's Hospital Medical Center (CCHMC) Animal Care and Use Committee (IACUC). Cultured MLL-AF9 (MA9) cells ($1x10^6$) were transplanted by intrafemoral injection into non-irradiated 6-8 week old NOD/SCID mice. Mice were anesthetized with isofluorane before transplantation. Animals were checked daily by a technician in the animal laboratory. Animals were euthanized if they developed signs of distress. Euthanasia was performed by cervical dislocation under CO_2 asphyxiation as approved by the IACUC and consistent with the recommendations of the panel on Euthanasia of the Animal Veterinary Medical Association. Decitabine was administered via the intra-peritoneal route at a dose of 0.3mg/kg ($1mg/m^2$) 3X/week (Mon, Thurs, Fri) beginning in week 2 after transplantation. Bone marrow cells obtained after euthanasia were assessed for DNMT1 depletion by immuno-fluorescence and DNA damage by flow-cytometric measurement of γH2AX. Survival analyses were performed by Kaplan-Meier analyses with the log-rank tests and $p<0.05$ was considered to be statistically significant.

Wild type and $RUNX1^{+/-}$ C57BL/6 mice were a kind gift of Jim Downing and Noel Lenny of the Downing laboratory. The breeding protocol was approved by the Cleveland Clinic Animal Care and Use Committee (IACUC). Animals were euthanized by cervical dislocation after CO_2 asphyxiation. RNA isolation for RQ-PCR experiments was performed from bone marrow cells obtained after euthanasia. Bone marrow cells were also seeded in liquid culture under self renewal (50 ng/ml murine SCF and 100 ng/ml human TPO) or differentiation inducing condition (50 ng/ml murine SCF, 10 ng/ml murine IL-6 and 20 ng/ml human GCSF). Cell counts were performed every 48 h. Clonogenic progenitor assays (MethoCult GF M3434; Stem Cell Technologies) were performed in triplicate under self renewal and differentiation inducing conditions at a density 2×10^4 cells/ml methyl-cellulose. The number of colonies was scored after 10 days. After counting, colony cells were washed twice with PBS to remove methylcellulose and then subsequently replated under identical conditions.