The methyltransferase G9a regulates HoxA9-dependent transcription in AML

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Chromatin modulators are emerging as attractive drug targets, given their widespread implication in human cancers and susceptibility to pharmacological inhibition. Here we establish the histone methyltransferase G9a/EHMT2 as a selective regulator of fast proliferating myeloid progenitors with no discernible function in hematopoietic stem cells (HSCs). In mouse models of acute myeloid leukemia (AML), loss of G9a significantly delays disease progression and reduces leukemia stem cell (LSC) frequency. We connect this function of G9a to its methyltransferase activity and its interaction with the leukemogenic transcription factor HoxA9 and provide evidence that primary human AML cells are sensitive to G9A inhibition. Our results highlight a clinical potential of G9A inhibition as a means to counteract the proliferation and self-renewal of AML cells by attenuating HoxA9-dependent transcription.

[Keywords: histone methylation; hematopoiesis; leukemia; Hox genes]

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vances in delineating biological roles of G9a/GLP, a detailed characterization of these enzymes during hematopoiesis has not been reported.

Results

Selective requirement for G9a in hematopoietic progenitor cells

To confirm the expression of G9a in the hematopoietic system, we performed quantitative RT–PCR (qRT–PCR) analyses from FACS-purified hematopoietic subpopulations and detected high expression of G9a in hematopoietic stem and progenitor cells (HSPCs), at levels comparable with mouse ESCs, and the lowest expression in mature myeloid and lymphoid cells [Supplemental Fig. S1]. We then investigated the biological importance of G9a in the hematopoietic system using G9a<sup>−/−</sup> mice (Fig. 1A; Lehnertz et al. 2010) crossed with Vav-Cre transgenic mice to obtain G9a<sup>−/−</sup>; Vav-Cre and G9a<sup>fl/fl</sup>; Vav-Cre mice [G9a<sup>−/−</sup> (Vav) and G9a<sup>−/−</sup> (Vav), hereafter]. As expected [Stadtfeld and Graf 2005; Gan et al. 2010], Vav-Cre-mediated excision in G9a<sup>−/−</sup> (Vav) mice harboring a ROSA26-YFP [R26-YFP] Cre reporter was specific to hematopoietic cells and fully penetrant [Supplemental Fig. S2a–c]. Consistent with previous reports [Tachibana et al. 2002, 2005], deletion of G9a resulted in a characteristic reduction in GLP and H3K9me2 levels in bone marrow-derived macrophages [BMMs] (Fig. 1B). While no difference in colony-forming unit (CFU) numbers colonies in cytokine-containing methylcellulose medium. While no difference in colony-forming unit (CFU) numbers and phenotypes (Fig. 1D) was observed, the total size of individual colonies, most of which contained decreased (Fig. 1E). This was the result of a reduction in the penetrant (Supplemental Fig. S2a–c). Consistent with previous reports [Tachibana et al. 2002, 2005], deletion of G9a in lymphoid cells, were born at normal frequency, and did not display any overt hematological abnormalities [Lehnertz et al. 2010].

We first investigated the function of G9a-deficient progenitor cells and evaluated the ability of bone marrow cells from G9a<sup>−/−</sup> (Vav) and G9a<sup>−/−</sup> (Vav) mice to form colonies in cytokine-containing methylcellulose medium. While no difference in colony-forming unit (CFU) numbers (Fig. 1C) and phenotypes (Fig. 1D) was observed, the total cell output of G9a-deficient progenitors was drastically decreased (Fig. 1E). This was the result of a reduction in the size of individual colonies, most of which contained <500 cells (Fig. 1F).

To assess the activity of G9a-deficient HSCs, we generated mixed bone marrow chimeras with R26-YFP<sup>+</sup> labeled G9a<sup>−/−</sup> (Vav) or G9a<sup>−/−</sup> (Vav) cells in competition with R26-YFP<sup>+</sup>; G9a<sup>fl/fl</sup> cells (Fig. 1H; Supplemental Fig. S2d). Interestingly, we observed only a modest, non-significant difference in the relative output of G9a<sup>−/−</sup> (Vav) and G9a<sup>−/−</sup> (Vav) cells in the examined lineages 8 wk after transplantation. [Fig. 1I]. However, this trend was no longer detectable 18 wk after transplantation [Fig. 1I], suggesting that G9a is not essential for the function of long-term repopulating HSCs [LT-HSCs].

Loss of G9a impairs AML progression and leukemia stem cell (LSC) self-renewal in vivo

To investigate G9a function in AML cells, which partially resemble myeloid progenitors [Krivtsov et al. 2006], we generated leukemias from knockout and heterozygous HSPCs by retroviral expression of HoxA9 and Meis1 [A9M] [Supplemental Fig. S3; Kroon et al. 1998], two genes frequently overexpressed in human AML [Lawrence et al. 1999]. Importantly, G9a and GLP were expressed in G9a<sup>−/−</sup> (Vav); A9M cells, and G9a expression was entirely ablated in G9a<sup>−/−</sup> (Vav); A9M cells (Fig. 2A). While all recipients of G9a<sup>−/−</sup> (Vav); A9M control cells rapidly advanced to end-stage AML, only 10 of 15 recipients of G9a<sup>−/−</sup> (Vav); A9M cells succumbed to AML, albeit with delayed kinetics [median survival 111 d vs. 75 d] (Fig. 2B). In addition, clonal analysis of HoxA9/Meis1 proviral DNA in the G9a<sup>−/−</sup> (Vav) cohort revealed identical integration patterns in more than one recipient, indicative of a reduced repertoire of self-renewing LSC clones (Fig. 2C). To confirm this notion, we performed an in vivo limiting dilution assay [LDA] using bone marrow cells harvested from end-stage leukemic mice [Fig. 2D] and found that the frequency of LSCs among G9a<sup>−/−</sup> (Vav); A9M cells was reduced ~20-fold [Fig. 2E].

We also generated A9M leukemias from G9a<sup>fl/fl</sup>; Mx-Cre<sup>+</sup> mice, allowing the inducible deletion of G9a in vivo following plpC treatment [Supplemental Fig. S4a]. plpC treatment per se had no effect in primary recipients of G9a<sup>fl/fl</sup> (Mx); A9M cells [Supplemental Fig. S4b]. In contrast, deletion of G9a in secondary leukemia recipients of G9a<sup>fl/fl</sup> (Mx); A9M cells led to a dramatic reduction in circulating leukemic cells after 12 d (Fig. 2E,F) and a significantly increased median survival of plpC-treated mice [44 d vs. 23.5 d] (Fig. 2G). Importantly, G9a deficiency similarly delayed disease progression of MN1-induced or MLL-AF9-induced experimental AML [Supplemental Fig. S5a,b], suggesting that this enzyme acts through a general mechanism and plays a role in multiple leukemias.

G9a regulates expansion and differentiation of AML cells through its methyltransferase activity

We additionally assessed the effects of G9a deletion on AML cells in vitro. G9a<sup>−/−</sup> (Vav); A9M cells displayed a substantial growth impairment, with a >2.5-fold increase in population doubling time (~37 h vs. 14 h), resulting in a vastly reduced cumulative cell output compared with controls [Fig. 3A,B]. This correlated with significantly more cells in G<sub>1</sub>/G<sub>0</sub> but no increase in apoptosis [Fig. 3C; Supplemental Fig. S6]. Importantly, loss of p53 did not rescue this growth deficiency, indicating that a p53-dependent senescence pathway was not responsible for this phenotype [Fig. 3A]. To determine whether the catalytic activity of G9a was required for efficient AML cell proliferation, we reintroduced wild-type or a methyltransferase-inactive mutant of G9a [H1166K] into G9a<sup>−/−</sup> (Vav); A9M cells using an MSCV-ires-GFP [MIG] retroviral vector [Fig. 3D]. Infection with MIG-G9a<sup>WT</sup> conferred a competitive growth advantage to transduced [GFP<sup>+</sup>] over untransduced (GFP<sup>−</sup>) cells as indicated by their rapid takeover of the culture. In contrast, control-infected [empty MIG] and MIG G9a<sup>H1166K</sup>-infected cells displayed no growth advantage or even a slight disadvantage, respectively [Fig. 3E,F]. Likewise, reintroduction of G9a<sup>WT</sup> but not G9a<sup>H1166K</sup> in G9a<sup>−/−</sup> (Vav); A9M cells restored
Figure 1. Characterization of G9a-deficient hematopoiesis. (A) Schematic representation of the G9a knockout strategy. Exons 4–20 were flanked by loxP sites to delete the central region of the gene and result in a frameshift in the SET domain coding region. Mice were routinely genotyped by PCR as shown. (B) Efficient inactivation of the targeted G9a locus in BMMs from G9a<sup>+/−</sup> (Vav) mice. Whole-cell lysates from BMMs were analyzed by Western blot. The absence of G9a and a characteristic decrease in GLP and H3K9me2 levels were observed. (C) Normal number of colony-forming cells (CFCs) in G9a<sup>+/−</sup> (Vav) mice. Whole bone marrow cells (2 × 10<sup>4</sup>) were plated in methylcellulose-based medium containing SCF, IL3, IL6, and Epo. The numbers of colonies at day 8 of culture were comparable between the G9a<sup>+/−</sup> (Vav) and G9a<sup>+/+</sup> (Vav) groups. (D) Normal distribution of CFC types in G9a<sup>+/−</sup> (Vav) mice. The relative distribution of megakaryocyte/erythrocyte (MegE), granulocytic (G), macrophage (M), granulocyte/macrophage (GM), and granulocyte/erythrocyte/macrophage/megakaryocyte (GEMM) CFCs was assessed in methylcellulose cultures from G9a<sup>+/−</sup> (Vav) and G9a<sup>+/+</sup> (Vav) bone marrow cells. No significant differences in the presence of CFUs were detectable in the absence of G9a. (E) Decreased cellular output of G9a-deficient progenitors. The total cell number of 8-d cultures starting from 2 × 10<sup>4</sup> G9a<sup>+/−</sup> (Vav) and G9a<sup>+/+</sup> (Vav) whole bone marrow cells was assessed. G9a<sup>+/−</sup> (Vav) cultures consistently yielded four to five fewer cells in total and in average per colony. A representative experiment is shown; n = 4. (F) Representative CFU-GMs of G9a<sup>+/−</sup> (Vav) and G9a<sup>+/+</sup> (Vav) origin are shown. (G) G9a is required for the activity of highly proliferative progenitors. G9a<sup>+/−</sup> (Vav) and G9a<sup>+/+</sup> (Vav) colony sizes were estimated and scored as low (<500 cells), intermediate (500–5000 cells), and high-proliferative (>5000 cells) categories. Highly proliferative clones are essentially absent in G9a<sup>+/−</sup> (Vav) bone marrow. (H) Experimental strategy to assess HSC function in the absence of G9a. Bone marrow cells [2 × 10<sup>6</sup>] from G9a<sup>+/−</sup> (Vav), R26-YFP<sup>−</sup> or G9a<sup>+/−</sup> (Vav), R26-YFP<sup>−</sup> test mice were mixed at a 50:50 ratio with YFP<sup>−</sup> competitor (G9a<sub><i>fl/fl</i></sub>) bone marrow cells and transplanted into lethally irradiated congenic CD45.1 hosts. The relative chimera in recipient mice was assessed by FACS analysis using YFP fluorescence and lineage-specific surface makers as indicated in Supplemental Figure S2d. (I) Summary of competitive bone marrow transplantation experiment 8 and 18 wk post-transplant. One representative experiment of two experiments is shown, individual bone marrow recipients are represented by dots, and significant differences by unpaired t-tests are indicated. One recipient of G9a<sup>+/−</sup> (Vav)/G9a<sub><i>fl/fl</i></sub> cells was sacrificed due to dermatitis 10 wk post-transplant.
Figure 2. G9a deficiency affects AML progression in vivo. (A) Efficient abrogation of G9a expression in G9a<sup>−/−</sup> (Vav) AML cells. 5-FU-activated G9a<sup>+/+</sup> (Vav) and G9a<sup>−/−</sup> (Vav) HSPCs were infected with a HoxA9-ires-Meis1 PGK-Neo-expressing MSCV vector and G418-selected for 5 d in vitro. Whole-cell lysates were analyzed by Western blot. No remaining G9a expression and reduced GLP and H3K9me2 levels were detectable. Four independent A9M cell lines for each genotype were analyzed. (B) Decreased AML penetrance and increased disease latency in the absence of G9a. HSPCs of the indicated genotypes were infected with HoxA9/Meis1 as described above and transplanted at a dose of 5 × 10<sup>5</sup> cells per irradiated CD45.1 recipient. Proportion of disease-free survival is plotted. (n = 14 for control; n = 15 for knockout cohort; <i>P</i> < 0.0001 using log rank test). (C) Proviral integration patterns in DNA of G9a<sup>+/+</sup> (Vav) and G9a<sup>−/−</sup> (Vav) mice suggest that G9a<sup>−/−</sup> (Vav) leukemias contain a limited repertoire of initiating cells. Genomic DNA from bone marrow cells was digested with SpeI, transferred to a nylon membrane, and hybridized with a Neo probe to detect individual integrants. (D) G9a<sup>−/−</sup> (Vav) leukemia shows reduction in LSC frequency. Bone marrow cells from leukemic mice shown in A were transplanted at increasing dilution (10<sup>5</sup> to 10<sup>3</sup> cells) in lethally irradiated secondary recipients to assess frequency of LSCs. (Left panel) G9a<sup>+/+</sup> (Vav) leukemia was used as control. (Right panel) G9a<sup>−/−</sup> (Vav) leukemia. (E) LDA to estimate LSC frequencies. Survival ratios are listed and were analyzed using an LDA software (WEHI-ELDA). Estimated LSC frequencies [indicated] decrease 20-fold in the absence of G9a. (F,G) Requirement for G9a in AML maintenance. Leukemic G9a<sup>+/+</sup> (Mx); A9M bone marrow cells (10<sup>5</sup>) from a diseased primary recipient were transplanted into irradiated secondary recipients. Control mice (G9a<sup>+/+</sup> (Mx)) were left untreated, whereas test mice (G9a<sup>−/−</sup> (Mx)) received four injections of ipiC to stimulate Cre expression and abrogate G9a expression. The abundance of peripheral AML cells was assessed by CD45.2 staining in recipients 12 d after ipiC treatment. G9a inactivation in the G9a<sup>−/−</sup> (Mx) cohort resulted in a significant decrease of peripheral AML cells. Representative FACS plot is shown in F; statistical summary is shown in G [n = 6 for untreated; n = 7 for treated; <i>P</i> < 0.0001 using unpaired <i>t</i>-test]. (H) A significant delay in AML progression in the G9a-depleted cohort (G9a<sup>−/−</sup> (Mx)) was observed [n = 6 for untreated; n = 7 for treated; <i>P</i> < 0.0001 using log rank test].
Figure 3. G9a activity determines the proliferation rate of mouse AML cells. (A) Impaired proliferation of AML cells deficient in G9a. 
A9M cells of the indicated genotypes were generated as in Figure 2A. The resulting leukemia cell lines were maintained in culture to assess their growth kinetics. G9a-deficient cultures were characterized by severely reduced proliferation. Compound deletion of p53 did not rescue this growth defect. Each individual experiment originating from distinct donor bone marrow is represented by a separate curve. (B) The average population doubling times of cultures from A was calculated and plotted. A >2.5-fold increase in net doubling time was observed in G9a-deficient cultures. (C) HoxA9/Meis1-expressing G9a+/− (Vav) cells display a decrease in cell cycle progression, indicated by a significant reduction in EdU incorporation compared with G9a+/− (Vav) control cultures. (D) Schematic representation of the used MSCV-based rescue constructs. A9M cells were infected with either empty MIG retrovirus or MIG-expressing wild-type or mutant G9a. (E,F) Rescue of G9a+/− (Vav); A9M cell growth by wild-type but not methyltransferase-inactive mutant G9aH1166K in vitro. A proliferative advantage of transduced (GFP+) cells over the remainder of the culture was observed only in G9a+/−-infected samples. (G) G9a levels and activity determine AML disease progression in vivo. G9a+/− (Vav); A9M or G9a+/− (Vav); A9M cells were infected with the indicated retroviruses and transplanted into lethally irradiated recipient mice. Reintroduction of G9aWT into G9a+/− (Vav); A9M cells restores normal AML progression. Ectopic expression of G9aWT in G9a+/− (Vav); A9M cells accelerates AML progression. n = 4 for each group; significant log rank P-values are indicated. (H) UNC0638-mediated G9a/GLP inhibition recapitulates the G9a+/− (Vav) phenotype. Treatment with 800 nM UNC0638 selectively inhibits the growth of A9M-expressing G9a−/− (Vav) but not G9a−/− (Vav) cells, demonstrating the specificity of this compound. (I) G9a inactivation results in myeloid differentiation of HoxA9/Meis1 leukemic cells. G9a+/− (Vav) and G9a+/− (Vav) were either control-treated (DMSO) or subjected to 1 µM UNC0638 for 5 d. Cytospin preparations indicate a loss of blast-like morphology and a concomitant increase in myeloid differentiation.
their ability to initiate AML in transplanted recipient mice [Fig. 3G]. Notably, ectopic expression of G9α\textsuperscript{WT} in G9α\textsuperscript{−/−} (Vav\textsuperscript{−/−}) A9M cells significantly accelerated AML progression, demonstrating that elevated levels of G9α increase AML aggressiveness in vivo [Fig. 3G]. These results suggested that catalytic inhibition of G9α should mimic the phenotype observed in G9α\textsuperscript{−/−} (Vav\textsuperscript{−/−}) cells. Indeed, treatment with the G9α/GLP inhibitor UNC0638 [Vedali et al. 2011] inhibited the growth of G9α\textsuperscript{−/−} (Vav\textsuperscript{−/−}) A9M but not G9α\textsuperscript{−/−} (Vav\textsuperscript{−/−}) A9M cells in vitro [Fig. 3H]. Furthermore, whereas G9α\textsuperscript{−/−} (Vav\textsuperscript{−/−}) A9M cells exhibited a characteristic blast-like morphology with a high nucleus to cytosol ratio and the absence of granular cytosolic structures, both G9α-deficient and UNC0638-treated AML cells displayed a significant degree of myeloid differentiation [Fig. 3I]. Taken together, these results indicate that the methyltransferase activity of G9α is critical to maintain high proliferation rates and the incomplete differentiation characteristic of AML cells.

**G9a regulates HoxA9-dependent gene expression**

G9α was previously linked to the transforming activity of Evi-1/PDm3 [Goyama et al. 2009], a Zn finger containing H3K9-specific monomethyltransferase [Pinheiro et al. 2012] whose expression is independently correlated with poor prognosis in AML. Since Evi-1 is critical for the function of HSCs as well as normal and transformed progenitors [Goyama et al. 2008], whereas G9α is not required for HSC function, we thus reasoned that G9α likely plays important roles independently of its connection with Evi-1. A number of considerations led us to hypothesize that G9α may exert its role in AML cells by facilitating HoxA9-dependent gene expression. First, although HoxA9 plays important roles in HSPCs, HoxA9-deficient mice are viable and, compared with Evi-1 mutants, display a relatively mild hematopoietic phenotype [Lawrence et al. 1997, 2005] that in some respects resembles that of G9α\textsuperscript{−/−} (Vav\textsuperscript{−/−}) mice. Second, HoxA9 elicits its oncogenic activity in AML cells by enforcing self-renewal and impairing myeloid differentiation, both of which were affected in G9α\textsuperscript{−/−} (Vav\textsuperscript{−/−}) AML cells. Third, overexpression of wild-type but not catalytically dead G9α accelerates the pathogenesis of HoxA9/Meis1-driven AML, suggesting that endogenous levels of G9α are limiting in this model and that it may play a direct role in HoxA9-regulated transcription mediated through its methyltransferase activity. To assess this hypothesis, we first asked whether a physical interaction between HoxA9 and G9α takes place. To this end, we performed coimmunoprecipitation studies using Flag- and HA-tagged versions of G9α and HoxA9, respectively. Indeed, we could detect a robust interaction between G9α and HoxA9 following immunoprecipitation with anti-Flag or anti-HA antibodies [Fig. 4A], suggesting that G9α is recruited to sites of HoxA9-dependent transcription. Next, we investigated whether the methyltransferase activity of G9α is required to facilitate HoxA9-dependent gene expression in mouse AML cells. To this end, we performed microarray analysis to assess changes in gene expression in HoxA9- and Meis1-expressing leukemic cells treated with UNC0638 for 5 d in comparison with mock-treated controls [Supplemental Fig. S7a]. We then compared this data set to that of a study that used an estrogen receptor-coupled version of HoxA9 (HoxA9-ER) together with Meis1 to generate mouse AML cells. These cells were initially cultured in the presence of 4-hydroxy-tamoxifen [4OHT] to allow HoxA9 function and then transferred to medium lacking 4OHT for 5 d prior to measuring HoxA9-dependent gene expression [Supplemental Fig. S7b, Huang et al. 2012]. Strikingly, gene set enrichment analysis (GSEA) revealed a highly significant overlap between genes that responded to UNC0638 treatment in HoxA9/Meis1 and those that responded to 4OHT withdrawal in HoxA9-ER/Meis1 cells [Fig. 4B,C]. This was consistent with a decrease in the expression of LSC-associated genes and an increase in granulocyte-specific genes in response to UNC0638 or 4OHT withdrawal in the respective data sets [Supplemental Fig. S7c,d], confirming the loss of a self-renewal signature and increased myeloid differentiation in both of these conditions. To assess whether the overlap between G9α-dependent and HoxA9-dependent gene expression changes was due to a direct effect on HoxA9 target genes or a secondary effect due to increased differentiation in both conditions, we restricted GSEA only to genes that had been shown to be bound by HoxA9 in their vicinity and whose expression either decreased or increased by >1.5-fold upon 4OHT withdrawal in HoxA9-ER/Meis1-expressing cells [Huang et al. 2012]. Importantly, we found that HoxA9-occupied genes respond to UNC0638 treatment and HoxA9 withdrawal [by removal of 4OHT] predominantly in the same way and that this overlap is highly significant in both directions [Fig. 4C,D]. Taken together, these data demonstrate that G9α partakes in the regulation of HoxA9-dependent gene expression in AML cells, suggesting that the observed phenotypes in UNC0638-treated AML cells are at least partially caused by the attenuation of a HoxA9-dependent gene signature.

**Sensitivity to G9A/GLP inhibition is conserved in human AML specimens**

To assess whether G9α inhibition also affects the growth of human AML cells, we cultured primary human AML cells [normal karyotype, NPM1\textsuperscript{WT}/FLT3\textsuperscript{ITD}] in the presence or absence of UNC0638 for up to 7 d. Reminiscent of our observation with murine A9M cells, UNC0638 inhibited human AML cell proliferation [Fig. 5A–C] and triggered differentiation. This was evident morphologically [Fig. 5D] and from an increase in the mast cell surface marker FceR1α [Fig. 5E]. In addition, UNC0638 treatment of human mobilized CD34\textsuperscript{+} HSPCs plated in methylcellulose led to a characteristic reduction in colony size identical to that observed in murine G9α\textsuperscript{−/−} (Vav\textsuperscript{−/−}) progenitor cultures [Supplemental Fig. S8]. To perform a more representative assessment of G9α's requirement in human AML proliferation, we tested the response of 15 additional and genetically diverse primary AML specimens toward
UNC0646, an improved version of the UNC0638 G9A/GLP inhibitor (Liu et al. 2011). We detected growth-suppressive activity of UNC0646 in all tested samples, albeit to varying degrees, with IC_{50} values ranging from 0.58 μM to 3.73 μM (Fig. 5F; Supplemental Fig. S9). Together, these results indicate that our findings in murine AML models can be extended to humans, demonstrating that G9A/GLP-dependent methylation is an important determinant of human AML proliferation.

**Discussion**

Our data demonstrate that G9a/GLP-dependent methylation plays an important role in the efficient repression of terminal differentiation programs in AML and thus for efficient LSC self-renewal and proliferation. Strikingly, LSCs exhibit a selective dependency on G9a compared with normal HSCs. This is consistent with the notion that oncogenic mutations of IDH1/2 (Mardis et al. 2009) result in elevated levels of histone marks like H3K9me2 deposited by G9A and GLP via inhibition of corresponding histone demethylases (Lu et al. 2012). Based on the results presented in this study, it is tempting to speculate that in an AML context, mutant IDH1/2 might specifically modulate target gene expression of HOXA9 and/or other transcription factors such as EVI-1, MYB, or MYC, whose roles in the pathogenesis of AML are at least in part mediated by G9A. While this would predict a more pronounced sensitivity of IDH1/2 mutant AML cells to inhibitors of G9A/GLP or other methyltransferases, testing of much larger cohorts of genetically characterized human AML specimens is necessary to substantiate this hypothesis.
Furthermore, it remains controversial to what extent global H3K9me2 increases during cellular differentiation (Wen et al. 2009; Lienert et al. 2011), particularly since elevated H3K9me2 and H3K9me3 often correlate with the defective differentiation found in cancerous cells (Lu et al. 2012). This controversy is further spurred by the observation that G9A/GLP inhibition can help to maintain the undifferentiated state of human HSCs under ex vivo culture conditions (Chen et al. 2012). Our data suggest that the cellular functions of G9a-dependent histone methylation are highly context-dependent and aid the stabilization of transcription programs that are specified by developmental transcription factors such as HoxA9 under normal or malignant circumstances.

From a practical perspective, this study reveals a novel and specific G9a function that is relevant to human biology and disease. By showing that G9a/GLP deletion selectively affects the proliferation of AML cells without detectable adverse effects on HSC function, our data emphasize the pharmacological inhibition of G9A/GLP as a potential targeted therapy.

**Materials and methods**

**Mouse strains**

G9a<sup>fl</sup> mice (Lehnertz et al. 2010), Vav-Cre mice (Stadtfeld and Graf 2005), and R26-YFP mice (Ye et al. 2003) were described earlier, and Mx-Cre mice were obtained from Jackson Laboratory.
All strains were maintained on a pure C57/B6 background and used between 6 and 12 wk of age. All procedures were conform with institutional guidelines.

**Antibodies for Western analysis**

Antibodies used for Western analysis were anti-G9a (clone A8620A, R&D Systems; clone C6H3, Cell Signaling Technology), anti-GLP (clone B0422, R&D Systems), anti-H3H9me2 (ab1220, Abcam), anti-H3 (ab1791, Abcam; clone C6H3, Cell Signaling Technology), anti-Flag (clone M2, Sigma-Aldrich), and anti-HA (clone 12CA5, Roche Applied Science).

**Progenitor growth assay**

Bone marrow cells were plated at 2 × 10⁴ cells per milliliter culture. Methylcellulose medium (M3434, Stem Cell Technologies) was either purchased or generated in-house. CFUs were scored at day 8 after plating.

**Competitive bone marrow transplantation experiments**

Bone marrow cells from age- and sex-matched mice were mixed 1:1 with YFP control (G9a+/+/+) bone marrow cells. We transplanted 4 × 10⁶ cells into lethally irradiated CD45.1 hosts. After 8 and 18 wk, we determined the ratios of YFP-negative (G9a+/−/−) versus YFP-positive (G9a+/−/+) or G9a+/−/− cells in donor-derived (CD45.2+) granulocytes (Gr1+, Mac1+), monocytes (Gr1−, Mac1+), T cells (CD3+), and B cells (B220+) using standard FACS antibodies on a BD LSRII.

**Bone marrow cell transduction and generation of mouse AML models**

MSCV HoxA9-ires-Neos PGK-Neo construct was obtained from Mark Kamps (University of California at San Diego). Retroviral technologies was either purchased or generated in-house. CFUs were extracted, cryopreserved, and then transplanted into lethally irradiated secondary recipient mice. Mx-Cre mice were extracted, cryopreserved, and then transplanted into sublethally irradiated secondary recipient mice. Mx-Cre+ AML cells were cultured in serum-free conditions in the presence of SR1 (Alichem, catalog no. 41864) and UM729 (Sauvageau 2013). UNC0638/UNC0646 were added to growth medium from a 5 mM DMSO stock solution.

**Gene expression and statistical analyses**

G9a-dependent gene expression was assessed by comparing DMSO- and UNC0638-treated A9M cells [800 nM, 5 d, three biological replicates per condition]. RNA was extracted with Trizol and analyzed on Affimetric Mouse Gene 1.0 chip (accessible on Gene Expression Omnibus [GEO], GSE53894). HoxA9-dependent gene expression files [Affimetric Mouse Genome 430 2.0 chip] (Huang et al. 2012) were downloaded from GEO (GSE12199). CEL files were processed using the ExpressionFileCreator (RMA method) and PreprocessDataSet modules in the GenePattern suite (Broad Institute). GSEA analysis was done using log2 transformed expression values and the “difference of classes” setting with the provided gene sets (Supplemental Table 1). All further statistical analyses were done using Flowjo (Treestar) and Graphpad Prism.

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