Meningioma-1 (MN1) overexpression is frequently observed in patients with acute myeloid leukemia (AML) and is predictive of poor prognosis. In murine models, forced expression of MN1 in hematopoietic progenitors induces an aggressive myeloid leukemia that is strictly dependent on a defined gene expression program in the cell of origin, which includes the homeobox genes Hoxa9 and Meis1 as key components. Here, we have shown that this program is controlled by two histone methyltransferases, MLL1 and DOT1L, as deletion of either Mll1 or Dot1l in MN1-expressing cells abrogated the cell of origin–derived gene expression program, including the expression of Hoxa cluster genes. In murine models, genetic inactivation of either Mll1 or Dot1l impaired MN1-mediated leukemogenesis. We determined that HOXA9 and MEIS1 are coexpressed with MN1 in a subset of clinical MN1hi leukemia, and human MN1hi/HOXA9hi leukemias were sensitive to pharmacologic inhibition of DOT1L. Together, these data point to DOT1L as a potential therapeutic target in MN1hi AML. In addition, our findings suggest that epigenetic modulation of the interplay between an oncogenic lesion and its cooperating developmental program has therapeutic potential in AML.
Meningioma-1 (MNI) overexpression is frequently observed in patients with acute myeloid leukemia (AML) and is predictive of poor prognosis. In murine models, forced expression of MNI in hematopoietic progenitors induces an aggressive myeloid leukemia that is strictly dependent on a defined gene expression program in the cell of origin, which includes the homeobox genes Hoxa9 and Meis1 as key components. Here, we have shown that this program is controlled by two histone methyltransferases, MLL1 and DOT1L, as deletion of either Mll1 or Dot1l in MNI-expressing cells abrogated the cell of origin–derived gene expression program, including the expression of Hoxa cluster genes. In murine models, genetic inactivation of either Mll1 or Dot1l impaired MNI-mediated leukemogenesis. We determined that Hoxa9 and Meis1 are coexpressed with MNI in a subset of clinical MNIhi leukemia, and human MNIhi/Hoxa9hi leukemias were sensitive to pharmacologic inhibition of DOT1L. Together, these data point to DOT1L as a potential therapeutic target in MNIhi AML. In addition, our findings suggest that epigenetic modulation of the interplay between an oncogenic lesion and its cooperating developmental program has therapeutic potential in AML.

Introduction

The meningioma-1 (MNI) gene is frequently overexpressed in acute myeloid leukemia (AML) and is associated with a poor prognosis (1–5). High MNI expression occurs across multiple cytogenetic and molecular subgroups of AML (4–6). Two distinct subtypes of AML are negatively associated with high MNI expression levels: AML with mutations in nucleophosmin 1 (NPM1) (1–5) and AML with a translocation of the mixed lineage leukemia gene, MLL (4, 6). The highest expression levels of MNI have been reported in patients with an inversion of chromosome 16 (inv[16]), and 100% of inv(16) AML overexpress MNI (4, 6). In apparent contradiction to the poor outcome associated with high MNI expression, inv(16) AML has a favorable prognosis. However, inv(16) AML represents only a small subgroup of MNI hi AML. A second subgroup associated with higher-than-average MNI expression levels is AML with a complex karyotype (4). Outcomes for AML with a complex karyotype, as well as MNI hi AML as a whole, are poor.

MNI was first described as part of a translocation in meningioma (7). MNI is a transcriptional coactivator that cooperates with the nuclear receptors for retinoic acid (RAR) (8) and vitamin D (9), possibly through direct binding of other coactivators such as RAC3 and p300/CBP (8). In AML, MNI is frequently overexpressed and occasionally fused to TEL as part of the rare MNI-TEL translocation (10). Exactly how MNI contributes to leukemogenesis is still not fully understood. A dominant negative effect on RAR/TEL signaling has been described for the MNI-TEL fusion (11), but this mechanism may not apply to MNI overexpression. Importantly, MNI has little structural similarity to any other protein (12, 13). Pharmacologic targeting of high MNI expression in AML has so far remained elusive.

MNI overexpression in murine hematopoietic progenitors induces a rapidly fatal myeloid leukemia, reflecting the strong transforming ability of MNI (6, 14–16). Forced expression of MNI induces proliferation and a block in differentiation, mapped to the N- and C-terminus of MNI, respectively (13, 16). The developmental window, during which MNI is able to induce leukemia, is narrow and well defined; neither purified hematopoietic stem cells (HSCs) nor cells beyond the common myeloid progenitor (CMP) can serve as the cell of origin (14). Heuser et al. pinpointed the transcriptional requirements for the cell of origin in the MNI leukemia mouse model. Progenitors that can serve as a target cell for MNI-mediated transformation are characterized by a distinct gene expression profile that is shut down at the transition from CMP to granulocyte macrophage progenitor (GMP). Key components of this program include the Hoxa cluster genes Hoxa9 and Hoxa10, as well as Meis1. Forced expression of Hoxa9/10 and Meis1 rendered GMPs as well as HSCs susceptible to MNI-mediated transformation. This suggests that MNI alone is unable to induce the full progenitor program (including the later Hoxa cluster)
required for transformation. However, expression of Hoxa9/10 and Meis1 remains high in MN1-AML cells with phenotypic and morphologic characteristics beyond the CMP stage; thus, MN1 may prevent the developmentally appropriate shutdown of these important loci at the CMP-to-GMP transition. Despite the ability to induce leukemia as a single hit in murine CMPs, forced expression of MN1 failed to fully transform human umbilical cord blood cells. However, cotransduction of MN1 and NUP98-HOXD13 resulted in full transformation to AML. This confirms the central role of homeobox transcription factors and their collaboration with MN1 in AML leukemogenesis (17).

The later Hoxa cluster genes and Meis1 are also critical direct-binding targets of fusions of the mixed lineage leukemia gene, MLL. MLL rearrangements occur in both myeloid malignancies and acute lymphoblastic leukemia (ALL) and are associated with a poor prognosis, particularly in infant ALL. We recently showed that the histone methyltransferase DOT1L is absolutely required in MLL-AF9 leukemia and that the mechanism involves a specific dependence of MLL fusion target gene expression on functional DOT1L (18). Based on these data, a small molecule inhibitor of DOT1L is currently in phase I/II clinical development for MLL-rearranged leukemia (19, 20). DOT1L methylates histone 3 on lysine 79 (H3K79); it is the predominant H3K79 methyltransferase and responsible for monomethylation (me1), dimethylation (me2), and trimethylation (me3) of H3K79 (21). H3K79 methylation is present on MLL fusion target genes in MLL-rearranged leukemia but also on the later Hoxa cluster genes and Meis1 in normal hematopoietic progenitors at the developmental stage where these genes are highly expressed (18). The later Hoxa cluster and Meis1 are also regulated in a DOT1L-dependent manner in non-MLL-rearranged CALM-AF10 (22) and NUP98-NSDI (23) fusion-driven leukemia.

Given the poor outcome associated with high MN1 expression and the ability of MN1 to act as a strong oncogenic driver, MN1 could represent an attractive target for therapeutic intervention. However, no small molecule inhibitor for MN1 has been described so far. Based on the dependence of MN1-driven leukemia on HOXA9 expression, we hypothesized that leukemias driven by MN1 are sensitive to DOT1L inhibition via downregulation of the cooperating later HOXA cluster genes. This would suggest that DOT1L could be a therapeutic target in MN1+ AML.

Results

Loss of Dot1l in normal early hematopoietic progenitors leads to downregulation of a distinct gene expression program. We aimed to delineate early gene expression changes that occur after genetic inactivation of Dot1l in normal hematopoiesis. To this end, we crossed conditional Dot1l+/– mice into the Mxl-Cre model, which allows rapid and precise excision of exon 5 of the Dot1l gene (which contains most of the active site) after 3 doses of polyinosinic:polycytidylic acid (pI:pC) (Supplemental Figure 1, A and B; supplemental material available online with this article; doi:10.1172/JCI80825DS1). Induced Dot1l+/– Mxl-Cre mice developed pancytopenia similar to data previously reported for conditional Dot1l inactivation models using tamoxifen-inducible systems (Figure 1A and refs. 24, 25). In the Mxl-Cre model, loss of functional Dot1l was confined to the hematopoietic system, and the high efficiency of the Mxl-Cre promoter allowed analysis of cell-autonomous gene expression changes at a defined early time point. We isolated Lin–Sca-1+c-Kit+ (LSK) cells 6 days after pI:pC injection. The interferon response elicited by pI:pC treatment has been shown to lead to a temporary loss of quiescence in the HSC compartment and distorts the ability to isolate HSC/progenitors using flowcytometry.

Figure 1. The MN1 cooperating program is enriched in Dot1l WT versus KO LSK cells. (A) Total WBC, hemoglobin (Hb), and platelet (Plt) count in Dot1l+/+ (fl/fl, n = 10) and Mxl-Cre Dot1l+/– (–/–, n = 9) mice 3 weeks after the induction of Cre. Two-sided t test Dot1l+/+ vs. Dot1l+/–, *P < 0.05. (B) Heatmap of expression array data of sorted LSK cells from Dot1l+/+ (fl/fl) and Mxl-Cre Dot1l+/– (–/–) mice 12 days after induction of Cre. Shown are all probe sets/genes with differential expression at P < 0.05 (393 genes), as well as a list of the top 30 differentially expressed probe sets and Meis1; n = 6 mice per group. (C) Venn diagram of genes associated with H3K79 dimethylation in LSK cells by ChIP-Seq (18) and genes downregulated after loss of Dot1l in LSK cells. (D) GSEA showing enrichment of the CMP/MN1 program in Dot1l+/+ vs. Dot1l+/– LSK cells. NES and P value according to ref. 43.
Figure 2. Loss of Dot1l leads to decreased growth, increased differentiation, and apoptosis of MN1-driven CMP-derived murine leukemia cells. (A) Serial replating of MN1-transformed CMPs (MN1CMP-T) after Cre-induced loss of Dot1l. Left plot: number of colonies per 500 plated cells; right plot: total cell number. n = 5 independent experiments; two-sided t test Dot1l+/− vs. Dot1l−/−. Error bars represent ±SEM. (B) Serial replating of MN1-driven murine leukemias (MN1CMP-L) after Cre-induced loss of Dot1l. Left plot: number of colonies per 500 plated cells; right plot: total cell number. Dot1l−/−: bulk population from 3 independent experiments; Dot1l+/−: 2 bulk populations and 4 individually picked clones (due to outgrowth of nondeleted cells) from 3 independent experiments. Two-sided t test Dot1l+/− vs. Dot1l−/−. Error bars represent ±SEM. (C) Methylcellulose colony and leukemia cell morphology (Wright-Giemsa stain) of MN1-driven murine leukemias (MN1CMP-L) 14 days after transduction with Cre. Representative of 3 independent experiments. (D) CD11b expression in MN1-driven murine leukemias (MN1CMP-L) 3 weeks after deletion of Dot1l. Dot1l−/−: bulk population from 3 independent experiments; Dot1l+/−: 2 bulk populations and 4 individually picked clones (due to outgrowth of nondeleted cells) from 3 independent experiments. Two-sided t test Dot1l+/− vs. Dot1l−/−. Error bars represent ±SEM. (E) Apoptosis (Annexin staining) in MN1-driven murine leukemias (MN1CMP-L) 3 weeks after deletion of Dot1l. Dot1l−/−: bulk population from 3 independent experiments; Dot1l+/−: 2 bulk population and 4 individually picked clones (due to outgrowth of nondeleted cells) from 3 independent experiments. Two-sided t test Dot1l+/− vs. Dot1l−/−. Error bars represent ±SEM. (F) Cell cycle distribution (EdU incorporation/DAPI staining) in MN1-driven murine leukemias (MN1CMP-L) 3 weeks after deletion of Dot1l. Dot1l−/−: bulk population from 3 independent experiments; Dot1l+/−: 2 bulk population and 4 individually picked clones (due to outgrowth of nondeleted cells) from 3 independent experiments. Two-sided t test Dot1l+/− vs. Dot1l−/−. Error bars represent ±SEM.

ric markers. However, these effects were resolved after 5 days (26), and flowcytometric analysis of plpC-injected animals performed 6 days after the last dose showed a clearly distinguishable LSK population (Supplemental Figure 1C). We performed gene expression analyses comparing plpC-injected animals vs. littermates (Supplemental Figure 1C). We defined the set of genes with decreased expression in Dot1l+/− LSK cells as “DOT1L-dependent in LSK” (Supplemental Table 2). As expected, the majority of the genes that were dependent on DOT1L in LSK cells were associated with high levels of H3K79 dimethylation downstream of the transcription start site (as determined by ChIP sequencing [ChIP-Seq] in ref. 18 and Figure 1C). Gene set enrichment analysis indicated that this gene set has significant overlap with genes regulated by DOT1L in MLL-rearranged leukemias, including the key MLL-fusion downstream target genes Hoxa9 and Meis1 (Supplemental Figure 1D). In addition, many of these genes are downregulated at the LSK-to-GMP transition (Supplemental Figure 1E).

The MN1 leukemogenic gene expression program is dependent on functional DOT1L in LSK cells. Heuser et al. (14) reported that a specific gene expression program in CMPs overlapped with the gene expression program defining MN1 leukemias, and key components of this CMP program cooperated with MN1 to cause myeloid leukemia. We asked whether the MN1 gene expression program (i.e., high in CMP vs. GMP, and MN1 vs. GR1/CD11b+) in ref. 14, “CMP/MN1 program”), is dependent on DOT1L in normal early hematopoietic progenitors. Indeed, gene set enrichment analysis (GSEA) demonstrated a strong enrichment of the CMP/MN1 program gene set in the DOT1L-dependent gene set in normal LSK cells (increased in Dot1l+/− vs. Dot1l−/−) (Figure 1D), as well as in the DOT1L-dependent genes in MLL-AF9 leukemias (Supplemental Figure 1F).
MN1-induced CMP-derived AML is dependent on functional Dot1l. Hoxa9/Meis1 expression in the cell of origin is critically important for the ability of MN1 to induce AML. Based on our findings showing that Hoxa9, Hoxa10, and Meis1 expression are dependent on functional DOT1L in early hematopoietic progenitors, we asked whether this dependency on DOT1L was preserved in MN1 leukemias. We introduced the human MN1 cDNA into sorted Dot1lfl/fl CMPs to establish in vitro transformed MN1CMP-T. Deletion of Dot1l through introduction of Cre (Dot1l<–/–MN1CMP-T) resulted in reduced cell numbers and colonies in serial replating assays (Figure 2A). We also injected MN1-transduced Dot1lfl/fl CMPs into recipient mice to establish Dot1l conditional leukemias (MN1CMP-L). Excision of exon 5 of Dot1l in MN1CMP-L isolated from moribund mice again resulted in decreased replating efficiency and decreased cell numbers (Figure 2B). Dot1l<–/– colonies were smaller (Figure 2C, left panel), and Dot1l<–/–MN1CMP-L cells showed morphologic signs consistent with increased differentiation (Figure 2C, right panel). This was reflected in an increase in the expression of the myeloid differentiation marker CD11b in Dot1l<–/–MN1CMP-L (Figure 2D). Loss of Dot1l also resulted in increased apoptosis (Figure 2E) and a decrease in the fraction of cycling cells (Figure 2F). Similar results were observed in Dot1l<–/–MN1CMP-T cells (Supplemental Figure 2, A–C). While the smaller colony size and increased differentiation mimicked the effect of loss of Dot1l in MLL-rearranged leukemias, we observed several subtle differences between the two models. Loss of Dot1l in MLL-rearranged leukemias causes a minimal increase in apoptosis, while apoptosis in Dot1l<–/– MN1 leukemias was more pronounced. More importantly, while we were unable to isolate viable and proliferating Dot1l<–/– MLL-AF9 cells beyond the third replating, serial replating of Dot1l<–/– MN1 leukemias was inefficient but possible.

We next analyzed the effect of loss of functional DOT1L on in vivo MN1-leukemias. Dot1lfl/fl MN1CMP-T were transduced with Cre (or control) and injected into primary recipients to interrogate leukemia initiation. To assess the effect of loss of DOT1L in leukemia maintenance, Dot1lfl/fl MN1CMP-L were isolated from moribund mice, transduced with Cre (or control), and transplanted into secondary recipients. While control mice succumbed to leukemia within 3–4 weeks, Dot1l<–/–MN1CMP-T or MN1CMP-L–injected animals experienced significantly decreased leukemic burden (Figure 3, A and C) and prolonged survival (Figure 3, B and D). All leukemias that eventually did develop in Cre-transduced cohorts were found to contain at least one floxed (unexcised) Dot1l allele by genotyping (Supplemental Figure 3) and had thus escaped full genetic inactivation of Dot1l.

Loss of DOT1L leads to downregulation of the MN1-cooperating program in MN1-transformed CMPs. We next determined the H3K79 methylation status of the MN1-bound genes using ChIP-Seq for H3K79me2 in MN1-driven leukemias. Indeed, high levels of H3K79 dimethylation were observed close to the transcription start site of the gene set that was previously published to be bound by MN1 (ref. 14, Figure 4, A and B, and Supplemental Table 2). We next asked whether loss of functional Dot1l in CMP-derived MN1-transformed cells resulted in downregulation of the CMP/MN1 program, including the key loci Hoxa9 and Meis1. We performed quantitative PCR (qPCR) analysis of MN1CMP-T 21 days after excision of exon 5 of Dot1l and found substantial downregulation of Hoxa9 (several 100-fold) (Figure 4C). Meis1, although also affected, was downregulated less consistently and to a lesser extent (0–5 fold, not statistically significant by qPCR). In order to evaluate gene expression changes on a whole transcriptome scale, we performed RNA-Seq of MN1CMP-T 7 days after introduction of Cre. Similar to the loss of Dot1l in other model systems, a defined gene set was found to have decreased expression after loss of Dot1l (Figure 4D and Supplemental Table 3). The later Hoxa cluster genes (Hoxa7, Hoxa9, Hoxa10, and Hoxa11) were among the most dysregulated genes. The previously defined CMP/MN1 program showed significant enrichment in Dot1lfl/fl vs. Dot1l<–/–MN1CMP-Ts, suggesting dependence of this program on functional DOT1L. The majority of these genes are associated with H3K79 methylation and bound by MN1 (Figure 4F). H3K79me2 ChIP-Seq tracks for the Hoxa cluster are shown in Figure 4G.
We also assessed enrichment of the genes dependent on DOT1L in MN1CMP-T (“Dot1l-dependent in MN1”) (Supplemental Table 2) in normal LSK vs. GMP, LSK Dot1lfl/fl vs. Dot1l−/−, and MLL-AF9 leukemia Dot1lfl/fl vs. Dot1l−/− data sets (Supplemental Figure 4, A–C); we found enrichment in normal LSK (vs. GMP or Dot1l−/− LSK) and Dot1lfl/fl MLL-AF9 leukemias (vs. Dot1l−/−). Finally, we asked whether the Dot1l-dependent in MN1 signature was also enriched in a second MN1-driven murine leukemia gene expression profile (16). Indeed, significant enrichment in MN1-transformed CMPs vs. normal CMPs was confirmed in this independent data set (Supplemental Figure 4D).

**MN1-HSC-T growth and Hoxa9/Meis1 expression are independent of DOT1L in vitro.** We isolated CMPs for transduction with MN1 in the experiments described above based on published results indicating transformed CMPs are the most efficient cell of origin in this model: MN1-transduced CMPs readily caused leukemia in recipient mice within 30–90 days, while MN1-transduced HSCs did not (14). Despite their inability to cause leukemia in mice, MN1-transduced HSC were able to serially replate. This was attributed to a lower expression of Hoxa9 in HSC-derived leukemias compared with CMPs — enough to allow in vitro immortalization but not enough to cause leukemia in an in vivo model (14). We asked whether DOT1L was also required for the (lower) Hoxa9 expression in MN1-transformed HSCs.

We isolated HSC-enriched populations from donor mice using two well-established flow cytometric approaches, LT-HSCs and LSK-SLAM. As previously reported, introduction of MN1 conferred serial replate potential to HSCs. We termed MN1-transduced...
HSCs that were maintained in vitro MN1HSC-T (transformed). To our surprise, MN1HSC-T grew very well in vitro in complete absence of functional DOT1L (Figure 5A and Supplemental Figure 5, A–C), and Dot1l−/− MN1HSC-T were capable of forming blast-like colonies in methylcellulose (Figure 5B, left panel). In contrast to MN1-transformed LT-HSCs, MLL-AF9–transformed LT-HSCs required functional DOT1L. Dot1l−/− MLL-AF9HSC-T formed less cellular and more dispersed colonies than Dot1lfl/fl MLL-AF9HSC-T, similar to what was previously observed with lineage-depleted or LSK-derived MLL-AF9 transformed cells (Figure 5B, right panels, and ref. 18). MLL-AF9–transformed LT-HSCs readily caused leukemia in mice, consistent with previously published results (27), and resulting leukemias were also DOT1L dependent (Supplemental Figure 5D).

We next investigated the transcriptional consequences of loss of Dot1l in MN1HSC-T and MN1-CMP-T. MN1-transformed HSCs had previously been reported to express lower levels of Hoxa9 than MN1-transformed CMPs (14), and we confirmed this result (Supplemental Figure 5E, white bars). Of note, albeit lower, Hoxa9 was still clearly detectable in MN1HSC-T. Consistent with the lack of phenotypic changes, we did not find any statistically significant changes in Hoxa9 and Meis1 expression in MN1HSC-T after inactivation of Dot1l (Figure 5C). DOT1L-independent Hoxa9 expression and growth were exclusively observed in vitro. Transplantation experiments confirmed that HSCs are inferior to CMPs as cell of origin for MN1-driven AML (Supplemental Figure 5F). Leukemias that did grow in vivo after injection of a large cell dose displayed Hoxa9 expression levels and regulation similar to CMP-derived leukemias (Supplemental Figure 5, G and H) and required functional DOT1L (Supplemental Figure 5, I and J). In addition, secondary transplants of MN1HSC-L were also DOT1L dependent (Supplemental Figure 5, K and L) and revealed no substantial differences in penetrance, latency, phenotype, or clinical presentation to MN1-CMP-L (Supplemental Figure 5, M–P).

High Hoxa9 expression is not universally associated with DOT1L dependence. The results from the MN1HSC-T suggest that Hoxa9 expression can be regulated in two distinct manners contingent on cellular or developmental context: DOT1L dependent and DOT1L independent. We asked whether DOT1L-independent Hoxa9 expression is observed in human leukemias. To this end, we screened a small panel of leukemia cell lines for MN1 and Hoxa9 expression (Figure 6, A and B). In this panel, we identified the Mutz3 cell line as having high MN1 (consistent with previous reports; ref. 28) and high Hoxa9 expression. In addition, high Hoxa9 expression at a level comparable or greater than the MLL-rearranged control cell lines (MV4-11 and Molm14) was found in the early T cell precursor (ETP) ALL cell line Loucy, and in the AML cell line KG1 and its subline KG1a. We inhibited H3K79 methylation in KG1, KG1a, Loucy, and Mutz3 cells using the DOT1L inhibitor EPZ004777 (19). Despite high levels of Hoxa9 expression (Figure 6A) and efficient decrease of the H3K79 methyl mark by Western blotting (Figure 6B), cell cycle, or apoptosis (Supplemental Figure 6, A and B). On a transcriptional level, there was no difference in Hoxa9 expression with or without DOT1L inhibition (Supplemental Figure 6C). Despite a profound decrease in global H3K79 methylation, KG1 and KG1a retained some residual ChIP signal on the HOXA cluster (data not shown). We therefore cannot exclude that residual low-level H3K79me2 contributed to maintaining Hoxa9 expression in these cells. However, ChIP-Seq over the HOXA cluster in Loucy cells demonstrated the complete absence of H3K79 methylation, yet Hoxa9 expression was not affected (Figure 6D). Hoxa9 expression is therefore independent of DOT1L in defined cellular contexts. In contrast, the MN1-hoxa9w HOXA9w AML cell line Mutz3 was very sensitive to DOT1L inhibition, as demonstrated by decreased growth, increased apoptosis, and decreased cell cycle and HOXA9 expression (Figure 6, G–J).

**Genetic inactivation of Mll1 impairs MN1-driven leukemogenesis.** We next asked what could explain the DOT1L dependence of the MN1 leukemogenic program, since not all HOXA9 cluster expression appears to be DOT1L dependent. We had previously noted that WT MLL1 and DOT1L regulate very similar programs in LSK cells. We therefore asked whether MN1 cooperates with WT MLL1,
Figure 6. **MN1hi/HOXA9hi** Mutz3 cells respond to DOT1L inhibition while several other cell lines with high HOXA9 expression are unaffected. (A and B) qPCR for HOXA9 (A) and MN1 (B) in HOXA9 expressing non–MLL-rearranged (non–MLL-r) cell lines: Loucy (early T cell precursor ALL), KG1 (AML), KG1a (AML), and Mutz3 (AML). MLL-rearranged controls: MV4;11 (AML), Molm14 (AML). Negative control (NC): Jurkat. Each bar represents fold-change compared with Molm14 (set to 1); n = 3. Two-sided t test Dot1l−/− vs. Dot1lfl/fl. Error bars represent ±SEM; *P < 0.01 (ANOVA). (C) Western blot showing H3K79 dimethylation in Loucy, Mutz3, KG1, and KG1a cell lines exposed to the indicated concentration of the DOT1L inhibitor EPZ004777 (EPZ). KG1 and KG1a express high levels of the efflux pump ABCB1/MDR1/P-GP, requiring blockade of the drug transported with verapamil or CSA to achieve efficient decrease in H3K79 methylation. (D) IGV tracks of Loucy, Mutz3, and Molm14 (Molm) treated with DMSO control (C) or 2 μM EPZ (E) over the HOXA9 locus confirm complete reduction of H3K79 dimethylation to background levels in treated cells. (E–G) Exposure of Loucy (E), KG1 and KG1a (F) to EPZ at the indicated concentrations. Shown are fold-expansion over a 14-day culture period (serial cell counts and Trypan Blue staining; n = 3 independent experiments performed in duplicate; two-sided t test; error bars represent ±SEM). (G–J) Exposure of Mutz3 to EPZ at the indicated concentrations. Shown are fold-expansion over a 14-day culture period (serial cell counts and Trypan Blue staining) (G), apoptosis (Annexin staining) (H), cell cycle (% cells in S-phase, EdU incorporation) (I), and HOXA9 expression (qPCR, fold change compared with DMSO set to 1) (J). n = 3 independent experiments performed in duplicate. Two-sided t test. Error bars represent ±SEM; *P < 0.05, **P < 0.01.
Figure 7. Loss of MLL1 leads to decreased growth, increased apoptosis, and decreased in vivo leukemogenic activity of MN1-driven CMP-derived murine leukemia cells. (A) Serial replating of MN1-driven murine leukemias (MN1CMP-L) after Cre-induced loss of MLL1. Left plot: number of colonies per 500 plated cells; right plot: total cell number. Bulk population from 2 independent experiments; two-sided t test in Mll1fl/fl vs. Mll1–/–. Error bars represent ±SE; *P < 0.05. (B) Apoptosis (Annexin staining) in MN1-driven murine leukemias (MN1CMP-L) 1 week after deletion of MLL1. Bulk population from 2 independent experiments. Two-sided t test in Mll1fl/fl vs. Mll1–/–. Error bars represent ±SE; *P < 0.05. (C) Cell cycle distribution (EdU incorporation/DAPI staining) in MN1-driven murine leukemias (MN1CMP-L) 3 weeks after deletion of MLL1. Bulk population from 2 independent experiments. Two-sided t test in Mll1fl/fl vs. Mll1–/–. Error bars represent ±SE; *P < 0.05. (D) Survival of recipients of 100,000 MN1-driven CMP-derived leukemias (MN1CMP-L) transduced with Cre (Mll1–/–) or control (Mll1fl/fl) vector (leukemia maintenance). n = 10 per group; P < 0.0001 (Mantel-Cox). --, leukemia originating from Mll1–/– Mll1fl/fl mice; **, leukemia originating from Mll1–/– Mll1fl/fl (Supplemental Table 1 in ref. 14) strongly enriched in CMPs compared with differentiation stage matched controls. (E) GSEA showing enrichment of the DOT1L-dependent in MN1 leukemia program in Mll1fl/fl vs. Mll1–/– MN1CMP-L. NES and P value according to ref. 43. (F) GSEA showing enrichment of the CMP/MN1 program in Mll1fl/fl vs. Mll1–/– MN1CMP-L. NES P value according to ref. 43.

explaining the dependence on DOT1L. We transduced CMPs from Mll1 conditional KO mice (29) with MN1 and established Mll1fl/fl MN1 leukemias. Similar to what we observed in the Dot1l conditional model, Cre-mediated inactivation of Mll1 resulted in decreased colony formation and cell growth, as well as increased apoptosis (Figure 7, A–C). Results from in vitro transformed cells also support a critical functional role for MLL1 (Supplemental Figure 7A). In vivo recipients of Mll1–/– MN1 leukemias had significantly prolonged survival compared with recipients of Mll1fl/fl MN1 leukemias (Figure 7D and Supplemental Figure 7B). We next asked whether MLL1 and DOT1L regulated similar gene expression programs in MN1 leukemias. Indeed, the top 200 downregulated genes after genetic inactivation of Dot1l in MN1-transformed cells were strongly enriched in the genes downregulated upon genetic inactivation of Mll1 (Figure 7E and Supplemental Tables 2 and 3). Similarly, the most dysregulated genes in MN1-transduced CMPs compared with differentiation stage matched controls (Supplemental Table 1 in ref. 14) strongly enriched in Mll1fl/fl versus Mll1–/– MN1 leukemias (Figure 7F). The gene expression program that requires functional MLL1 in MN1 leukemias also substantially overlaps with the DOT1L-dependent program in MLL-AF9-driven leukemias and in normal LSK cells (Supplemental Figure 7, C and D). Taken together, our data in the Mll1 conditional loss of function model suggest that MLL1 and MN1 coregulate a DOT1L-dependent leukemogenic gene expression program.

High HOXA9 expression is observed in a subgroup of AML patient samples with high MN1 expression. Results from our Dot1l and Mll1 conditional mouse model and cell line data suggest that MN1-driven leukemias are dependent on high levels of HOXA9 expression, which in turn is regulated by MLL1 and DOT1L. This raises the possibility that targeting DOT1L could have therapeutic efficacy in MN1-driven AML. However, high MN1 expression in clinical AML is observed over a broad range of phenotypic, cytogenetic, and molecular subgroups, a heterogeneity that is not well captured in the retroviral MN1-overexpression mouse model or a single cell line. In order to investigate a potential role for DOT1L in clinical MN1-driven AML, we first asked whether HOXA9 and MEIS1 are coexpressed with MN1 in a substantial number of primary AML patient samples. We performed qPCR analysis of MN1, HOXA9, and MEIS1 from RNA of 25 primary AML patient samples (patient characteristics are summarized in Supplemental Table 4). MN1 is shown dichotomized at the median, the most commonly used cut-off to correlate MN1 with cytogenetics and outcome (Figure 8A, left axis, and refs. 1, 2, 5). Whenever possible, we confirmed MN1 expression levels with a second, standardized qPCR-based assay that reports ratios of MN1/ABL1 (Supplemental Figure 8A). We observed HOXA9 expression in 6 of 12 AML samples with high MN1 expression (Figure 8A, right axis). Elevated MEIS1 expression was observed in all HOXA9-expressing samples (Supplemental Figure 8, B and C). Moderately high MEIS1 expression was also
Figure 8. HOXA9 expression and sensitivity to DOT1L inhibition in MN1™ AML patient samples. (A) qPCR analysis of MN1 (left axis) and HOXA9 (right axis) in 25 primary patient AML samples. MN1 expression values are shown as fold-enrichment compared with normal CD33+ myeloid progenitors and plotted dichotomized at the median (median = 70-fold overexpression). HOXA9 values are plotted as fold-enrichment compared with AML25 (MLL-rearranged, with known high HOXA9 expression set to 1). Error bars represent ±SEM of 2–3 technical replicates. n.d., not detected. (B) MN1 and HOXA9 expression by genotype in Wouters Leukemia data set (Oncomine). Full legend: 0, not determined (90); 1, +8 (20); 2, -5/7(Q) (29); 3, -q (6); 4, 1q23 (10); 5, complex (13); 6, failure (12); 7, MDS -q(Q) (2); 8, MDS -Y (2); 9, MDS complex (3); 10, normal (187); 11, other (53); 12, abn(3q) (2); 13, idv(16) (34); 14, t(15;17) (21); 15, t(6;9) (6); 16, t(8;21) (35); 17, t(9;22) (2). n = 526 AML samples. (C–F) Exposure of primary patient AML samples to the DOT1L inhibitor EPZ004777 at the indicated concentrations. AML12 (MLL-rearranged [MLL-r], positive control), AML38 (high MN1/HOXA9, complex karyotype with 5q-), AML 40 (high MN1/HOXA9, complex karyotype with 5q-), AML51 (inv[16], high MN1/no HOXA9), and AML24 (AML/ETO, intermediate high MN1/no HOXA9). Shown are fold-expansion over a 14-day culture period (serial cell counts and Trypan Blue staining; error bars represent duplicate counts) (C). Wright-Giemsa stain on cytospin of AML 40 (high MN1/HOXA9, complex karyotype with 5q-) treated with DMSO or EPZ004777 (D). HOXA9 expression in AML 38 and 40 (high MN1/HOXA9) treated with DMSO or EPZ004777. Error bars over technical replicates; *P < 0.05 (two-sided t test) (E). Summary of cell growth of 3 inv(16) AML patient samples treated with DMSO or EPZ004777 (F). n = 3; *P < 0.05 (two-sided t test).
observed in several HOXA9+ samples, including those with inv(16) (Supplemental Figure 8, B and C). Correlation with cytogenetics revealed that 3 samples with high MNI/HOXA9 expression had a complex karyotype with loss of 5q and/or 7q sequences (AML 38, 19, and 40). On the other hand, we found that 6 of 12 AML samples with high MNI expression had no detectable HOXA9/MEIS1 expression. The highest MNI expression level in this group was observed in 2 samples with inv(16) (AML 2 and 6), which has previously been shown to be universally associated with MNI overexpression (4, 6). Analysis of a well-annotated publicly available data set confirmed our results in a larger cohort of patients (30). The highest MNI expression was found to be associated with two distinct cytogenetic subgroups, inv(16) and 5q-/7q-. As in our smaller cohort, HOXA9 was overexpressed in 5q-/7q-, but not inv(16) AML (Figure 8B and Supplemental Figure 8D). AML with complex karyotype and 5q-/7q- often arises from myelodysplastic syndrome and is associated with poor outcome.

Two MNI+/HOXA9hi human AML samples are sensitive to DOT1L inhibition. We next asked whether MNI+/HOXA9hi AML samples are sensitive to pharmacologic inhibition of DOT1L. Viably frozen cells were available from 2 patients (AML 38 and AML 40). Primary AML samples were maintained in culture on a feeder layer as recently described (31, 32) and exposed to the DOT1L inhibitor EPZ004777 in vitro (19). In AML 38 and 40, EPZ004777 induced a dose-dependent decrease in cell growth and in the fraction of cycling cells, as well as an increase in apoptosis (Figure 8C and Supplemental Figure 8E). The observed effect was in a range comparable to AML 12 (MLL-rearranged). We also observed a dose-dependent upregulation of CD14, as well as a decrease in the nucleus/cytoplasm (N:C) ratio and increased vacuolization on cytosin consistent with differentiation (Figure 8D and Supplemental Figure 8E; differentiation was not assessed for sample AML 38). The observed phenotypic effects were associated with downregulation of HOXA9 (Figure 8E).

The very high MN1 levels in inv(16) AML also raised the questions of whether the cooperation between MNI and DOT1L is more universal and whether DOT1L might play a role in this subtype. We therefore included 3 inv(16) primary patient samples in our analysis. Serial cell counts for AML 51 are shown in Figure 8C. Drug response for all 3 primary samples is summarized in Figure 8F and suggests sensitivity to DOT1L inhibition in inv(16) AML. EPZ004777 administered together with verapamil to overcome MDR1-mediated exports also resulted in decreased growth and viability of the inv(16) cell line Me1 (Supplemental Figure 8, G and H). The mechanism for the effect of EPZ004777 in inv(16) AML is almost certainly different, given the low levels of HOXA cluster expression typically observed in this subtype of AML. We performed transcriptome analysis of EPZ004777-treated and control inv(16) primary patient samples to delineate which gene sets are downregulated after DOT1L inhibition. We observed downregulation of gene sets associated with growth and cell cycle, consistent with the phenotypic results. Finally, we tested the susceptibility of AML 24 (AML/ETO) to DOT1L inhibition and found it to be unaffected (Figure 8C). This sample displayed moderately high MNI expression—above the median but not as high as the inv(16) and 5q-/7q-/complex karyotype samples. Furthermore, this sample did not have any detectable expression of HOXA9.

Discussion

A previous study has identified a developmental gene expression program in CMPs that is downregulated at the transition to GMPs and is shared with MNI-driven murine leukemia. Key components of this program cooperate with forced expression of MNI to cause AML (14). Our results suggest that expression of this program is regulated by two specific epigenetic modifiers, the histone methyltransferases MLL1 and DOTIL. CMP-derived MNI-transformed leukemias responded to conditional genetic inactivation of both Mll1 and Dotil with an increase in apoptosis and differentiation and a decrease in the fraction of cycling cells, as well as a decrease in ability to form leukemia in vivo. On a transcriptional level, loss of both MLL1 and DOTIL results in the downregulation of similar gene sets. These changes were associated with a collapse of the “MNI susceptibility program,” which included, as key players, the later HOXA cluster genes (HOXA7-13). Our data suggests a model wherein the aberrant expression of HOXA cluster genes (particularly HOXA9) beyond the normal developmental stage of a CMP can be caused by aberrant activation of MLL1 via translocation, partial tandem duplication, or overexpression of the transcriptional coregulator MN1. In each case, persistent expression of this program is dependent on DOT1L. A pharmacologic inhibitor of DOT1L is currently in clinical trials for leukemia carrying MLL-rearrangements (MLL-translocations and partial tandem duplications [ref. 20], ClinicalTrials.gov, NCT02141828).

Interestingly, MN1-transduced HSCs gain serial replating capabilities in vitro but are inferior to CMPs in causing in vivo leukemia in recipient animals. This phenotype has been linked to a lower expression of Hoxa9 in MNI-transduced HSCs (14). It is in clear contrast to cell-of-origin requirements in the MLL-AF9-driven mouse leukemia model; forced expression of MLL-AF9 induces high-level Hoxa9 expression and in vivo leukemogenic potential in cell-of-origin populations spanning the developmental stages of LT-HSC through GMPs, where the Hoxa9 cluster is silenced (27, 33, 34). It appears thus that MLL-AF9 is capable of directly increasing or reactivating the expression of later HOXA cluster genes in GMPs, while the transforming potential of MN1 is dependent on the endogenous expression levels of HOXA cluster genes in the cell of origin (14). The discrepancy in the efficiency with which MN1 and MLL-AF9 are able to cause full leukemic transformation in HSCs is probably more complex. We confirmed the lower Hoxa9 expression in HSC-derived MN1-transformed cells. Interestingly, there is no difference in Hoxa9 expression in normal HSCs and uncommitted progenitors (data not shown and ref. 35); thus, the failure of forced MN1 expression to cause leukemia has different causes in GMPs and HSCs.

Somewhat unexpectedly, we found Hoxa9 expression in HSCs immortalized by forced expression of MN1 to be independent of DOT1L. Hoxa9 is overexpressed in about 50% of AML (36), and most recent data has suggested that normal as well as malignant later HOXA cluster expression may require DOT1L independent of the expression of a leukemogenic fusion protein that could mediate direct recruitment (23, 37, 38). We find that functional DOT1L is not universally required for high HOXA9 expression. The myeloid cell line KG1 and its subline KG1a, as well as the T-ALL cell line Loucy, all express HOXA9 at a level similar or
higher to MLL-rearranged AML cell lines, yet HOXA9 expression — as well as cell growth and viability — are not affected by a substantial decrease (KG1, KG1a) or complete obliteration of H3K79 methylation globally and on the HOXA locus. Therefore, HOXA9 expression does not automatically imply DOT1L dependence. This underscores the need for in-depth mechanistic studies to identify subsets of AML that might be candidates for modulation of H3K79 methylation as a therapeutic approach.

The murine AML model caused by forced expression of MN1 in CMPs is consistently associated with overexpression of HOXA9 and Meis1, which are critical for leukemogenesis. In contrast, human AML with high MN1 expression is considerably less uniform in patients. MN1 expression does not occur in two distinct clusters, but rather in a continuum, and is found across multiple phenotypic and cytogenetic subgroups. This poses considerable difficulties in defining a clinically meaningful cut-off, reflected in the inconsistencies of what constitutes high MN1 in several studies that investigated the prognostic impact of MN1 expression levels. We provide quantitative expression levels in a subset of samples in our cohort, which suggest that levels of >10 copies over ABL may be associated with biologically relevant MN1 expression levels and DOT1L sensitivity. Similarly, expression levels at or above that of the Mutz3 cell line may constitute a good preliminary definition of high MN1. Confirmatory experiments in a larger cohort will be required to develop a precise and predictive clinical test for clinically relevant high MN1 expression.

Analysis of publicly available AML data sets, as well as our own patient samples, suggests that about 50% of AML patients with high MN1 expression also express high levels of HOXA9. This constellation is enriched in AML with a complex karyotype and 5q/7q abnormalities (30), an AML subtype with poor outcomes, and stellation is enriched in AML with a complex karyotype and 5q/7q abnormalities (30), an AML subtype with poor outcomes. This subtype comprises about 50% of AML patients with high MN1 expression, which was confirmed by the response of human AML with high MN1/HOXA9 expression in a cell line and patient samples to pharmacologic inhibition of DOT1L. These results suggest that DOT1L inhibition should be explored further in this poor prognostic subtype of AML.

In summary, we found that deletion of either Mll1 or Dot1l profoundly compromises MN1-driven AML in a murine model; applicability of this finding to at least a subset of MN1+ human AML is confirmed by the response of human AML with high MN1/HOXA9 expression in a cell line and patient samples to pharmacologic inhibition of DOT1L. These results suggest that DOT1L inhibition should be explored further in this poor prognostic subtype of AML.

Methods

For primer sequences, antibodies, and detailed experimental procedures, please refer to the Supplemental Methods.

Human samples. The samples from AML patients were obtained from diagnostic procedures at the University of Colorado Hospital (protocol 06-0720), with patient informed consent according to the Declaration of Helsinki and institutional review board approval from all participating centers.

qPCR analysis of human HOXA9 and MN1 in AML patient samples. Expression of MN1 and HOXA9 was determined using Taq-man primer/probes. Fold-change of MN1 is shown compared with normal CD33+ myeloid progenitors from two normal volunteers. HOXA9 is not expressed in normal CD33+ myeloid progenitors. Fold-change of HOXA9 was calculated compared with MLL-rearranged AML25. Absolute quantification of MN1 was performed using the MN1 Ipsogen kit (QIAGEN).

Dot1l and Mll1 KO mice, breeding. Animals were maintained at the Animal Research Facility at the University of Colorado Anschutz Medical Campus. Animal experiments were approved by the Internal Animal Care and Use Committee. Dot1l (18) and Mll1 (29) conditional KO mice were previously described and were maintained on a fully backcrossed C57BL/6 background.

Generation of transformed murine cells and leukemia. The MN1 cDNA was a gift from Ellen C. Zwarthoff (Erasmus University, Rotterdam, the Netherlands) and was cloned in the MSCV-IRES-GFP (MIG) plasmid. Ecotropic retroviral vectors containing murine MNI-IRES-GFP, Cre-IRES-pTomato (Cre), and MSCV-IRES-pTomato (MIT) were generated by cotransfection of 293 cells. Lin Sca-1-c-Kit+ CD48 CD150+ (SLAM), Lin Sca-1-c-Kit+CD34 FLK2 (LT-HSC), Lin Sca-1-c-Kit+ (LSK), or Lin Sca-1-c-Kit+CD34-FcR+ (CMP) cells were transduced with MN1-GFP and maintained with supplemental cytokines. After 2–7 days, GFP+ cells were sorted and transduced with Cre or MIT. Two to 3 days after transduction, GFP+/pTomato+ cells were sorted and transplanted into syngeneic irradiated recipients at 105 cells/mouse. For secondary transplants, blast colonies were allowed to grow out from sorted GFP+ BM cells. Leukemic cells were transduced with Cre or MIT, sorted, and transplanted.

Biochemical assays (apoptosis, cell cycle analysis, Western blotting, and qPCR). Cell growth and viability were followed by serial cell counts. Apoptosis and cell cycle analysis were performed using the Annexin-staining from BD Biosciences — Pharmingen and a Click-IT Edu kit (Molecular Probes, Invitrogen).

Cell growth assays for murine MN1 AML. For colony assays, sorted transduced leukemia cells were plated in methylcellulose M3234 containing IL3, IL6, and SCF at 1,000 cells per plate in duplicate and replated weekly at 500 cells/plate. Dot1l deletion was verified by PCR at each replating.

Cell growth assays and DOT1L inhibition for patient samples. Patient AML samples were maintained as described by Klco et al.

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(31). The DOT1L inhibitor EPZ004777 or DMSO control was added at the indicated concentrations. Cells were washed and replated in fresh compound every 3-4 days.

ChIP. ChIP for H3K79me2 in murine MN1 leukemias and human cell lines was performed using rabbit polyclonal antibodies from Abcam (catalog ab3594) similarly as described (42). ChIP DNA libraries were made following Illumina ChIP-seq library preparation kit and subjected to sequencing as below.

RNA amplification and gene expression array. RNA was isolated from sorted Dot1lfl/fl or Dotll−/− LSK or Mmx1-CMP-T cells using Trizol (Invitrogen) or RNeasy mini columns (QIAGEN). RNA for array analysis was amplified, labeled, and hybridized to Affymetrix 430 2.0 murine microarrays. RNA for RNA-Seq was submitted to the University of Colorado Denver genomics core for library preparation and sequencing.

Statistics. Array data was analyzed using GenePattern (http://www.broadinstitute.org/cancer/software/genepattern/). Raw sequences obtained from RNA-Seq were trimmed and mapped to mm9 using Short-read Nucleotide Alignment Program (GSNAP). Gene expression was calculated using CUFFLINKS (http://cole-trapnell-lab.github.io/cufflinks/install/); differential gene expression was determined using ANOVA. GSEA was performed using www.broadinstitute.org/gsea. Shown are the normalized enrichment scores for the top gene sets enriched in DMSO-treated cells. Curated list of gene sets from MSigdb (Myc/E2F/proliferation associated datasets) were also used for GSEA. For genes associated with K79me2, ChIP-Seq, and Input reads were each combined into a single Bam file. MACS2 was used to identify peaks, which were intersected with mm9 RefSeq genes using Bedtools (intersectBed). Peaks were visualized using Integrated Genome Viewer (IGV) software. Venn diagrams were generated using BioVenn.

Gene expression and ChIP-Seq data was deposited at the NCBI Gene Expression Omnibus (GEO GSE54498, Expression changes after loss of Dotll in murine LSK; GSE76750, ChIP-Seq and RNA-Seq).

Statistical analysis of colony and cell numbers, cell cycle, apoptosis, percent expression of markers associated with differentiation, and qPCR analysis was carried out using two-tailed Student’s t tests. Statistical analysis of survival was carried out using Kaplan Meyer estimates (Prism 5 software). A P value of less than 0.05 was considered statistically significant.

Study approval. The murine studies were reviewed and approved by the University of Colorado Health Sciences Center Animal Care and Use Committee, University of Colorado Denver – Anschutz Campus, Aurora, Colorado, USA. The human studies were reviewed and approved by the Colorado Multiple Institutional Review Board (COMIRB), University of Colorado Denver – Anschutz Campus, Aurora, Colorado, USA. Samples were collected with patient informed consent prior to the procedure and in accordance with the Declaration of Helsinki.

Author contributions
SSR, KMB, and TN designed experiments. SSR, JNH, MB, BS, and KMB conducted experiments. DAP, QW, and CTJ provided patients samples, data, and technical help. PE provided Mll1fl/fl mice and technical help. AUS and TN performed bioinformatics analysis. PE, DAP, SAA, and CTJ provided assistance in designing experiments and critically reviewed the manuscript. SSR, KMB, and TN wrote the manuscript. RMP and SRD provided EPZ004777 and assistance in designing experiments, confirmed DOT1L dependence of Mutz3 and MDRI-mediated drug efflux of EPZ004777 in independent experiments (data not included), and critically reviewed the manuscript.

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