Histone H3 Lysine 79 Methyltransferase Dot1 Is Required for Immortalization by MLL Oncogenes

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Abstract

Chimeric oncoproteins resulting from fusion of MLL to a wide variety of partnering proteins cause biologically distinctive and clinically aggressive acute leukemias. However, the mechanism of MLL-mediated leukemic transformation is not fully understood. Dot1, the only known histone H3 lysine 79 (H3K79) methyltransferase, has been shown to interact with multiple MLL fusion partners including AF9, ENL, AF10, and AF17. In this study, we utilize a conditional Dot1 deletion model to investigate the role of Dot1 in hematopoietic progenitor cell immortalization by MLL fusion proteins. Western blot and mass spectrometry show that Dot1-deficient cells are depleted of the global H3K79 methylation mark. We find that loss of Dot1 activity attenuates cell viability and colony formation potential of cells immortalized by MLL oncoproteins but not by the leukemic oncoprotein E2a-Pbx1. Although this effect is most pronounced for MLL-AF9, we find that Dot1 contributes to the viability of cells immortalized by other MLL oncoproteins that are not known to directly recruit Dot1. Cells immortalized by MLL fusions also show increased apoptosis, suggesting the involvement of Dot1 in survival pathways. In summary, our data point to a pivotal requirement for Dot1 in MLL fusion protein-mediated leukemogenesis and implicate Dot1 as a potential therapeutic target. Cancer Res; 70(24); 10234–42. ©2010 AACR.

Introduction

In addition to their critical function regulating normal gene expression, chromatin-modifying enzymes are also important mediators of neoplastic processes. In this report, we focus on the histone H3 lysine 79 (H3K79) methyltransferase Dot1 and its participation in the immortalization of hematopoietic stem cells by leukemic oncopgenes resulting from rearrangements of the mixed lineage leukemia (MLL) gene.

DOT1 (for disruptor of telomere silencing) was originally identified in a genetic screen for high-copy suppressors of telomere silencing in Saccharomyces cerevisiae (1). The Dot1 protein is highly conserved from yeast to mammals (in which it has been designated Dot1L in humans and Dot1l in mice), suggesting an important role in the biology of eukaryotes (2). Mouse Dot1L generates 5 alternatively spliced variants producing isoforms designated Dot1a–e, with Dot1a sharing 84% identity and 88% similarity with human Dot1L (3). Dot1L is a unique histone methyltransferase (HMT) in two respects. First, it is the sole enzyme that methylates lysine residue 79 of histone H3 that is located in the globular domain, not the histone tail, of the protein (4–6). Second, unlike other HMTs, the catalytically active site of Dot1 lacks a SET domain (7).

In mammals, the function of Dot1-mediated H3K79 methylation is not completely understood. H3K79 methylation is coupled to transcriptionally active genes in a number of mammalian cell lines (8), making Dot1L a positive regulator of transcription. Moreover, hypomethylated H3K79 is associated with regions of repressed chromatin (6, 9). On the other hand, Dot1 and H3K79 methylation has been shown to be directly involved in repression of several genes including the epithelial sodium channel gene αENaC and the connective tissue growth factor gene CTGF in the mouse kidney (10, 11), as well as in the developing cerebral cortex where Dot1 represses the transcription factor encoding Tbr1 gene (12). Thus, the significance of Dot1L methylation of H3K79 seems to be locus dependant.

In the case of αENaC and Tbr1, the activity of Dot1 is positively affected by AF9/MLLT3, which has been shown to bind directly to Dot1 (10, 12). AF9 was first identified as one of the many known fusion partners of the MLL oncoprotein. The t(9;11)(p22q23) translocation that gives rise to the MLL-AF9...
oncoprotein is among the most commonly encountered chromosomal rearrangements in patients with so-called MLL leukemia involving the MLL gene at chromosome band 11q23 (13). The mechanism by which MLL fusion proteins result in leukemic transformation has not been fully elucidated, but in the case of MLL-AF9, aberrant recruitment of Dot1 to MLL-regulated genes has been proposed as one possibility (14). Complicating this model, many other MLL fusion proteins have no structural or functional similarities to AF9 and are unlikely to directly recruit Dot1. Nevertheless, in leukemia cell lines expressing functionally diverse MLL fusion proteins, dimethylated H3K79 is enriched in the promoter regions of genes that are typically overexpressed in MLL leukemias (15). This raises the possibility that Dot1 may be a relevant therapeutic target for MLL leukemias. To explore whether inhibition of Dot1 represents a valid approach to treat MLL leukemias, we tested the effects of genetic ablation of Dot1 function in murine hematopoietic cells immortalized by distinct MLL oncoproteins.

**Materials and Methods**

**Generation of Dot1f/f and Dot1f/f; mouse lines**

A Dot1 target vector was constructed using a recombinering method (16, 17). Briefly, an approximately 14-kb DNA fragment spanning from intron 1 to intron 7 of the murine Dot1 gene was retrieved from BAC clone RP23-164H17 and converted into a plasmid. A LoxP site and a LoxP-FRT-neo-LoxP-FRT cassette were subsequently introduced into intron 4 and intron 5, respectively, generating the final target vector (WZPL493) for conditional deletion of exon 5. The process involved multiple steps and generated 9 intermediate constructs. The detailed cloning strategy, mouse genotyping, Southern blot analysis, and mating schemes are described in Supplemental Materials and Figs. S1–S3. 

**Retrovirus production and myeloid progenitor immortalization assay**

The oncoproteins MLL-AF9, MLL-GAS7, MLL-AFX, and E2a-Pbx1 were all cloned into MSCV-based retroviral vectors containing a neomycin resistance gene as previously described (18–21). The MLL-GAS7, MLL-AFX, and E2a-Pbx1 constructs were provided by Dr. Michael Cleary (Stanford University, Stanford, CA). The bicistronic MSCV-Cre-IRESGFP (Cre-GFP) and the MSCVpuro-Cre (Cre-puro) were provided by Dr. Jiwang Zhang (Loyola University Chicago) and the GFP control vector MigR1 was provided by Dr. Warren Beer (University of Pennsylvania Philadelphia, PA). The MSCVpuro control vector was from Clontech.

Eco Phoenix packaging cells were transfected with retroviral vectors using CalPhos reagent (Clontech). Supernatants were collected 2 and 3 days later, concentrated by centrifugal concentrating filters (Centricon-70; Millipore) and stored at −80°C. Bone marrow was isolated from the leg bones of Dot1f/f and Dot1f/f; mice that had been cared for in accordance with an institutionally approved animal use and care committee protocol (University of Texas Medical School at Houston). c-kit+ progenitor cells were collected using the EasySep kit (StemCell Technologies) and transduced with retrovirus by spinoculation as we have previously described (21). Cells were plated in methylcellulose medium (M3234; StemCell Technologies) supplemented with growth factors: 50-ng/mL stem cell factor (SCF), 10-ng/mL interleukin-3 (IL-3), 10 ng/mL IL-6, and 10-ng/mL granulocyte macrophage-colony stimulating factor (GM-CSF; Invitrogen). Antibodies were added to the medium for selection of transduced cells [1 mg/mL geneticin (Hyclone)]. For secondary transduction, 105 to 106 onco-gene-immortalized cells were transduced with retrovirus by spinoculation and cultured for 2 days in liquid medium before sorting of GFP+ cells by fluorescence-activated cell sorting (BD FACSAria; BD Biosciences). GFP+ cells were plated in either M3234 methylcellulose or liquid medium supplemented with growth factors as above. Colony-forming units (CFU) per 10,000 cells were enumerated 5 to 7 days after plating in methylcellulose. Cells collected after culture in methylcellulose medium were processed by cytospin and stained with Hema 3 (Fisher).

**RT-PCR analysis**

Total RNA was purified using an RNasy mini kit (Qiagen), and 1 μg was reverse-transcribed using Superscript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen) according to the manufacturer’s instructions. Ten nanograms of cDNA was used in each real-time PCR reaction, which was carried out in triplicate using Taqman probes and the 7300 Real Time PCR System. TaqMan probes for Hoxa9 (Mm00439364_m1) and GAPDH were purchased from Applied Biosystems and Integrated DNA Technologies, respectively. Expression levels of Hoxa9 relative to that of GAPDH were calculated using a comparative Ct method.

**Microscopy and image acquisition**

Digital photomicrographs of colonies and individual cells were obtained using a Leica inverted microscope with a Canon PowerShot digital camera and an Olympus AX80 microscope with a Qimaging Retiga 4000R CCD camera, respectively.

**Histone extraction, Western blot, and liquid chromatography–mass spectrometry**

Ten nanograms of histone extract (see Supplemental Materials) was separated by SDS–4%–20% PAGE and either transferred to a nitrocellulose membrane or stained with Coomassie blue R250. For Western blot, antihistone H3 (Abcam), anti-dimethyl–H3K79 (Millipore), and anti-tri-methyl–H3K79 (Abcam) antibodies were used as primary antibodies. Signals were detected with enhanced chemiluminescence using standard protocols.

For liquid chromatography–mass spectrometry (LC-MS), trypsin-digested samples were analyzed with a Thermo-Fisher LTQ- XL linear ion trap mass spectrometercoupled with an Eksigent nanoLC. Detailed procedures are provided in Supplemental Materials.

**Flow cytometric analysis**

The PE-Annexin V apoptosis kit (BD) was used to label GFP+–transduced cells cultured in liquid medium for analysis.
of apoptosis. Flow cytometry was carried out with a FACS-Canto flow cytometer (BD) and the data were analyzed with FlowJo software. In addition, cells maintained in liquid culture were analyzed at serial time points for GFP expression.

Results

Generation of Dot1f/f and Dot1f/fΔ mice

To understand the biological functions of Dot1l in MLL oncogene-mediated immortalization and cell survival, mice carrying a floxed Dot1l allele were created. As Dot1l null mice exhibit an embryonic lethal phenotype (22), and also confirmed in this study, we generated a Dot1l conditional target vector using the recombineering method (16, 17). Exon 5 of Dot1l was flanked by two LoxP sites, and an FRT-flanked G418 resistance gene (neo) cassette was inserted between exon 5 and exon 6 (Fig. 1; Supplementary Fig. S1). It is anticipated that excision of exon 5 upon Cre-mediated recombination will create a Dot1l null allele. Deletion of exon 5 leads to loss of the sequence encoding the methyltransferase domain as well as a frameshift of the downstream coding region. The resulting transcript is predicted to generate a truncated protein that contains only residues 1–87 (of a 1,543-amino acid wild-type Dot1a protein). Therefore, Dot1l function and thus cellular hematopoietic progenitor cells are immortalized by different MLL oncoproteins.

Dot1f/f hematopoietic progenitor cells are immortalized by different MLL oncoproteins

There are more than 50 known MLL fusion partners, and, not surprisingly, a unified model of MLL-induced leukemia has been difficult to formulate. However, experimental evidence suggests that one of at least three general functional properties can be attributed to the majority of the fusion partners. First, several fusion partners that are normally found in the cytoplasm contain dimerization domains and promote homodimerization of their respective MLL fusion proteins. The significance of homodimerization has been confirmed both by blocking the ability of the fusion partners to self-associate and by fusing artificial homodimerization domains to the amino-terminus of MLL (23–25). Another class of fusion partners comprises bone fide transcription factors and transcriptional coactivators including forkhead family members as well as CBP and p300 (26–29). Next is a collection of proteins that assemble to recruit the transcriptional elongation complex P-TEFb. Included in this group of fusion partners is AF4 and its family members, LAF4 and AF5q31, as well as AF9 and its close homologue, ENL (30, 31). As noted above, AF9 (as well as ENL) is also capable of recruiting Dot1l. However, recent evidence indicates that AF9 cannot simultaneously bind P-TEFb and Dot1l, and, importantly, it seems that recruitment of P-TEFb is an essential initiating event in leukemic transformation (15).

We selected representative MLL fusion proteins from each of the 3 classes described above to immortalize c-kit+ hematopoietic precursor cells collected from Dot1f/f mice.
murine hematopoietic precursors with virus expressing these genes has been shown to efficiently immortalize cells in serial replating assays (20, 28, 31). We selected c-kit^+ cells harvested from the bone marrow of Dot1^Flox/Flox mice and transduced the cells with retrovirus expressing MLL-GAS7, MLL-AFX, or MLL-AF9, or another leukemia-associated oncogene E2a-Pbx1, which is also known to transform myeloid progenitors in vitro (32, 33). All four leukemic oncogenes rendered Dot1^Flox/Flox hematopoietic precursor cells from 3 individual animals capable of continued proliferation after 3 successive rounds of replating in methylcellulose-based medium (Supplementary Fig. S4 and data not shown). Therefore, introduction of loxP sites flanking the Dot1 loci had no apparent effect on the ability of these oncogenes to immortalize hematopoietic precursors.

**Loss of Dot1 has profound effects on MLL-AF9–immortalized cells**

We next evaluated the consequences of Dot1 ablation in these oncogene-immortalized hematopoietic cells. Inactivation of Dot1 was achieved by deleting exon 5 through Cre-mediated recombination. Oncogene-immortalized cells were transduced with a retrovirus expressing a bicistronic Cre recombinase–GFP transcript (Cre-GFP). The GFP-expressing parent vector MigR1 served as a control (GFP). Following transduction, GFP-positive cells were selected by FACS, plated in methylcellulose-based medium containing growth factors, and incubated for one week before colonies were enumerated and genotyped by PCR (Fig. 2A). Figure 2B depicts the number of distinct colonies that developed in MLL-AF9– and GFP-transduced cells. In the case of MLL-AF9–immortalized cells, introduction of the Cre recombinase led to a dramatic reduction in the number of viable colonies when compared with controls. The same result was observed in a serial replating colony assay in which the excision of Dot1 was mediated by a retrovirus expressing Cre recombinase in conjunction with a puromycin selection gene (Supplementary Fig. S5). In addition, the colonies that did develop from Cre-expressing cells were morphologically distinct. In contrast to the compact, cell-dense colonies of cells transduced with the empty GFP vector, cells transduced with Cre recombinase formed diffuse, ill-defined colonies (Fig. 2B). GFP^+ sorted cells were subjected to cytospin and stained with Wright–Giemsa. A large proportion of Dot1^− deleted (Dot1^Flox/−) MLL-AF9–immortalized cells showed condensed and fragmented nuclei characteristic of apoptotic cells (Fig. 2C). In addition, in contrast to the small and uniform morphology of immortalized Dot1^Flox/Flox cells, Dot1^Flox/− cells have large and highly vacuolated cytosol.

In the case of Dot1^Flox/Flox cells immortalized by MLL-GAS7 and MLL-AFX, introduction of Cre recombinase also resulted in diminished colony numbers and changes in colony morphology similar to MLL-AF9–immortalized cells; however, the overall effect of loss of Dot1 in these cells was less pronounced (Fig. 2B; Supplementary Fig. S4). In concordance with the colony counts, the proportion of morphologically apoptotic cells in Dot1^Flox/− cells was decreased in the case of MLL-GAS7 or MLL-AFX compared with that of MLL-AF9 (Fig. 2C). Strikingly, Cre-mediated gene excision of Dot1^Flox/− had no apparent effect on colony number or morphology in cells immortalized by E2a-Pbx1 (Fig. 2B and C; Supplementary Fig. S4).

A separate set of Cre-GFP or GFP-transduced cells were maintained in liquid culture and analyzed by flow cytometry. Cells immortalized by MLL-AF9, MLL-GAS7, and MLL-AFX revealed a reduction in GFP^+ cells from day 3 to day 5 after Cre-GFP transduction whereas cells transduced with the GFP control vector maintained substantial GFP^+ populations (Fig. 2D; Supplementary Fig. S5). For example, among MLL-AF9–immortalized cells, GFP^+ cells decreased from 14% at day 3 to 4% at day 5 after GFP-Cre transduction. These fractions were only slightly decreased from 33% to 30% in GFP-transduced control cells. Cre-dependent reduction of GFP^+ cells was not observed in E2a-Pbx1–immortalized cells as evidenced by the similar percentage of GFP^+ cells in both groups. This indicates that many of the MLL oncogene–immortalized cells are lost upon Cre-mediated excision of Dot1.

We then verified the status of the Dot1 loci in the surviving cells. After transduction with GFP-Cre or GFP control vector, cells were GFP-sorted and either harvested immediately (designated day 0) or following culture in methylcellulose (designated day 8). Genomic DNA was isolated and subjected to PCR amplification using primer sets (P7/P9 or P9/P10) specific for either the floxed allele or the excised Dot1 allele (Dot1^Flox; Fig. 1D; Supplemental Materials). Figure 2E reveals that immediately following transduction (day 0), only the excised allele of Dot1 can be detected in sorted Cre-GFP–transduced cells. However, the same GFP-sorted cells grown in methylcellulose for 8 days show varying amounts of the excised allele. Importantly, in cells immortalized by MLL-AF9 and by MLL-AFX in which Cre-GFP was expressed, a significant number of the surviving cells retain the floxed allele consistent with a strong selective pressure against loss of Dot1 under these conditions. In fact, the floxed Dot1 allele predominated over the excised allele in surviving MLL-AF9–immortalized cells. In contrast, the floxed allele could not be detected in cells immortalized either by MLL-GAS7 or by E2a-Pbx1 following Cre expression indicating complete or near-complete excision of Dot1. These data suggest that many of the MLL-AF9- and MLL-AFX–immortalized cells that survive 8 days after introduction of Cre recombinase retain at least 1 functional (floxed) allele of Dot1. On the other hand, cells immortalized by MLL-GAS7 and E2a-Pbx1 survive even after loss of all detectable functional Dot1. In the case of MLL-GAS7–immortalized cells, however, survival is nevertheless compromised by Dot1 deletion as shown in Figs. 2B–D and Supplementary Figs. S4 and S5.

The promoter region of one of the most widely studied MLL target genes, HOXA9, is hypermethylated at H3K79, and overexpression of HOXA9 is a hallmark of MLL leukemias (15). We measured transcript abundance of Hoxa9 in cells immortalized by MLL-AF9 and E2a-Pbx1 to determine whether loss of Dot1 function downregulated expression of this gene. Immortalized cells were transduced with GFP-Cre or the GFP control.
Figure 2. Cre-mediated deletion of Dot1l impairs survival in mouse hematopoietic progenitor cells immortalized by MLL-GAS7, MLL-AFX, and MLL-AF9 but not by E2a-Pbx1. A, experimental scheme to evaluate the effect of Dot1l deletion shows the time points when CFU activity, genotype (by PCR), or Hoxa9 expression (by qRT-PCR) was examined. All experiments were carried out using cells from Dot1lf/+ mice unless specified. B, CFUs per 10⁴ cells and representative colony morphologies (20× magnification) of cells immortalized by the indicated fusion oncogenes after Cre-mediated Dot1l deletion. Error bars indicate SD from 3 independent experiments with the exception of E2a-Pbx1, which is from 2 independent experiments. Each independent experiment was conducted in duplicate. Scale bar, 1 mm. C, Wright–Giemsa stain of GFP-sorted cells 6 days after transduction. Representative cells are enlarged in insets to show morphologic details. Magnification is 400× and scale bars are 40 μm. Arrowheads indicate cells with condensed, fragmented nuclei. D, percentage of GFP+ cells at 3 or 5 days after GFP or Cre-GFP transduction in cells expressing indicated oncogenes. Nontransduced cells were used as controls (gray fill) to determine GFP positivity. Cells from Dot1lf/+ mice were used in all samples with the exception of the MLL-AFX-immortalized cells, which originated from Dot1lf/+ mice. The percentage of GFP+ cells is shown on each histogram. E, Dot1l genomic status was examined by PCR at days 0 and 8 in methylcellulose culture. Arrowhead, floxed allele at 510 bp; open arrowhead, deleted allele at 378 bp. F, relative expression levels of Hoxa9 after Cre-mediated Dot1l deletion. Expression levels are normalized to GAPDH and expressed relative to GFP-transduced cells (set to 100%). Error bars indicate the SD of analyses carried out in triplicate.
Nevertheless, the reduction in Hoxa9 expression may be of greater biological significance in cells that require this gene for leukemic transformation. As described below, ChIP analysis of methylated H3K79 is not possible as this modification is absent in cells lacking Dot1.

**Dot1 deletion abolishes histone H3 lysine 79 methylation**

Dot1 is the only known histone modifying enzyme that methylates histone H3 at the lysine 79 residue. In yeast, more than 90% of nucleosomes are methylated by Dot1 (34). Given the ubiquitous expression of Dot1 and the embryonic lethal phenotype of Dot1-deficient mice, we investigated whether viable immortalized cells in which Dot1 had been experimentally excised lacked all H3K79 methyltransferase activity. We observed that E2a-Pbx1–immortalized cells with deletion of Dot1 survived under continuous culture conditions. Therefore, a single colony of E2a-Pbx1–immortalized cells with PCR-confirmed Dot1\(+/\Delta\) genotype was expanded in liquid culture and analyzed for H3K79 methylation. First, histones were extracted, separated by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies to total histone H3, dimethyl H3K79, and trimethyl H3K79. As the available antibody against monomethyl H3K79 (ab2886; Abcam) nonspecifically recognizes histone H3, its application to detect monomethyl H3K79 was omitted (22). Figure 3B shows that both Dot1\(+/\) and Dot1\(+/\Delta\)-immortalized cells have equal amounts of histone H3. Dimethyl H3K79 and trimethyl H3K79 are easily detected in the Dot1\(+/\) cells immortalized by E2a-Pbx1; however, these protein modifications could not be detected by Western blot in Dot1\(+/\Delta\) cells (Fig. 3B).

Second, histones extracted from Dot1\(+/\) and Dot1\(+/\Delta\)-immortalized cells were separated by SDS-PAGE, excised from a Coomassie-stained gel (Fig. 3A), and subjected to LC-MS analysis. The relative abundance of the H3 peptide was manually set to 100. H3 peptide, YRPGTVALR; K79me0, K79me1, and K79me3 represent the unmodified H3 K79, and dimethyl (EIAQDFKTDLR) H3K79 peptides, respectively.

![Figure 3. Dot1 deletion abolishes histone H3K79 methylation. A, Coomassie-stained SDS-PAGE gel of histone extract with histone H3 band indicated. B, histone H3K79 methylation status in E2a-Pbx1–immortalized cells obtained from Dot1\(+/\) or Dot1\(+/\Delta\) backgrounds was examined by Western blot using antibodies specific for dimethyl (K79me2) or trimethyl (K79me3) marks. The total histone H3 serves as a loading control. C, SDS-PAGE–purified histone H3 from E2a-Pbx1–immortalized cells with Dot1\(+/\) or Dot1\(+/\Delta\) genotype was subjected to LC-MS analysis. The relative abundance of the H3 peptide is depicted in the results of this analysis.](https://www.aacrjournals.org/doi/fig/10.1158/0008-5472.CAN-10-2298/Fig3.jpg)
previously been shown to represent only 0.1% of the total H3K79 peptides (Fig. 3C; ref. 22). Nevertheless, using 2 independent analyses, there was no evidence of H3K79 methylation in Dot1l/D cells immortalized by E2a-Pbx1. These findings indicate that the gene product of the excised Dot1l allele lacks methyltransferase activity. Furthermore, Dot1l is not absolutely required for cell survival at least in the setting of hematopoietic cells immortalized by E2a-Pbx1.

Loss of Dot1l triggers apoptosis in MLL-AF9-, MLL-GAS7-, and MLL-AFX–immortalized cells

Colony-forming assays indicate that loss of Dot1l in cells immortalized by MLL-GAS7, MLL-AFX, and MLL-AF9 impairs survival. Flow cytometric analysis of cells transduced with Cre-GFP showed a significant reduction in GFP+ cells following Cre-mediated recombination in cells immortalized by any of these three oncogenes. However, the percentage of GFP+ cells was unaffected in the case of immortalization by E2a-Pbx1 (Fig. 2D; Supplementary Fig. S5). Microscopic analysis of Dot1lΔΔ cells, in particular those immortalized by MLL-AF9, showed a number of cells with condensed, fragmented nuclei that are characteristic of apoptotic cells (Fig. 2C). Embryonic stem cells derived from Dot1lΔΔ murine blastocysts can be maintained in cell culture but do exhibit an increased rate of apoptosis (22). We tested whether there were differences in the frequency of apoptotic in immortalized progenitor cells transduced by GFP-Cre compared with controls. For this, cells were labeled with Annexin-V and 7-amino-actinomycin D (7-AAD) and analyzed by flow cytometry. Figure 4 depicts representative plots of the four different oncogene-immortalized hematopoietic cells. The percentage of Annexin-V-positive, 7-AAD-negative cells that are indicative of apoptosis is significantly increased among cells immortalized by MLL-GAS7, MLL-AFX, and MLL-AF9 five days following transduction with Cre-GFP compared with GFP-transduced cells. Again, no meaningful difference among similarly treated cells immortalized by E2a-Pbx1 was found. Thus, reduced colony numbers and loss of GFP+ cells can at least partly be attributed to apoptosis when Dot1l function is lost in cells immortalized by these MLL oncogenes.

Discussion

The large diversity of MLL fusion partners poses a challenge to developing a unified model of the pathobiology of MLL leukemia. Despite this, gene expression signatures of MLL leukemias are remarkably similar and can be reliably used to distinguish leukemias with MLL rearrangements from other subtypes (35). Mistargeting of Dot1 by the MLL-AF10 oncoprotein was described by Zhang and colleagues. They proposed that Dot1-catalyzed chromatin modifications of loci normally regulated by wild-type MLL contribute to leukemogenesis (36). The importance of Dot1 in MLL leukemias was given additional weight when it was found that the MLL fusion partners AF9, ENL, and AF17 are components of a novel Dot1L-containing complex (DotCom; ref. 37). Our previous studies have shown that Dot1l (mouse isoform a) directly interacts with AF9 and AF17 in multiple assays (10, 38). More
evidence that Dot1 may play a central role in many MLL leukemias has been provided by other groups as well. Hematopoietic precursor cells are not immortalized when transduced with a mutant MLL-ENL that encodes an ENL moiety that does not bind Dot1. Furthermore, H3K79 methylation is abundant in the promoter regions of genes that are over-expressed in cells transformed by MLL-ENL as well as MLL-AF4 (39–41).

To directly test the requirement for Dot1 in an experimental model of MLL leukemia, we have generated a new Dot1 conditional knockout mouse (Dot1f/f) with a floxed exon 5. As a consequence of Cre-mediated Dot1 deletion, the majority, if not all of the Dot1a function, including the methyltransferase activity and the AF9/AF17-binding domain, which maps to residues 479–659 of Dot1a (10, 38), is eliminated. Although there are several other isoforms of Dot1l in the mouse, the function of Dot1b should also be completely disrupted because its translation start site is found in exon 5 (3). The translation start sites for Dot1c–e have not been determined, but the transcripts would lack exon 5 and the resulting isoforms would not possess H3K79 methyltransferase activity. Our results indicate that this is, in fact, the case. There is no detectable H3K79 methyltransferase activity in Dot1f/f cells.

Zhang and colleagues were the first to experimentally show the requirement for Dot1 in at least one type of MLL fusion-mediated leukemia (36). Expression of a dominant-negative allele of Dot1l inhibited the proliferation of mouse hematopoietic precursor cells immortalized by MLL-AF10. This artificial mutant expresses a full-length Dot1l protein in which the catalytic site has been inactivated. In contrast to our findings, if not all of the Dot1a function, including the methyltransferase activity and the AF9/AF17-binding domain, which maps to residues 479–659 of Dot1a (10, 38), is eliminated. Although there are several other isoforms of Dot1l in the mouse, the function of Dot1b should also be completely disrupted because its translation start site is found in exon 5 (3). The translation start sites for Dot1c–e have not been determined, but the transcripts would lack exon 5 and the resulting isoforms would not possess H3K79 methyltransferase activity. Our results indicate that this is, in fact, the case. There is no detectable H3K79 methyltransferase activity in Dot1f/f cells.

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