LYMPHOID NEOPLASIA

Dnmt3b is a haploinsufficient tumor suppressor gene in *Myc*-induced lymphomagenesis

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Key Points

- Dnmt3b acts as a haploinsufficient tumor suppressor in Myc-induced lymphomas.

The drivers of abnormal DNA methylation in human cancers include widespread aberrant splicing of the *DNMT3B* gene, producing abnormal transcripts that encode truncated proteins that may act as dominant negative isoforms. To test whether reduced *Dnmt3b* dosage can alter tumorigenesis, we bred *Dnmt3b*$^{+/-}$ mice to *Eμ-Myc* mice, a mouse model susceptible to B-cell lymphomas. *Eμ-Myc/Dnmt3b*$^{+/-}$ mice showed a dramatic acceleration of lymphomagenesis, greater even than that observed in *Eμ-Myc* mice that express a truncated DNMT3B isoform found in human tumors, DNMT3B7. This finding indicates that *Dnmt3b* can act as a haploinsufficient tumor suppressor gene. Although reduction in both *Dnmt3b* dosage and expression of DNMT3B7 within the *Eμ-Myc* system had similar effects on tumorigenesis and DNA hypermethylation, different molecular mechanisms appear to underlie these changes. This study offers insight into how de novo DNA methyltransferases function as tumor suppressors and the sensitivity of *Myc*-induced lymphomas to DNA methylation. (*Blood*. 2013;121(11):2059-2063)

Introduction

Cancer cells are characterized by abnormal DNA methylation, causing aberrant activation of some genes and silencing of others.[1] Three DNA methyltransferases (DNMTs) catalyze DNA methylation in eukaryotic cells DNMT1, DNMT3A, and DNMT3B.[2] Our laboratory and others discovered aberrant splicing of the *DNMT3B* gene in human cancer cells that produces transcripts encoding truncated proteins that may act as dominant negative isoforms.[3,4] Transgenic mice expressing *DNMT3B7*, an aberrant transcript expressed most commonly in human cancers, have little cancer predisposition on their own.[5] However, when crossed to *Eμ-Myc* mice, which are susceptible to B-cell lymphomas,[6] *Eμ-Myc/DNMT3B7* mice showed accelerated mediastinal tumorigenesis, with overall increases in global DNA methylation and alterations in site-specific DNA methylation.[5]

DNMT3B7 transgenic mice demonstrated developmental defects including subaortic ventriculoseptal defects,[5] reminiscent of those in *Dnmt3b* knockout mice.[7,8] Given the similar phenotype between *DNMT3B7* transgenic mice and *Dnmt3b* knockout mice, we hypothesized that DNMT3B7 acts as a dominant negative isoform of full-length Dnmt3b. Recent work has demonstrated that inactive DNMT3B isoforms can alter the enzymatic activity of full-length DNMT3B in vitro.[9] Because we observed acceleration of tumorigenesis in *Eμ-Myc* mice with *DNMT3B7* introduction, we tested whether *Dnmt3b* gene dosage (*Dnmt3b*$^{+/-}$) could influence *Myc*-induced lymphomagenesis.

Study design

Mice and monitoring of tumors

Dnmt3b$^{+/-}$ knockout mice[7] and *Eμ-Myc* mice,[6] both on a pure C57Bl/6 background, were interbred, and at least 20 mice of each *Dnmt3b* genotype carrying the *Eμ-Myc* transgene were monitored for lymphoma development.[5]

Chromatin immunoprecipitation

Chromatin immunoprecipitation assays were performed using a Myc (Millipore, Billerica, MA) or Dnmt3b antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Real-time polymerase chain reaction (PCR) primers are listed in supplemental Table 1.

Detailed materials and methods are included in "Supplemental Materials."

Results and Discussion

Given the acceleration of Myc-induced lymphomagenesis in the presence of the dominant-negative DNMT3B7 protein,[5] we tested directly whether reducing *Dnmt3b* levels would influence tumorigenesis in *Eμ-Myc* mice.[6] We found dramatic acceleration in mediastinal lymphomagenesis in *Eμ-Myc/Dnmt3b*$^{+/-}$ mice (Figure 1A), whereas the incidence of peripheral lymphomas was not significantly different

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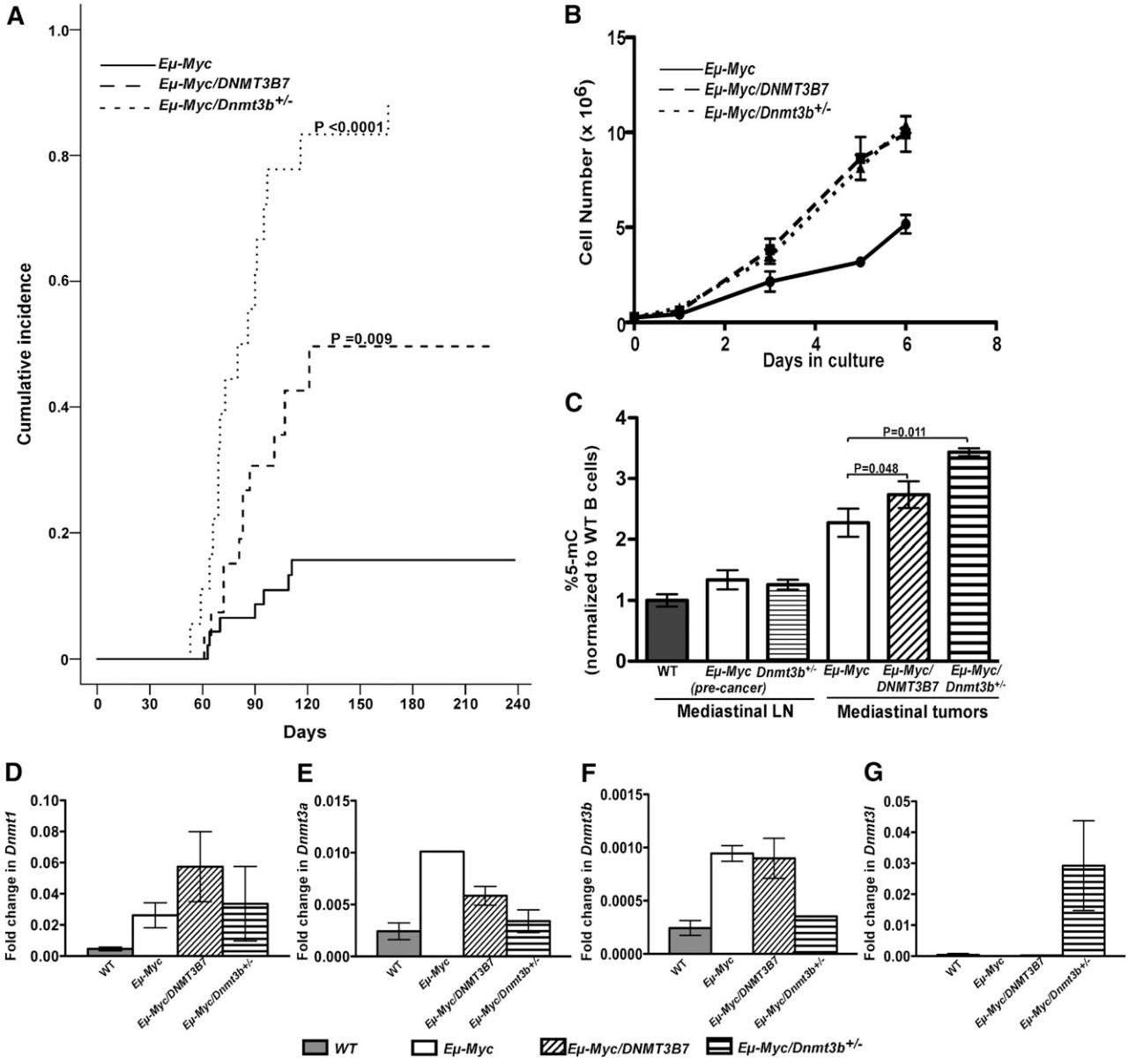

Figure 1. Incidence of mediastinal lymphomas and changes in DNA methylation in *Eμ-Myc*, *Eμ-Myc/DNMT3B7*, and *Eμ-Myc/Dnmt3b*$^{+/-}$ mice. (A) *Eμ-Myc/Dnmt3b*$^{+/-}$ (dotted line) vs *Eμ-Myc/DNMT3B7* (dashed line) and *Eμ-Myc* mice (solid line). Cumulative incidence curves were compared using the K-sample test (*Eμ-Myc* vs *Eμ-Myc/Dnmt3b*$^{+/-}$: $P < .0001$; *Eμ-Myc* vs *Eμ-Myc/DNMT3B7*: $P = .009$).[5] (B) Growth curves of cell lines established from mediastinal lymphomas of *Eμ-Myc* (solid line), *Eμ-Myc/DNMT3B7* (dashed line), and *Eμ-Myc/Dnmt3b*$^{+/-}$ mice (dotted line). Curves were compared using 2-way analysis of variance (ANOVA) ($P = .012$). (C) Total 5-methylcytosine levels were quantified by liquid chromatography-electrospray ionization/tandem mass spectometry,[10] $n \geq 4$ for each genotype. DNA methylation levels were normalized to wild-type (WT) peripheral blood B cells (gray bar). The 2-tailed Student *t* test was used to compare global DNA methylation. (D-G) Relative quantification of *Dnmt* levels, normalized to *β-Actin* levels. *Dnmt1* (D), *Dnmt3a* (E), *Dnmt3b* (F), and *Dnmt3l* (G) levels in WT lymph node (LN) (gray bar) and *Eμ-Myc* (white bar), *Eμ-Myc/DNMT3B7* (hatched bar), and *Eμ-Myc/Dnmt3b*$^{+/-}$ (striped bar) cell lines. *P* values were calculated by ANOVA, using WTLN as the control.

(supplemental Figure 1A). Overall lymphoma incidence was dramatically accelerated in both *Eμ-Myc/DNMT3B7* and *Eμ-Myc/Dnmt3b*$^{+/-}$ mice (supplemental Figure 1B). Similar to the *Eμ-Myc/DNMT3B7* mice, mediastinal tumors isolated from *Eμ-Myc/Dnmt3b*$^{+/-}$ mice were composed of pre-B or mature B-lymphocytes (supplemental Figure 2), and the mice had high leukocyte counts (supplemental Table S2). Cell lines generated from mediastinal tumors recapitulated the enhanced growth (Figure 1B). Cell-cycle profile analysis demonstrated that the growth difference arose from *Eμ-Myc* cells accumulating in the S-phase, and *Eμ-Myc/DNMT3B7* and *Eμ-Myc/Dnmt3b*$^{+/-}$ cells overcoming this block in progression from S to G2 phase (supplemental Figure 3). There were no significant differences in apoptosis in the 3 cell lines (data not shown). The *Dnmt3b* cDNA expressed from the remaining allele was wild-type in sequence (data not shown), establishing that *Dnmt3b* has a haploinsufficient tumor suppressor function in the *Eμ-Myc* mouse model.

Our observation is consistent with recent work demonstrating that complete ablation of *Dnmt3b* in T-lymphocytes promotes proliferation and accelerates lymphomagenesis in the context of *MYC* overexpression.[11] Conditional deletion of *Dnmt3a* in a mouse model of lung tumorigenesis also promotes tumor growth and progression.[12] Heterozygous *DNMT3A* mutations, often in exons encoding the catalytic domain, have been found in myeloid neoplasms.[13-15] Taken together, these data suggest that the de novo DNA methyltransferases DNMT3A and DNMT3B function as haploinsufficient tumor suppressors.

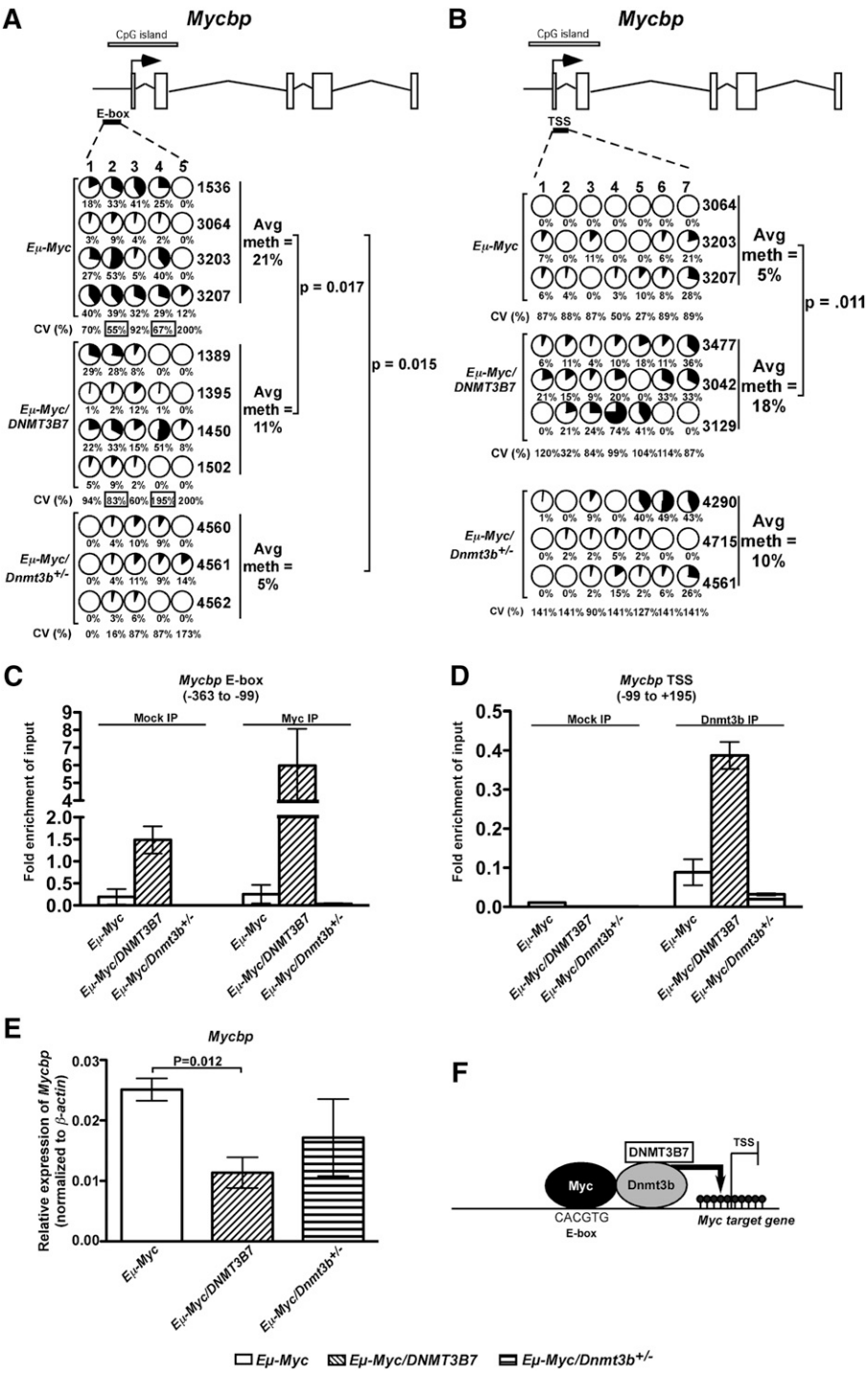

Figure 2. DNA methylation changes and binding of Myc and Dnmt3b at *Mycbp* promoter elements. (A) DNA methylation changes measured by bisulfite sequencing around the E-box upstream of *Mycbp* TSS. Schematic diagram for *Mycbp* is shown with exons represented by vertical rectangles, and the location of the CpG island shown with a horizontal shaded rectangle. The black arrow indicates the TSS. The smaller, black horizontal rectangle indicates the location of CpGs analyzed for changes in DNA methylation. Each row represents DNA methylation in a single lymphoma, indicated to the right. Numbers across the top indicate specific CpG dinucleotides in a region of the CpG island. Changes in DNA methylation are indicated by shaded circles, with the shading indicating average amount of DNA methylation at each CpG; numbers below represent percent methylated cytosine. Average percent methylation is indicated to the right, and *P* values calculated using the 2-tailed Student *t* test. Coefficient of variance (CV) was used to calculate the heterogeneity of DNA methylation at each CpG within tumors of a single genotype. The average CV is given as a percentage underneath each CpG position. CV values with a black box around them denote $P < .05$ for *Eμ-Myc/DNMT3B7* or *Eμ-Myc/Dnmt3b*$^{+/-}$ tumors relative to *Eμ-Myc* tumors. (B) DNA methylation changes as measured by bisulfite sequencing in the *Mycbp* TSS. Detailed description is as outlined in (A). (C) Fold enrichment of Myc in chromatin from at least 3 independent *Eμ-Myc*, *Eμ-Myc/DNMT3B7*, and *Eμ-Myc/Dnmt3b*$^{+/-}$ cell lines. The plot shows real-time PCR data for the region around the *Mycbp* E-box (−363 to −99 relative to *Mycbp* TSS) from Myc-immunoprecipitated or control-immunoprecipitated chromatin normalized to input. (D) Fold enrichment of Dnmt3b in chromatin from at least 3 independent *Eμ-Myc*, *Eμ-Myc/DNMT3B7*, and *Eμ-Myc/Dnmt3b*$^{+/-}$ cell lines. The plot shows real-time PCR data for the region around the Mycbp TSS (−99 to +195 relative to *Mycbp* TSS) from Dnmt3b-immunoprecipitated or control-immunoprecipitated chromatin normalized to input. (E) Expression of Mycbp in *Eμ-Myc*, *Eμ-Myc/DNMT3B7*, and *Eμ-Myc/Dnmt3b*$^{+/-}$ tumors. Relative expression of *Mycbp* was measured in all *Eμ-Myc* tumor types and expressed normalized to *β-Actin*. (F) Proposed model for the increased acceleration of lymphomagenesis induced by hypermethylation in *Eμ-Myc/DNMT3B7* mice. The presence of an unmethylated CpG in the Myc-binding E-box promotes Myc binding, which then assembles a complex that includes Dnmt3b and DNMT3B7. The complex locks into position at the *Mycbp* TSS and leads to hypermethylation around the TSS.

We found a progressive increase in the global 5-methylcytosine levels in *Eμ-Myc*, *Eμ-Myc/DNMT3B7,* and *Eμ-Myc/Dnmt3b*$^{+/-}$ tumors (Figure 1C).[5] Because *Dnmt3b*$^{+/-}$ transgenic mice themselves do not demonstrate any overt DNA methylation defect,[7] this was an unexpected observation. A CpG island hypermethylation signature was defined in a mouse model of *MYC*-induced lymphomas,[16] supporting the hypothesis that MYC drives DNA methylation. DNA methylation levels in *B1* repetitive elements, major and minor satellites, and retroviruses were similar across all *Eμ-Myc* genotypes (data not shown). The expression of *Dnmt1*, *Dnmt3a, Dnmt3b*, and *Dnmt3l* did not differ significantly between the *Eμ-Myc*, *Eμ-Myc/DNMT3B7*, and *Eμ-Myc/Dnmt3b*$^{+/-}$ cell lines; however, almost all *Dnmts* increased in expression with *Myc* overexpression (Figure 1D-G). In a proliferative germinal center B-cell model, repatterning of DNA methylation correlated with gene expression changes and upregulation of *DNMT1* expression.[17] Chromosomal abnormalities in *Eμ-Myc/Dnmt3b*$^{+/-}$ tumors were not significantly different compared with *Eμ-Myc* tumors (supplemental Table 3).

Because virtually all human tumors and cell lines express aberrant *DNMT3B* transcripts, we focused on testing whether the molecular mechanism of tumorigenesis in *Eμ-Myc/DNMT3B7* and *Eμ-Myc/Dnmt3b*$^{+/-}$ tumors was similar. We hypothesized that

Myc, Dnmt3b, and DNMT3B7 could form a complex when DNMT3B7 was expressed, because Myc binds to Dnmt3a and Dnmt3b,[18] and DNMT3B7 binds to full-length Dnmt3b.[5] We hypothesized that this complex could bind at Myc-binding E-box (es) upstream of the transcriptional start site (TSS), leading to hypermethylation and gene repression. Because Myc binds preferentially at hypomethylated E-boxes,[19] we predicted hypomethylation around the E-box of the Myc target gene tested.

To test this hypothesis, we examined DNA methylation in promoters of genes that are known Myc targets and are repressed in *Eμ-Myc/DNMT3B7* tumors (see "Supplemental Methods"). *Mycbp*, which encodes a protein that binds c-Myc and enhances its ability to activate transcription, was hypomethylated around the E-box in both *Eμ-Myc/DNMT3B7* and *Eμ-Myc/Dnmt3b*$^{+/-}$ tumors relative to *Eμ-Myc* tumors (Figure 2A). We observed more heterogeneity in DNA methylation levels of particular CpG dinucleotides in *Eμ-Myc/DNMT3B7* tumors relative to the *Eμ-Myc* tumors as we had observed previously,[5] in contrast to *Eμ-Myc/Dnmt3b*$^{+/-}$ tumors that failed to demonstrate this heterogeneity (Figure 2A). The *Mycbp* TSS was hypermethylated in *Eμ-Myc/DNMT3B7* tumors relative to *Eμ-Myc* tumors, a feature not shared by *Eμ-Myc/Dnmt3b*$^{+/-}$ tumors (Figure 2B).

A chromatin immunoprecipitation assay around the E-box demonstrated enrichment of Myc binding in the *Eμ-Myc/DNMT3B7* cell lines and absence of Myc binding in both *Eμ-Myc* and *Eμ-Myc/Dnmt3b*$^{+/-}$ cell lines (Figure 2C). We also found Dnmt3b enrichment at the *Mycbp* TSS in *Eμ-Myc/DNMT3B7* cell lines, a phenomenon not observed in *Eμ-Myc* and *Eμ-Myc/Dnmt3b*$^{+/-}$ cell lines (Figure 2D). Consistent with DNA methylation patterns around the TSS, there was *Mycbp* repression in *Eμ-Myc/DNMT3B7* tumors, but not in *Eμ-Myc/Dnmt3b*$^{+/-}$ tumors (Figure 2E). We also observed enrichment of Myc binding at the *Mycbp* TSS and increased enrichment of Dnmt3b binding at the *Mycbp* E-box (supplemental Figure 4). Similar results were seen for *Gadd45a* and *Maf*, which are other Myc targets (supplemental Figures 5A and 6). *Gadd45*a repression in *Eμ-Myc/DNMT3B7* and *Eμ-Myc/Dnmt3b*$^{+/-}$ tumors (supplemental Figure 5B) provides a mechanism for the hypermethylation observed in these tumors. Taken together, our data indicate that *Eμ-Myc/DNMT3B7* tumors have increased Myc binding at the *Mycbp* E-box, which enhances recruitment of Dnmt3b to form a repressive complex at its promoter, leading to *Mycbp* repression (Figure 2F), a mechanism distinct from that present in *Eμ-Myc/Dnmt3b*$^{+/-}$ tumors.

Overall, our observations suggest that Myc-induced lymphomas are exquisitely sensitive to DNA methylation, which may suggest that targeting epigenetic pathways may be particularly effective for MYC-induced hematopoietic malignancies. Epigenetic silencing of *RASSF1A,* a tumor suppressor gene, has been shown to occur via HOXB3-mediated induction of DNMT3B, which binds MYC and polycomb repressor at the *RASSF1A* promoter in multiple human cancer cell lines.[20] The ability of JQ1, an inhibitor of BRD4-mediated reading of the histone code, to suppress myeloid leukemia cell growth further supports the importance of epigenetic therapy.[21] Finally, our findings, combined with the identification of heterozygous *DNMT3A* mutations in myeloid malignancies, suggest that both de novo DNA methyltransferases can act as haploinsufficient tumor suppressors.

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Authorship

Contribution: A.V. designed and performed experiments, analyzed data, and wrote the manuscript; J.B.L., M.H.Z., M.K., M.S., E.M.D., and P.A.L. performed experiments and analyzed data; J.A., M.M.L.B., and A.R.K. helped design experiments; L.A.G. conceived the study, analyzed data, and edited the manuscript.

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