Myelodysplastic syndromes (MDS) are clonal hematopoietic stem cell disorders characterized by dysplastic blood cell morphology, ineffective hematopoiesis and a high rate of transformation to acute myeloid leukemia (AML). Epigenetic dysregulation underpins the pathogenesis of MDS, with recurrent mutations in epigenetic regulators including TET2 (20%), ASXL1 (14%), DNMT3A (12%), EZH2 (6%) and IDH1/2 (5%). EZH2, or Enhancer of zeste homolog 2, is a histone methyltransferase and member of the highly conserved polycomb group of proteins, with important roles in regulating gene expression to coordinate self-renewal and differentiation of hematopoietic stem cells (HSCs). EZH2 loss-of-function mutations have an adverse effect on prognosis in MDS. Herein, we describe an in vivo model of attenuated acceleration to leukemia transformation with Ezh2 deletion in a mouse model of MDS.

EZH2, together with other core subunits EED, SUZ12, and RBBP4 form the polycomb repressor complex 2 (PRC2) complex responsible for the repressive tri-methylation modification of lysine 27 on histone 3 (H3K27me3). EZH1 and 2 are the only histone methyltransferases responsible for the H3K27 mark in mammals and functional redundancy exists with EZH1. EZH2 has important roles in maintaining HSC identity via repression of differentiation genes. EZH2 mutations are usually loss-of-function in a broad range of myeloid malignancies including MDS, myeloproliferative neoplasms (MPNs) and AML. In MDS, mutations occur within EZH2 in 6% of cases, however PRC2 is dysregulated in a larger subset of MDS (potentially 25–30% of cases) via gene deletion (del7q36.1) (3–4%), ASXL1 mutation (20%), which inhibits PRC2 function, or mutations in other PRC2 components (1–2%). EZH2 mutations occur rarely in de novo AML (~2%), but are relatively enriched in AML arising from a precedent MDS (9%). EZH2 mutations are a poor prognostic indicator in MDS overall, including low-risk MDS, where EZH2 mutation defines a subset with adverse clinical outcomes.

In mouse models, loss of Ezh2 leads to fatal defects in fetal hematopoiesis, although inductive loss in adult mice leads to a milder phenotype including retained self-renewal of HSCs that are able to engraft in secondary recipients. This may reflect increased dependency on Ezh2 in highly proliferative fetal HSCs in the liver, compared to quiescent adult HSCs or may reflect Ezh1 compensation. However, the complete loss of PRC2 activity in Eed knockout mice, leads to pancytopenia, defective differentiation and inability to compete with wild-type cells in competitive transplants, demonstrating integral roles of PRC2 signaling in hematopoiesis. Inducible Ezh2 knock-out mice develop hematological malignancies with MDS, MDS/MPN and AML, recapitulating key phases of human disease including a cytopenic phase which progresses at variable latencies to acute leukemia between 6 and 14 months. Additionally, epigenetic dysregulation appears to be an important contributor to the Upregulated Hox genes, a common mechanism in human disease. Additionally, epigenetic dysregulation is evidenced by its frequent fusions with epigenetic regulators.

Attenuated Acceleration to Leukemia after Ezh2 Loss in Nup98-HoxD13 (NHD13) Myelodysplastic Syndrome

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Given the driving role of EZH2 dysregulation in MDS and its poor prognostic implications, we sought to study the effects of EZH2 loss-of-function in the NHD13<sup>T</sup> mouse model. As HOX gene overexpression is also observed in EZH2-deleted MDS/AML, we hypothesized that the additive upregulation of HOX genes might provide a mechanism of cooperation between EZH2 deletion and Nup98/HoxD13. In long-term survival studies, we found that Ezh2 deletion minimally accelerated leukemia development and death in NHD13<sup>T</sup> demonstrating limited contribution to disease pathogenesis in the context studied.

NHD13<sup>T</sup> mice were crossed with Ezh2<sup>fl/fl</sup> mouse expressing the polyinosinic-polycytidylic acid (poly(I:C))-inducible Mx1-cre transgene (MxT) to generate NHD13<sup>T</sup>;MxT;Ezh2<sup>fl/fl</sup> mice (Fig. 1A, Supplementary Digital Content, http://links.lww.com/HS/A40). Analysis of hematopoietic stem and progenitor cell (HSPC) subpopulations from non-leukemic mice demonstrated an expansion of multipotent progenitor (MPP) and progenitor cell (HSPC) subpopulations, which contain the granulocyte/macrophage (GM)-committed and lymphoid-committed MPPs, as found in NHD13<sup>T</sup>;Ezh2<sup>fl/fl</sup> and MxT;Ezh2<sup>fl/fl</sup> mice (Fig. 1H). This study demonstrates a contribution of Ezh2 loss-of-function to NHD13<sup>T</sup>-driven MDS and leukemia, however, there was only a mild acceleration of disease onset and similar spectrum of blood cancers. The effect of Ezh2 deletion in this model may be abrogated for a number of reasons. First, substantial levels of H3K27me3 were maintained in the absence of Ezh2 expression. From H3K27me3 chromatim immunoprecipitation (ChIP)-sequencing studies, ~79% of Ezh2 target loci had compensatory methylation mediated by Ezh1. Functionally, Ezh1 compensation attenuated the hematologic phenotype caused by complete PRC2 dysfunction as has been elegantly demonstrated in Ezh2 knockout versus Ezh1 knockout mice and Ezh2/Ezh2 double knockout mice. Given that Ezh2 and Ezh1 are the only known methyltransferases capable of H3K27 trimethylation in mammals, we presume the residual H3K27me3 demonstrated in the absence of Ezh2 was mediated by Ezh1 with consequent functional compensation for Ezh2 loss in our model, which we believe may have attenuated its phenotypic effects. Nonetheless, EZH1 mutations are not seen in myeloid malignancy and thus these compensatory mechanisms are also likely to be active in human MDS. Second, Ezh2 protein levels were markedly reduced in Ezh2<sup>T</sup> mice despite RNA expression showing a trendwise increase. Given the maintained H3K27me3 levels, it is likely that Ezh1 compensation was limiting effects of loss of Ezh2. Third, epigenetic mutations, including those in EZH2, do not occur late in MDS pathogenesis. In our study, Ezh2 deletion was induced relatively late in disease pathogenesis after MDS features such as thrombocytopenia and leucopenia were already present in NHD13<sup>T</sup>. The timing of deletion may have also abrogated the influence of Ezh2 deletion in altering the course of NHD13<sup>T</sup> disease and is an inherent limitation of the transgenic NHD13 model. Finally, Ezh2 loss has been previously shown to upregulate HOX clusters, including HOXA genes, via reduction of H3K27me3 repression in human MDS. The potential overlap in mechanisms of transformation with NHD13<sup>T</sup> and Ezh2 loss through Hox gene dysregulation and lack of further de-
Figure 1. Long-term effects of Ezh2 deletion in NHD13<sup>3</sup> leukemia. A) Breeding and experimental schema for long-term survival assessment. PB, peripheral blood. B) Western blot demonstrating Ezh2 and H3K27me3 protein levels in Ezh2<sup>D/D</sup>, NHD13<sup>3</sup> and NHD13<sup>3</sup>; Ezh2<sup>D/D</sup> leukemias compared to kit-enriched wild-type bone marrow from adult C57BL/6J mice (WT), relative to an Actin loading control. Leukemic bone marrow samples of each genotype were taken from selected mice listed in Supplementary Table 1 (Supplemental Digital Content, http://links.lww.com/HS/A40) and loaded in the listed order. C) Ezh2 and Ezh1 RNA expression levels as measured by qPCR in Ezh2<sup>D/D</sup>, NHD13<sup>3</sup> and NHD13<sup>3</sup>; Ezh2<sup>D/D</sup> leukemias compared to wild-type whole bone marrow (WT WBM) and WT kit-enriched bone marrow (WT kit). Samples were taken from the same mice as for B). D) Expression of Ezh2 targets as measured by qPCR. E) Peripheral blood counts prior to (baseline) and at 6 months post Poly (I:C). WCC, white cell counts. F) Kaplan-Meier survival curve after Poly (I:C) administration for each genotype. G) Proportions of disease types analyzed at time of cull for each genotype. H) HoxA9 expression by qPCR in Ezh2<sup>D/D</sup>, NHD13<sup>3</sup> and NHD13<sup>3</sup>; Ezh2<sup>D/D</sup> leukemias compared to WT WBM. Statistics for qPCR experiments show results of unpaired t tests. Statistics for peripheral blood analyses show results of ANOVA testing. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.
repression of HoxA9 in NHD13T;MxT;Ezh2Δ11A may explain the lack of in vivo synergy. MxT;Ezh2Δ11A mice in this model exhibited a highly penetrant leukemia with most mice succumbing to acute leukemia during the observation period with a median survival of 273 days. Our findings are most similar to the report by Simon et al where all mice exclusively developed T-ALL after a latency of approximately 10 months. In contrast, in other models using a tamoxifen-inducible Cre-ERT, Ezh2-deleted mice developed features of MDS, MPN and MDS/MPN although in 2 of these reports, had similar overall survival to wild-type controls over a period of 300 days’ observation. Two studies reported effects of Ezh2 deletion in combination with other mutations seen in myeloid malignancy, Runx1 mutation and Tet2 knockdown and demonstrated greater acceleration of hematological malignancy from Ezh2 deletion compared to the NHD13T background. In combination with Runx1 mutation, Ezh2 loss led to a median overall survival of 262 days compared to ‘not reached’ in either mutation alone over 10 months’ observation. Similarly on a Tet2 knockdown background, deletion of Ezh2 accelerated death (median ~180 days) compared with a ~300 day median overall survival in mice with either single mutation. Altogether, these studies highlight diverse, context-dependent outcomes of Ezh2 deletion in mouse models.

In conclusion, this study describes a model of EZH2 deletion in MDS, adding to existing literature on the cooperation of Ezh2 deletion in combination with other genetic aberrations in MDS pathogenesis. These findings suggest Ezh2 loss may have limited effects in the NHD13TΔ11A mice in vivo

References
