Polycomb repressive complex 2 (PRC2) suppresses Eμ-myc lymphoma

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• PRC1 and PRC2 have opposing activity in Eμ-myc lymphoma
• Inhibition of PRC2 leads to increased self-renewal in B cell progenitors

ABSTRACT

Deregulation of polycomb group (PcG) complexes PRC1 and PRC2 is associated with human cancers. While inactivating mutations in PRC2-encoding genes EZH2, EED and SUZ12 are present in T-ALL and in myeloid malignancies, gain-of-function mutations in EZH2 are frequently observed in B-cell lymphoma, implying disease-dependent effects of individual mutations. We show that, in contrast to PRC1, PRC2 is a tumor suppressor in Eμ-myc lymphomagenesis, as disease onset was accelerated by heterozygosity for Suz12 or by shRNA-mediated knockdown of Suz12 or Ezh2. Accelerated lymphomagenesis was associated with increased accumulation of B-lymphoid cells in the absence of effects on apoptosis or cell cycling. However Suz12-deficient B-lymphoid progenitors exhibit enhanced serial clonogenicity. Thus, PRC2 normally restricts the self-renewal of B-lymphoid progenitors, the disruption of which contributes to lymphomagenesis. This finding provides new insight regarding the functional contribution of mutations in PRC2 in a range of leukemias.
INTRODUCTION

Polycomb group (PcG) proteins are global transcriptional repressors first identified in *Drosophila* as silencers of *Hox* genes during development. Subsequent genome-wide studies showed that PcG proteins regulate genes involved in diverse cellular functions \(^1,2\). PcG proteins exist in two distinct protein complexes called Polycomb Repressive Complex 1 (PRC1) and 2 (PRC2). Mammalian PRC1 components include Bmi1, Mel18, Cbx2, 4, 7 and 8, Scmh1 and 2, Phc1/Rae28, Phc2 and 3, Ring1A and Ring1B; the complex is highly heterogeneous and its precise make-up varies depending on the cellular and developmental context. While Bmi1 is crucial for augmenting PRC1 activity, Ring1B is the enzyme that monoubiquitinates histone H2A at lysine 119 (H2AK119ub), a mark associated with transcriptional repression \(^3\). PRC2 mediates tri-methylation of histone H3 at lysine 27 (H3K27me3), another repressive mark. The main components in PRC2 are Suz12, Eed, and the enzymatic components Ezh2 and/or Ezh1. Eed and Suz12 are essential for PRC2 complex stability, while accessory factors Jarid2, Rbbp4 and 7, Phf1 and Mtf2 are required to modulate PRC2 function \(^4\).

PRC1 and PRC2 interact to control transcriptional activity at target loci. In the hierarchical-recruitment model \(^5\), PRC2-mediated H3K27me3 recruits PRC1 via the chromodomain of Cbx proteins, leading to H2AK119ub-induced transcriptional silencing. Studies have correlated PRC2 activity and H3K27me3 with PRC1 occupancy at target genes \(^1,6\), providing support for a role of PRC2/H3K27me3 in recruiting PRC1. However, other observations suggest that the hierarchical model may not always hold. For example, PRC1 can bind nucleosomes lacking N-terminal histone tails *in vitro* and can be recruited to targets in the absence of PRC2 \(^7,8\). Moreover, mice with a heterozygous loss-of-function mutation in *Suz12* display enhanced hematopoietic stem cell (HSC) activity \(^9\), while mice lacking PRC1 components have functionally compromised HSCs \(^10,11\).
PcG genes are deregulated in many human cancers. \textit{EZH2} and \textit{BMI1} are overexpressed in some breast cancers and colon cancers, and increased expression of \textit{EZH2} is associated with more aggressive disease in prostate cancers \cite{12}. Discovery of a gain-of-function mutation in \textit{EZH2} in follicular and diffuse large B-cell lymphoma \cite{13-15} strengthened the argument that PcG genes are oncogenic. Conversely, loss-of-function mutations and deletions in \textit{EZH2}, \textit{EED} and \textit{SUZ12} have been described in myelodysplastic syndromes (MDS) and T-cell acute lymphoblastic leukemia (T-ALL) \cite{16-19}. This suggests that PRC2 has a tumor suppressor role in specific hematological malignancies, a hypothesis supported in a mouse model where Ezh2 inactivation resulted in T-cell lymphoma \cite{20}. These studies suggest that aberrant PRC2 function contributes to tumorigenesis in a context-dependent manner and emphasise the need to define the underlying mechanisms via which altered PRC2 contributes to disease. Accordingly, we have compared and contrasted the contribution of PRC1 and PRC2 to \textit{Myc}-driven lymphomagenesis and show that in contrast to PRC1, PRC2 behaves as a tumor suppressor by restricting self-renewal of B cell progenitors.

\textbf{METHODS}

\textbf{Mice}

Experimental procedures were approved by the Walter and Eliza Hall Institute Animal Ethics Committee. \textit{Eμ-myc} \cite{21}, \textit{Suz12Plt8/+} \cite{9} and \textit{Bmi1+-} \cite{22} mice were maintained on C57BL/6 background.

\textbf{Analysis of hematopoietic cells by flow cytometry}

Single-cell suspensions from bone marrow, spleen and thymus were prepared in balanced salt solution (BSS; 150 mM NaCl, 3.7 mM KCl, 2.5 mM CaCl\textsubscript{2}, 1.2 mM MgSO\textsubscript{4}, 7.4 mM HEPES-NaOH, 1.2 mM KH\textsubscript{2}PO\textsubscript{4}, 0.8 mM K\textsubscript{2}HPO\textsubscript{4}) with 5\% FCS. Whole blood was collected for automated cell count (Advia3120, Bayer); for FACS analysis, erythrocytes were lysed in 150 mM NH\textsubscript{4}Cl, 0.1 mM EDTA, and 12 mM NaHCO\textsubscript{3}. Cells were stained with monoclonal antibodies to B220 [RA3-6B2], CD19 [1D3], CD25 [PC61], c-Kit [2B8], IgM [II/41], IgD [11-26], Mac-1 [M1-
Apoptosis and cell cycle analysis

BrdU was administered intra-peritoneally (0.1 mg/g bodyweight). BrdU incorporation in bone marrow and spleen was determined one hour after injection (BD Pharmingen). In vitro apoptosis assays of FACS-purified pro-B, pre-B and sIg+ B cells were performed as described previously 23.

Retrovirus production

The protocol for retrovirus production has been previously described 9. Briefly, retroviral supernatants were prepared by transfection of 293T cells with plasmids encoding viral envelope proteins and specific shRNAs in the LTR-miR30-SV40-GFP (LMS) vector that target Suz12 (CGCTCTTACTGCTGAGCGTATA), Ezh2 (CGCTCTTACTGCTGAGCGTATA) or a proprietary scrambled sequence (Nons) designed by Open Biosystems.

Adoptive transfer of Eμ-myc fetal livers

Ter119-depleted E13.5 Eμ-myc fetal livers were transduced with retroviral supernatants containing LMS-Suz12, LMS-Ezh2 or LMS-Nons, and cultured overnight at 37°C/5% CO2, as described previously 9,24. 3-4 x 10^5 cells were injected intravenously into lethally irradiated CD45Ly5.1 recipients (11 Gy; ^60^Co source) and mice were monitored for lymphoma development.
**In vitro culture of B-lymphocytes**

Unfractionated bone marrow cells and FACS-purified pro-B cells were either cultured on OP9 stroma supplemented with IL-7 (2% supernatant from an IL-7-producing cell line\textsuperscript{25}, or 5ng/mL murine rIL7 (PeproTech)), or in MethoCult M3630 (StemCell Technologies).

**Immunoblotting**

Cells and primary lymphomas were homogenised in RIPA buffer (1% NP-40, 0.1% SDS, 0.5% sodium decarboxylate, 150 mM NaCl, 50 mM Tris-HCl) containing Complete Protease Inhibitors (Roche). Proteins were separated by SDS-PAGE, transferred to PVDF membranes, and blotted with antibodies against Suz12 (P-15, Santa Cruz, or D39F6, Cell Signalling Technology (CST)), Ezh2 (07-689, Millipore, or AC-22, CST), GFP (A-11122, Invitrogen), H3K27me3 (07-449, Millipore, or C36B11, CST), H2AK119ub (D27C4, CST), histone H3 (AS3, Millipore), and actin (I-19, Santa Cruz).

**RNA extraction, cDNA synthesis and RT-qPCR**

RNA was extracted using RNeasy Mini or Micro Columns with DNase I treatment (Qiagen), and reverse-transcribed into cDNA with oligo-dT priming (Promega, Madison, USA) using Superscript III reverse transcriptase (Invitrogen). RT-qPCR was performed using Taqman probes to *c-myc* (Mm00487804\_m1), *Pax5* (Mm00435501\_m1), *Ebf1* (Mm00395519\_m1), *Tcfe2a* (Mm01175595\_m1), and *Hprt* (Mm00446968\_m1) in an ABI 7900HT PCR machine (Applied Biosystems). Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method.

**Statistical analysis**

Data were analysed using Prism GraphPad v6.0. A two-tailed Student’s t-test was performed in two-group comparisons. When comparing multiple groups, one-way ANOVA followed by Tukey’s post-hoc test was performed. Log-ranked (Mantel-Cox) test was used in survival studies. Progenitor
frequencies from limiting dilution assays were determined using the Extreme Limiting Dilution Assay (ELDA) software tool.

**Expression profiling of Eμ-myc and Eμ-myc/Suz12^{Plt8/+} lymphomas**

Total RNA from seven Eμ-myc/Suz12^{+/+} and five Eμ-myc/Suz12^{Plt8/+} FACS-purified lymphomas were sequenced at the Australia Genome Research Facility. An average of 12.2 million single-end 100bp sequence reads were obtained per lymphoma sample on an Illumina GA-II Sequencer. An average of 95% of the reads were successfully mapped to the mm9 mouse genome build using the Bioconductor package Rsubread. The RNA-Seq profiles were summarized using the featureCounts function to count the number of reads overlapping the exome of each gene. Differential expression analysis was performed using the Bioconductor package edgeR. Reads mapping to immunoglobulin, ribosomal RNA and mitochondrial protein genes were filtered out of the analysis. Genes on the X- and Y-chromosomes were also removed to avoid any confounding factors due to lymphomas arising from mice of different sexes. Read counts were TMM normalised to adjust for compositional differences between samples. Genewise estimates of biological variation were obtained using empirical Bayes moderated dispersions. Statistical significance was assessed using an exact test for negative binomial distributed data. To prepare for gene set testing, the read counts were transformed to approximately standard normal deviates using the zscoreNBinom function of the edgeR package. A battery of gene sets from the Broad Institute’s curated C2 molecular signatures database was tested using the CAMERA function of the limma package. This is a competitive gene set test which tests whether the genes in the gene set are significantly more up- or down-regulated compared to the other genes in the experiment. Human gene symbols were mapped to mouse orthologs using the Mouse Genome Database.
RESULTS

Suz12<sup>Plt8/+</sup> mice have elevated numbers of B-lymphocytes

Previous studies have demonstrated that mice carrying a heterozygous loss-of-function allele of Suz12, Ezh2 or Eed have enhanced hematopoietic stem cell activity and platelet production. However, the impact of these mutations has not been detailed in other hematopoietic cell types. Using Suz12<sup>Plt8/+</sup> mice to model hypomorphic PRC2 function, we found a consistent elevation in blood leukocytes relative to Suz12<sup>+/+</sup> mice at both 4 and 8 weeks of age (Figure 1), which was exclusively due to higher lymphocyte counts (Tables S1, S2).

Progenitor cell analysis in Suz12<sup>Plt8/+</sup> mice

The elevated numbers of B cells in Suz12<sup>Plt8/+</sup> mice prompted us to examine B cell development in detail at 4 and 8 weeks of age. The numbers of cells at various stages of B-lymphoid maturation appeared unaltered at both ages, although a modest increase in total B-lymphoid cell numbers was evident in 8-week-old Suz12<sup>Plt8/+</sup> mice (Figures 2A,B and Tables S3, S4). Enumeration of B- and T-cell subsets in the spleen and thymus of Suz12<sup>Plt8/+</sup> mice revealed no significant abnormalities (Tables S3, S4). There was a modest increase in the number of LSK cells and multi-potent progenitors (MPPs) in Suz12<sup>Plt8/+</sup> mice at weaning (Figure 2D), although this was less pronounced in adult mice (Table S3). Enumeration of lymphoid-primed multi-potent progenitors (LMPPs), and common lymphoid progenitors (CLPs) did not reveal significant differences between Suz12<sup>Plt8/+</sup> and Suz12<sup>+/+</sup> mice. Similarly, sub-fractionation of the CLP population into ALPs (Ly6D<sup>-</sup>; all-lymphoid progenitors) and BLPs (Ly6D<sup>+</sup>; B-cell primed progenitors) did not reveal any changes in Suz12<sup>Plt8/+</sup> mice (Figure 2C,D and Table S3). To determine whether impaired PRC2 function influenced the expression of the master B-cell regulators Pax5, Ebf1 and E2A in immature progenitors, as observed in Bmi1-knockout mice, we compared their expression in purified HSC, MPP, LMPP and CLP populations. There were no significant differences in the relative expression of these genes in all populations examined between Suz12<sup>+/+</sup> and Suz12<sup>Plt8/+</sup> mice (Figure S1).
**Suz12 deficiency results in increased clonogenicity of B-lymphoid progenitors**

The frequency (f) of B-lymphoid progenitors with proliferative potential was assessed by limiting dilution from unfractionated bone marrow cells. Suz12Plt8/+ mice contained a higher number of clonogenic progenitors than Suz12+/+ controls (Figure 3A). This observation was independently verified when equal numbers of bone marrow cells from 3-week-old Suz12+/+ and Suz12Plt8/+ mice were cultured in methylcellulose for development of B-lymphoid colonies (Figure 3B). Limiting dilution assay and methylcellulose cultures revealed no significant difference in clonogenicity of purified Suz12Plt8/+ pro-B cells (B220+ CD19+ c-Kit+ IgM-) relative to control (Figure 3C,D). However, when cells from primary methylcellulose cultures were re-plated, Suz12Plt8/+ cells generated more secondary colonies than Suz12+/+ controls and the difference in re-cloning potential was further enhanced upon a third round of re-plating, in which Suz12+/+ cells generated very few colonies, while Suz12Plt8/+ cells re-plated robustly (Figure 3D). Increased re-plating potential of Suz12Plt8/+ pro-B cells was confirmed in a separate experiment in which 20 individual colonies from primary cultures were re-plated individually into secondary cultures. The frequency and the absolute number of secondary colonies were significantly higher in Suz12Plt8/+ pro-B cells than control (Figure 3E).

There was a modest decrease in global H3K27me3 level in Suz12Plt8/+ pro-B cells compared to Suz12+/+ pro-B cells, which coincided with a slight decrease in Suz12 and Ezh2 levels (Figure S2A). This effect was more pronounced in pro-B cells expressing shRNA to Suz12, in which a profound reduction in Suz12, Ezh2 and global H3K27me3 levels was evident (Figure S2B). The level of H2AK119ub, the histone mark deposited by PRC1, was not altered in Suz12-deficient cells relative to total protein (Figure S2).

**PRC2 restricts Eμ-myc lymphomagenesis**
To address if heterozygosity of PcG genes contributes to B cell malignancy, \textit{Suz12}\textsuperscript{Plt8/+} mice or mice with a heterozygous deletion in the gene encoding the PRC1 protein Bmi1 were crossed with \textit{E\textmu-myc} transgenic mice. Consistent with previous observations \textsuperscript{38}, \textit{E\textmu-myc} mice developed lymphoma with a median onset of 103 days, while loss of one allele of \textit{Bmi1} prolonged survival (Figure 4A). In contrast, \textit{E\textmu-myc/Suz12}\textsuperscript{Plt8/+} mice showed accelerated onset of disease with a median survival of 72 days (Figure 4A). This suggests that unlike \textit{Bmi1}, which promotes lymphomagenesis in \textit{E\textmu-myc} mice, \textit{Suz12} functions as a tumor suppressor.

Analysis of moribund \textit{E\textmu-myc/Suz12}\textsuperscript{Plt8/+} mice revealed lymphomas typical of those observed in \textit{E\textmu-myc/Suz12}\textsuperscript{+/+} mice. All moribund mice presented with lymphadenopathy, splenomegaly (Figure S3A) and increased leukocyte numbers, accompanied by normal red blood cell counts and mild thrombocytopenia (Figure S3D-F). All lymphomas were of B-lymphoid origin, and the proportion of pre-B, sIg\textsuperscript{+} B or mixed pre-B/mature B lymphomas were consistent (Figure S3B). To verify malignancy, 2 x 10\textsuperscript{6} splenocytes from lymphoma-bearing mice were transplanted into non-irradiated recipients. As documented for \textit{E\textmu-myc} disease \textsuperscript{39}, lymphomas developed quickly in all recipients, but the disease latency did not differ between recipients of \textit{E\textmu-myc/Suz12}\textsuperscript{+/+} and \textit{E\textmu-myc/Suz12}\textsuperscript{Plt8/+} lymphomas (Figure S3C). \textit{E\textmu-myc/Suz12}\textsuperscript{Plt8/+} lymphomas displayed a variable but on average modest reduction in \textit{Suz12} levels compared to \textit{E\textmu-myc} lymphomas, while there was no consistent difference in \textit{Ezh2} levels (Figures 4B and S2C). Global H3K27me3 levels varied considerably between individual \textit{E\textmu-myc/Suz12}\textsuperscript{+/+} lymphomas, and a similar pattern was observed in \textit{E\textmu-myc/Suz12}\textsuperscript{Plt8/+} lymphomas, with no consistent differences between the two groups.

To independently confirm that reduced PRC2 activity accelerates \textit{E\textmu-myc} lymphoma, E13.5 \textit{CD45\textsuperscript{Ly5.2} E\textmu-myc} fetal liver cells were infected with GFP-tagged shRNAs targeting \textit{Suz12} (\textit{LMS-Suz12}), \textit{Ezh2} (\textit{LMS-Ezh2}) or a non-silencing control (\textit{LMS-Nons}), and transplanted into lethally irradiated \textit{CD45\textsuperscript{Ly5.1}} recipients (Figure S4A). The activity of the shRNAs was confirmed using
G1ME cells and primary pro-B cell cultures (Figures S2B and S4B). Mice reconstituted with PRC2-deficient cells had higher blood leukocyte numbers at 4 and/or 8 weeks post-transplantation (Figure S5A). Effective donor-derived contribution to mature blood cells was confirmed at eight weeks post-transplantation (Figure S5B). There was a preferential expansion in cells of the B-lymphoid lineage over T-lymphoid and myeloid lineages in recipients of LMS-Suz12 and LMS-Ezh2-expressing cells, which was not evident in the LMS-Nons control (Figure S5C-F).

Mice reconstituted with PRC2-deficient cells developed lymphomas faster than the control group: 19/20 mice from the LMS-Ezh2 group and 23/27 mice from the LMS-Suz12 group succumbed to lymphoma with a median onset of 100 days, while only 12/30 mice from the LMS-Nons group developed disease, with a median latency of 150 days (Figure 4C). All moribund mice developed either pre-B or B-cell lymphomas at similar frequencies (Figure S5G) and displayed leukocytosis and thrombocytopenia similar to unmanipulated Eμ-myc mice (Figure S5H-J). While only 5/12 LMS-Nons lymphomas expressed GFP, 21/23 LMS-Suz12 and 19/20 LMS-Ezh2 lymphomas were GFP+, indicating that PRC2-deficient cells were more lymphomagenic than the LMS-Nons control, which were no more likely to cause lymphoma than non-transduced (GFP−) cells. Immunoblotting analysis from FACS-purified lymphoma cells showed a significant reduction in Suz12, Ezh2 and global H3K27me3 levels in Suz12- and Ezh2-knockdown lymphomas relative to control (Figures 4D). These data confirm that PRC2 functions as a cell-autonomous tumor suppressor of Eμ-myc lymphoma.

**Expanded B-lymphopoiesis in pre-neoplastic Eμ-myc/Suz12Plt8/+ mice**

To understand the mechanisms behind accelerated lymphoma onset, we examined changes in cellular pathways in pre-neoplastic mice, a distinct phase in 3-4 week-old mice defined by the lack of transplantable tumor cells. Lymphomas failed to develop in mice transplanted with 10⁶ splenocytes from 3-4 week-old Eμ-myc/Suz12Plt8/+ mice, confirming that a true pre-neoplastic phase
also exists in these mice. Quantitative RT-PCR analysis from purified bone marrow pre-B cells showed that the Suz12Plt8 mutation did not influence c-myc RNA levels (Figure S6), excluding the possibility that accelerated lymphomagenesis in Eμ-myc/Suz12Plt8/+ mice was simply due to increased transgene expression.

Pre-neoplastic Eμ-myc/Suz12Plt8/+ mice exhibited a 3-fold increase in blood leukocytes relative to control mice (Figure 5A), primarily due to increased lymphocytes (Table S2). In contrast, Eμ-myc/Bmi1+/− mice showed significant reduction in total leukocytes (Figures 5A) and lymphocytes (Table S2). Analysis of nucleated blood cells revealed a marked increase in total B-lymphoid cell numbers in Eμ-myc/Suz12Plt8/+ mice relative to Eμ-myc littermates, while a significant reduction in B-lymphoid cells was observed in Eμ-myc/Bmi1+/− mice (Figure 5B). While bone marrow cellularity was constant in the three groups (Table S4), Eμ-myc/Suz12Plt8/+ mice had increased number of B-lineage cells compared to Eμ-myc mice, due to elevated numbers of sIg− precursors (Figure 5C and Table S4). In contrast, Eμ-myc/Bmi1+/− mice had fewer B-lymphoid cells than in Eμ-myc controls (Figure 5C and Table S4). Eμ-myc mice exhibited significant splenomegaly, both in weight and cellularity, which was further exacerbated in Eμ-myc/Suz12Plt8/+ mice, while the opposite was observed in Eμ-myc/Bmi1+/− mice (Table S4). This was attributable to an increased number of total splenic B-lymphoid cells and specific subsets including pre-B, immature and mature B cells (Figure 5D). The numbers of T lymphocytes and myeloid cells were generally within the normal range in mice of all genotypes (Table S4).

**Suz12 deficiency has no influence on apoptosis or cell cycle of Eμ-myc B-lymphoid cells**

Pre-neoplastic Eμ-myc/Suz12Plt8/+ mice had an increased frequency of B-cell progenitors in their bone marrow as assessed by limiting dilution assay (Figure 6A), as well as an increased number of cells capable of forming B-cell colonies in methylcellulose (Figure 6B). The Suz12Plt8 mutation did not influence BrdU uptake in vivo in pro-B, pre-B or sIg+ B-cells in bone marrow or spleen of Eμ-
myc mice (Figure 6C). While bone marrow pre-B and slg+ B-cells from \( \text{E}_\mu\text{-myc} \) mice died rapidly in the absence of cytokines, the rate of apoptosis was equivalent in \( \text{E}_\mu\text{-myc/Suz12}^{Plh8/+} \) and \( \text{E}_\mu\text{-myc/Bmi1}^{+/+} \) mice (Figures 6D,E). It is therefore unlikely that changes in the cell cycle or apoptotic mechanisms contribute significantly to the increased number of B cell progenitors evident in pre-leukemic \( \text{E}_\mu\text{-myc/Suz12}^{Plh8/+} \) mice.

**Gene expression analysis of PRC2-deficient lymphomas**

A gene-level differential expression analysis revealed 35 genes up-regulated (Table S5) and 32 genes down-regulated (Table S6) in \( \text{E}_\mu\text{-myc/Suz12}^{Plh8/+} \) lymphomas. Competitive gene set analysis was used to interpret the differential expression patterns in terms of molecular pathways, using the curated gene set collection of the molecular signatures database. Of the 3235 gene sets tested, 111 were significantly altered in \( \text{E}_\mu\text{-myc/Suz12}^{Plh8/+} \) lymphomas (Table S7). Many of the gene sets were derived from hematological and epithelial tumor studies. For example, genes involved in the progression from benign adenoma to malignant hepatocellular carcinomas were up-regulated (p-value = 0.010) \(^{41}\), while genes that are normally up-regulated during the transition from pro-B to pre-B cells were down regulated in \( \text{E}_\mu\text{-myc/Suz12}^{Plh8/+} \) lymphomas (p-value = 0.036) \(^{42}\).

**DISCUSSION**

While a role for deregulation of PRC2 in multiple cancer contexts is compelling, the diversity of molecular lesions in PRC2 components implies that PRC2 can contribute to tumorigenesis via multiple mechanisms. To better understand the role of PRC1 and PRC2 in hematological malignancies, we have compared the effect of compromising each complex in the \( \text{E}_\mu\text{-myc} \) transgenic mouse model of B lymphoma. In striking contrast to the previously established effects of heterozygosity of the PRC1 gene \( Bmi1 \), which delays disease onset \(^{38}\), a loss of function allele of \( \text{Suz12} \) accelerated B lymphoma in the \( \text{E}_\mu\text{-myc} \) model. Similar observations were found in chimeric mice reconstituted with \( \text{Suz12}- \) or \( Ezh2 \)-knockdown \( \text{E}_\mu\text{-myc} \) fetal liver cells. It is known that \( \text{E}_\mu\text{-myc} \)
myc B-lymphoid cells have increased rates of cell cycling and deregulated apoptosis \cite{23,40}; however, these parameters were not measurably different in PRC2-compromised Eμ-myc mice. The enhanced self-renewal capacity of B-lymphoid progenitors evident in Suz12Plt8/+ mice is likely responsible for the expansion of the B cell lineage in these animals and the accelerated disease onset in Eμ-myc mice that have impaired PRC2 activity.

While studies in *Drosophila* led to proposal of the initiator-mediator model, which posits that PRC1 is sequentially recruited to PRC2 targets to execute gene silencing \cite{5}, PRC1 and PRC2 clearly have opposing functions in Eμ-myc-driven lymphomagenesis. The concept that the actions of PRC1 and PRC2 can diverge from the initiator-mediator model in specific circumstances is supported by several in vitro studies. For example, PRC1 can be recruited to targets in PRC2-deficient ES cells during X-inactivation \cite{7}, and genome-wide ChIP studies showed PRC1 and PRC2 can occupy distinct loci \cite{43}. The recent identification of a novel PRC1 complex that monoubiquitinates histone H2A independent of H3K27me3/PRC2 in mouse ES cells \cite{44} provides a potential mechanism for PRC2-independent gene regulation by PRC1. These observations imply that polycomb complexes regulate gene expression via multiple mechanisms in context-dependent manners.

Previously, we demonstrated that PRC1 and PRC2 regulate distinct targets in hematopoietic stem cells (HSCs) \cite{24}. Although these genes represent a small proportion of total PRC-responsive genes, they can have a profound influence on HSC functions. The contrasting roles of PRC1 and PRC2 in Eμ-myc-driven lymphomagenesis suggest that these complexes may also regulate distinct targets in lymphoid progenitors. Another alternative is that some target genes are particularly sensitive to the dosage of either PRC1 or PRC2, or to individual complex components. For example, the tumor suppressor gene *Cdkn2a* is very sensitive to changes in the level of PRC1 \cite{11} but it is less responsive to inhibition of PRC2 \cite{24}. Our results suggest that inhibition of PRC2 results in enhanced self-renewal in lineage-committed progenitors that speeds up the course of Eμ-myc disease. Further
analysis of the genomic occupancy and activity of PRC1 and PRC2 will be required to determine why inhibition of these complexes results in such distinct outcomes.

Mutations that disrupt PRC2 have been identified in human lymphoid malignancy, but the precise role of these mutations remains unclear. While activating mutations in \textit{EZH2} are common in diffuse large B-cell lymphoma \textsuperscript{13-15}, loss-of-function mutations and deletions of \textit{EZH2} and \textit{SUZ12} have been described in human T-ALL \textsuperscript{18,19}, and recently \textit{Ezh2} was shown to be critical for T-ALL suppression in mouse models \textsuperscript{20}. These observations mirror those obtained with our results in \textit{Eµ-myc} lymphoma, which emphasizes that PRC2 function is indeed context-dependent. \textit{EZH2} expression is high in B lymphoid progenitors, declines during B cell maturation, and then is up-regulated again during affinity maturation of activated germinal-centre B cells \textsuperscript{45}. Thus, while PRC2 activity may restrict the proliferative potential of B lymphoid progenitors via effects on self-renewal, it may be that gain-of-function mutations work to stimulate proliferation specifically in more mature B cells.

Loss of \textit{EZH2} has also been identified in myelodysplastic syndrome (MDS) \textsuperscript{16}. Intriguingly, ectopic expression of \textit{Ezh2} in mice also results in myeloid malignancies \textsuperscript{46}, and PRC2 is required for the maintenance of self-renewal in MLL-AF9-driven myeloid leukemias \textsuperscript{47}. These studies collectively show that maintenance of PRC2 activity within a defined normal range is essential, as either reduced or excess activity predisposes to malignancy. Modulation of epigenetic regulators, including EZH2, is an exciting and rapidly developing area in cancer therapy \textsuperscript{48,49}. It is important to consider that PRC2 is clearly acting as a tumor suppressor in some contexts, which has important implications for how to approach this complex therapeutically.

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AUTHORSHIP CONTRIBUTIONS

SCWL, WSA and IJM designed experiments and wrote the manuscript. SCWL, CDH, HSL and RSA performed experiments and analysed data. BP, AL and GKS performed bioinformatics analyses. SLN and MEB provided reagents. DJH, SLN, MEB and GKS provided critical feedbacks to the manuscript. WSA and IJM edited manuscript and supervised research.

CONFLICTS OF INTEREST

All authors declare no competing financial interests.
REFERENCES


FIGURE LEGENDS

Figure 1. *Suz12Plt8/+* mice have elevated numbers of B-lymphocytes in the peripheral blood

Automated leukocyte counts from peripheral blood performed on 4 week-old (A) and 8 week-old (C) *Suz12+/+* and *Suz12Plt8/+* mice. Enumeration of blood B- and T-lymphocytes in *Suz12+/+* and *Suz12Plt8/+* at 4 weeks (B) and 8 weeks (D) of age. Data represent mean ± SD. A two-tailed Student’s t-test was used for comparison between the genotypes (* p<0.05, *** p<0.001).

Figure 2. Analysis of bone marrow progenitors and B-lymphocyte subsets in 4 week-old *Suz12Plt8/+* mice

Gating strategy (A) and number of viable cells (B) for various B lymphoid populations in the bone marrow of 4 week-old *Suz12+/+* and *Suz12Plt8/+* mice. Gating strategy (C) and number of bone marrow progenitors (D) in 4 week-old mice *Suz12+/+* and *Suz12Plt8/+* mice. Data represent mean ± SD. A two-tailed Student’s t-test was used for comparison between the genotypes (* p<0.05). Cell surface markers used to define various subsets are as follows: total B: B220+ CD19+; pre-pro B: CD11c- NK1.1- CD19- CD43+; pre-B-II: B220+ CD19+ cKit- CD25- IgM-; pro + preB: B220+ CD19+ cKit- IgM+; Immature B: B220low IgM+; recirculating B: B220high IgM+; LSK: Lineage- Sca1+ c-Kit+; CD150+: LSK CD150+; HSC: LSK Flt3-; MPP: LSK Flt3int; LMPP: LSK Flt3high; CLP: Lineage- Sca-1+ c-Kitint IL-7Rα+ Flt3+; ALP: CLP Ly6D-; BLP: CLP Ly6D+.

Figure 3. *Suz12Plt8/+* pro-B cells have enhanced self-renewal potential

(A) Limiting dilution analysis of *Suz12+/+* (n=8) and *Suz12Plt8/+* (n=4) bone marrow cells was performed to compare the frequency (f) of B lymphoid progenitors. Cells were cultured on OP-9 stroma with IL-7. The clonogenic cell frequency model fitted to each dilution series is shown by a solid straight line relating the log10 fraction of negative wells to the number of cells per well. Steeper slopes indicate higher frequencies of colony-forming cells. Broken lines show 95% confidence intervals. (B) Unfractionated bone marrow cells from 3 week-old *Suz12+/+* (n=6) and...
Suz12Plt8/+ (n=5) mice were cultured in methylcellulose with IL-7, and the numbers of colonies were scored 7 days later. Data represent means ± SEM. A two-tailed Student’s t-test was performed (* p<0.05). (C) Limiting dilution analysis showed no difference in progenitor frequencies of purified bone marrow pro-B cells (B220+ CD19+ c-Kit+ IgM-) from 3 week-old Suz12+/+ and Suz12Plt8/+ mice. (D) Self-renewal potential of Suz12+/+ (n=8) and Suz12Plt8/+ (n=8) pro-B cells was determined by serial re-plating of colonies in methylcellulose. Data represent means ± SEM. A two-tailed Student’s t-test was performed (* p<0.05, *** p<0.001). (E) Comparison of the clonogenic potential of individual primary colonies from Suz12+/+ and Suz12Plt8/+ pro-B cells, expressed both in frequency and total secondary colony numbers. Data represent means ± SEM. A two-tailed Student’s t-test was performed (** p<0.01, *** p<0.001).

Figure 4. PRC2 suppresses the development of B–cell lymphoma in Eμ-myc transgenic mice in a cell-autonomous manner.
(A) Lymphoma-free survival of Eμ-myc (circle), Eμ-myc/Suz12Plt8/+ (square) and Eμ-myc/Bmi1+/+ (triangle) mice. (B) Immunoblot analysis of primary lymphomas from Eμ-myc and Eμ-myc/Suz12Plt8/+ mice. (C) Lymphoma-free survival of lethally irradiated Ly5.1 recipient mice reconstituted with Eμ-myc fetal liver cells expressing shRNA to Suz12 (circle), Ezh2 (triangle), and a Nons control (square). (D) Immunoblot analysis of Suz12, Ezh2 and H3K27me3 from FACS-sorted donor-derived lymphoma cells expressing the indicated shRNAs.

Figure 5. Pre-neoplastic Eμ-myc/Suz12Plt8/+ mice have an expanded B-lymphoid compartment.
(A) Automated enumeration of peripheral blood leukocytes from pre-neoplastic Eμ-myc (n=17), Eμ-myc/Bmi1+/+ (n=8), and Eμ-myc/Suz12Plt8/+ (n=12) mice. Immunophenotypic characterisation of B-lymphocyte subsets from the peripheral blood (B), bone marrow (C) and spleen (D) of pre-neoplastic Eμ-myc, Eμ-myc/Bmi1+/+, and Eμ-myc/Suz12Plt8/+ mice. Data represent mean ± SD. One-way ANOVA followed by Tukey’s post-hoc test was used for pair-wise comparisons (* p<0.05, **
p<0.01, *** p<0.001). Cell surface markers used to define B-cell subsets are as follows: BM pro-B: B220⁺ CD19⁺ c-Kit⁺ IgM⁻ IgD⁻; BM pre-B-II: B220⁺ CD19⁺ c-Kit⁻ CD25⁺ IgM⁻ IgD⁻; BM pro + pre B, blood sIg⁻ B or spleen pre-B: B220⁺ CD19⁺ c-Kit⁻ IgM⁻ IgD⁻; spleen immature B: B220⁺ CD19⁺ c-Kit⁻ IgM⁺ IgD⁻; BM sIg⁺; spleen mature B: B220⁺ CD19⁺ c-Kit⁻ IgM⁺ IgD⁺.

**Figure 6.** Proliferation or spontaneous apoptosis are unchanged in pre-neoplastic B-lymphoid cells between Eµ-myc/Suz12⁺/+ and Eµ-myc/Suz12Plt8/+ mice.

(A) Limiting dilution analysis of unfractionated bone marrow cells from pre-neoplastic Eµ-myc/Suz12⁺/+ (n=4) and Eµ-myc/Suz12Plt8/+ (n=3) mice. (B) Unfractionated bone marrow cells from pre-neoplastic Eµ-myc (n=6) and Eµ-myc/Suz12Plt8/+ (n=7) mice were cultured in methylcellulose and scored 7 days later. Data represent means ± SEM. A two-tailed Student’s t-test was performed (* p<0.05). (C) BrdU incorporation in Eµ-myc (n=3) and Eµ-myc/Suz12Plt8/+ (n=3) cells 1 hour after BrdU injection (0.1 mg/mg bodyweight). The percentage of BrdU⁺ cells in bone marrow pro-B, pre-B and sIg⁺ B-cells, and in splenic pre-B and sIg⁺ B-cells were determined by FACS. Data represent means ± SD. A two-tailed Student’s t-test was used to determine statistical significance. In vitro survival assay was performed on cells from pre-neoplastic Eµ-myc, Eµ-myc/Bmi1⁺/-, and Eµ-myc/Suz12Plt8/+ mice. FACS-purified bone marrow pre-B (D) and sIg⁺ B cells (E) were cultured under conditions of cytokine deprivation. Cell viability was measured by Annexin-V and propidium iodide staining using flow cytometry. Three-week-old non-transgenic wildtype (+/+), Bmi1⁺/- and Suz12Plt8/+ mice were included as controls. Data represent means ± SEM at each time point. One-way ANOVA followed by Tukey’s post-hoc test was used to compare mice of the following genotypes: wildtype (+/+), Bmi1⁺/- and Suz12Plt8/+, either carrying the Eµ-myc transgene, or the corresponding non-transgenic controls.
Lee_Fig. 1.

A

Blood Leukocyte number (x10^3 cells/μL)

Suz12^+/+  Suz12^Plt8/+  ***

B

Absolute cell number (per μL blood)

Suz12^+/+ (20)  Suz12^Plt8/+ (18)  ***

C

Blood Leukocyte number (x10^3 cells/μL)

Suz12^+/+  Suz12^Plt8/+  ***

D

Absolute cell number (per μL blood)

Suz12^+/+ (6)  Suz12^Plt8/+ (7)  *

B-cells  T-cells
**Lee_Fig. 2.**

**A**

*SuZ12<sup>+/+</sup> SuZ12<sup>Plt8/+</sup>*

- **Total B cells**
  - 38.5
  - 42.3

- **Immature & recirculating B-cells**
  - **SuZ12<sup>+/+</sup>**
    - IgM: 3.37
    - B220: 35.6
  - **SuZ12<sup>Plt8/+</sup>**
    - IgM: 3.55
    - B220: 39.2

- **Pre-pro B cells**
  - **SuZ12<sup>+/+</sup>**
    - CD19: 2.63
  - **SuZ12<sup>Plt8/+</sup>**
    - CD19: 4.21

- **Pre-B-II cells**
  - **SuZ12<sup>+/+</sup>**
    - CD25: 28.7
  - **SuZ12<sup>Plt8/+</sup>**
    - CD25: 31

**B**

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**C**

- **Gated on Lin-ve**
  - **SuZ12<sup>+/+</sup>**
    - IL-7Rα: 4.88
  - **SuZ12<sup>Plt8/+</sup>**
    - IL-7Rα: 5.08

- **Gated on c-Kit<sup>int</sup>**
  - **SuZ12<sup>+/+</sup>**
    - Sca-1: 4.04
  - **SuZ12<sup>Plt8/+</sup>**
    - Sca-1: 4.01

- **Gated on CLP**
  - **SuZ12<sup>+/+</sup>**
    - CD150: 14.7
  - **SuZ12<sup>Plt8/+</sup>**
    - CD150: 17.7

- **Gated on LSK**
  - **SuZ12<sup>+/+</sup>**
    - CD150<sup>+</sup>LSK: 65.9
  - **SuZ12<sup>Plt8/+</sup>**
    - CD150<sup>+</sup>LSK: 65.4

**D**

- **Cell number (x10<sup>3</sup>)**
  - **SuZ12<sup>+/+</sup>**
    - 7
  - **SuZ12<sup>Plt8/+</sup>**
    - 7

- **Cell number (x10<sup>6</sup>)**
  - **SuZ12<sup>+/+</sup>**
    - 17
  - **SuZ12<sup>Plt8/+</sup>**
    - 18
**Figure 3.**

(A) Log fraction negative wells vs. No. of unfractionated BM cells per well for Suz12+/+ and Suz12Plt8/+ cells. The lines represent the linear regression for each group:

- Suz12+/+: \( f = 1/91 \)
- Suz12Plt8/+: \( f = 1/72 \)

(*** \( p = 0.00628 \))

(B) Colony numbers per 25,000 BM cells for Suz12+/+ and Suz12Plt8/+ cells. The plot shows a significant difference between the two groups.

(C) Log fraction negative wells vs. No. of purified Pro-B cells per well for Suz12+/+ and Suz12Plt8/+ cells. The lines represent the linear regression for each group:

- Suz12+/+: \( f = 1/2.04 \)
- Suz12Plt8/+: \( f = 1/2.67 \)

(\( p = 0.105 \))

(D) Total colony numbers for Suz12+/+ and Suz12Plt8/+ cells across 3 rounds of re-plating. The plot shows a significant increase in colony numbers for Suz12Plt8/+ cells.

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(E) Summary of re-cloning frequency and average colony numbers:

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<tr>
<td>Re-cloning frequency</td>
<td>(0.069 \pm 0.02)</td>
<td>(0.319 \pm 0.04**)</td>
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<tr>
<td>Average colony</td>
<td>(3.10 \pm 0.94)</td>
<td>(86.9 \pm 27.8**)</td>
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</table>
Lee_Fig. 4.

**A**

- €μ-myc (62)
- €μ-myc/Suz12\(^{Plt8/+}\) (44)
- €μ-myc/Bmi1\(^{+/−}\) (32)

**B**

- **Suz12**
- **Ezh2**
- **Actin**
- **H3K27me3**
- **Histone H3**

**C**

- LMS-Nons (12/30)
- LMS-Suz12 (23/27)
- LMS-Ezh2 (19/20)

**D**

- sh-Nons
- sh-Ezh2
- sh-Suz12

**Percent survival (%)**

**Tumour-free survival (Days)**
A

B

C

D

E

Lee_Fig. 6.
Polycomb repressive complex 2 (PRC2) suppresses Eμ-\( \text{myc} \) lymphoma