Opposing roles of polycomb repressive complexes in hematopoietic stem and progenitor cells

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Short title – Polycomb repressive complexes in HSCs
Abstract

Polycomb group (PcG) proteins are transcriptional repressors with a central role in the establishment and maintenance of gene expression patterns during development. We have investigated the role of Polycomb Repressive Complexes (PRCs) in hematopoietic stem cells (HSCs) and progenitor populations. We show that mice with loss of function mutations in PRC2 components display enhanced HSC/progenitor population activity, whereas mutations that disrupt PRC1 or PhoRC are associated with HSC/progenitor cell defects. Since the hierarchical model of PRC action would predict synergistic effects of PRC1 and PRC2 mutation, these opposing effects suggest this model does not hold true in HSC/progenitor cells. To investigate the molecular targets of each complex in HSC/progenitor cells, we measured genome-wide expression changes associated with PRC-deficiency, and identified transcriptional networks that are differentially regulated by PRC1 and PRC2. These studies provide new insights into the mechanistic interplay between distinct PRCs and have important implications for approaching PcG proteins as therapeutic targets.

Introduction

PcG proteins are epigenetic repressors important in the maintenance of transcriptional silencing. PcG proteins exist in 3 distinct complexes: PRC1, PRC2 and pleiohomeotic repressive complex (PhoRC). PRC2 has 4 constituent proteins: E(z), Su(z)12, Esc and Pcl in flies and Ezh2, Suz12, Eed and Phf1 in mammals (Fig. 1A). Ezh2 is the enzymatic component of PRC2, catalysing methylation of lysine 27 of histone H3 (H3K27)1-4, while Suz12 and Eed are required for complete function of Ezh2 and stable formation of the
complex. Phf1 influences the enzymatic specificity of Ezh2, promoting trimethylation of H3K27 (H3K27me3) in preference to dimethylation (H3K27me2). Dimethylation is broadly distributed throughout the genome and is thought to have a structural role, whereas H3K27me3 is enriched on promoters and associated with transcriptionally silent regions.

There has been a vast expansion in the number of PRC1 components in mammals. PRC1 components include Bmi1 and Mel18 (orthologues of Drosophila Psc), Cbx2, 4, and 8 (orthologues of Drosophila Pc), Scmh1 and 2 (orthologues of Drosophila Scm), Phc1/Rae28, Phc2 and 3 (orthologues of Drosophila Ph) and Ring1A and Ring1B (orthologues of Drosophila Ring) (Fig. 1A). Ring1A and Ring1B are the enzymatic components of PRC1 responsible for the mono-ubiquitination of histone H2A at lysine 119 (H2AK119Ub), other components such as Bmi1 and Mel18 are able to stimulate this activity. H2AK119Ub is also associated with transcriptional repression.

PhoRC is composed of the DNA binding transcription factor Yy1 (Drosophila Pho) and Sfmbt1 (Fig. 1A). PhoRC is not known to possess any enzymatic activity. Rather, Sfmbt binds methylated histone residues, and Yy1 is the only mammalian PcG protein with specific DNA binding activity, suggesting that PhoRC may direct PRC1 or PRC2.

Some years ago the chromodomain of Drosophila Polycomb was shown to bind H3K27me3, leading to the hierarchical recruitment model (reviewed), where PRC2 trimethylates H3K27, leading to PRC1 recruitment and ubiquitination of H2AK119.
Several lines of evidence support this prevailing model: (i) a high degree of overlap between sites bound by PRC1 and PRC2 in human and mouse cells\(^9\),\(^{10}\),\(^{19}\) (ii) the failure of \textit{Drosophila} E(z) mutants to recruit PRC1\(^20\); and (iii) a correlation between H3K27me3 levels and PRC1 recruitment and H2AK119Ub accumulation\(^21\). A growing number of reports now suggest that PRC1 and 2 may not always act in this fashion: PRC1 binds nucleosomes that lack N-terminal tails \textit{in vitro}\(^22\); PRC1 is recruited in PRC2 deficient mammalian cells\(^23\); and genome-wide ChIP studies show PRC2 bound to loci devoid of PRC1\(^19\) and vice versa\(^15\). These observations imply that the interaction between various PRCs is complex.

Genome-wide ChIP studies suggest PRCs play a role in ES cells by repressing key developmental transcription factors\(^8\),\(^9\), although they also play roles in adult neural, mammary and hematopoietic stem cells. HSCs deficient in Bmi1, Mel18 or Phc1/Rae28 have defective repopulating capacity that is partially explained by derepression of the \textit{Ink4a}/\textit{Arf} locus, which encodes the cyclin-dependent kinase p16\(^{\text{Ink4a}}\) and tumour suppressor p19\(^{\text{Arf}}\)\(^{24,25}\). Deletion of the \textit{Ink4a}/\textit{Arf} locus only partially rescues the \textit{Bmi1}\(^{-/-}\) phenotype, pointing to a broader role for PRC1 in regulating HSC function. In contrast to the role of PRC1, the PRC2 component Suz12 restricts HSC activity; reduced levels of Suz12 in HSCs result in enhanced HSC activity\(^26\), reminiscent of earlier studies using hypomorphic alleles of Eed\(^27\). While there is growing evidence that PRC1 and PRC2 have opposing activities in HSCs, the molecular bases for the differences remain undefined.
The study of PRC2 in HSCs has been limited by the embryonic lethality observed for null mutants. We have used the \( Mpl^{-/-} \) genetic background, which provides a sensitised environment to detect small changes in HSC activity in viable heterozygous animals\(^{28}\). \( c\text{-Mpl} \) is the receptor for thrombopoietin, a cytokine critical for both HSC function and platelet production\(^{29}\). Our previous studies have shown that peripheral blood platelet count is a surrogate marker for stem cell activity in these mice\(^{26,30}\). We reasoned that by combining the \( Mpl \) null sensitised background and mutations in one or more of the PRCs, we would be able to systematically test whether the PRCs act in synergy, and thus test whether the hierarchical model for PRC action holds true in hematopoiesis.

Here we report that mutation of \( \text{Ezh2, Eed or Suz12} \) enhances HSC/progenitor activity, suggesting that PRC2 as a complex restricts HSC/progenitor activity. We establish genetically that PRC1 and PRC2 have opposing roles in HSC/progenitor populations, similar to what is seen in progenitors by others\(^{27}\) and contrary to the prediction of the hierarchical model of PRC action. We have also shown that PhoRC, like PRC1, enhances HSC/progenitor function. Finally, we investigated the gene expression signatures that account for the opposing role of the PRCs in HSC/progenitors, and have identified genes that are differentially regulated between PRC1 and 2. This set of genes encompasses the transcriptional networks downstream of HoxA9 and C/EBP\(\alpha\), both of which are central to normal development of the hematopoietic system and are implicated in disease.
Materials and Methods

Mouse strains

All mice were on an inbred C57BL/6 background, unless otherwise stated. Mice carrying the \textit{Eed}^{3354} allele were purchased from Oak Ridge National Laboratories. The \textit{Eed}^{3354} point mutation was detected using an allelic discrimination assay (Table S5). The \textit{Ezh2}^{Del} allele was generated from the \textit{Ezh2}^{fl} allele and genotyped as previously described\textsuperscript{31}. Animal studies were approved by the Walter and Eliza Hall Institute Animal Ethics Committee.

Haematological analyses

Analyses were performed essentially as previously described\textsuperscript{26}. For bone marrow competitive transplants, test \textit{CD45}_{Ly5.2} bone marrow was mixed with an equal number or 10 fold excess of competitor \textit{CD45}_{Ly5.1} bone marrow cells, and 2 x 10\textsuperscript{6} nucleated cells injected into irradiated \textit{CD45}_{Ly5.1} / \textit{CD45}_{Ly5.2} or \textit{CD45}_{Ly5.1} recipients. For fetal liver LSK competitive transplants, 4 to 5 livers per genotype were pooled prior to sorting. 1000 test \textit{CD45}_{Ly5.2} LSK cells, 1000 competitor \textit{CD45}_{Ly5.1} LSK cells and 200,000 \textit{CD45}_{Ly5.1} / \textit{CD45}_{Ly5.2} filler bone marrow cells were injected into 4 lethally irradiated \textit{CD45}_{Ly5.1} / \textit{CD45}_{Ly5.2} recipients, per sorted LSK sample. Test and competitor contribution was analysed at 10 weeks post-reconstitution for peripheral blood, and 12 to 16 weeks for other organs.
**LSK analysis and fluorescence activated cell sorting**

Bone marrow LSK analysis was performed as previously described\textsuperscript{26}. Individual fetal livers were dissociated in isotonic buffer containing 5% (v/v) fetal calf serum (FCS, Hyclone). Samples for LSK enumeration were treated with red cell removal buffer, otherwise all cells were stained with rat monoclonal antibodies against Ter119, CD3, CD5, B220, Gr1 and CD8. Samples used for sorting were incubated with BioMag goat anti-rat IgG beads (Qiagen), and lineage marker positive cells removed using a Dynal magnet (Invitrogen). The lineage depleted cells, or all cells in the case of LSK enumeration, were stained with fluorophore-conjugated anti-rat IgG antibody, then monoclonal antibodies to c-Kit, Sca-1, and CD45\textsuperscript{ly5.1} and CD45\textsuperscript{ly5.2} where applicable. Cells were flow sorted on a FACSDiva, FACSaria (BD Biosciences) or MoFlo (Dako).

**shRNA mediated viral knockdown and transplantation of fetal liver cells**

E14.5 fetal livers were harvested and dissociated in isotonic buffer containing 5% FCS (Hyclone). Ter119 positive cells removed via magnetic bead depletion. Ter119 depleted cells were cultured in IMDM with 15% FCS and cytokines (10 ng/mL IL-6, 5 ng/mL Flt3 ligand, 50 ng/mL TPO and 100 ng/ SCF) for 4 hours. Retroviral supernatants were applied to culture dishes treated with RetroNectin (Takara Biosciences). Supernatant was removed and fetal liver cells cultured on the viral coated dishes in the presence of 4 µg/mL polybrene (Sigma). Fetal liver cells were washed off plates after 16 to 24 hours infection. The proportion of infected cells was determined by measuring GFP, and approximately 3-5 x 10\textsuperscript{5} viable nucleated cells were injected into each lethally irradiated...
recipient. The sequence of each shRNA is given in Table S5, and each was used in the LMS vector.

RNA extraction and qPCR
RNA was extracted from 5×10^3 – 1×10^6 sorted cells using RNeasy micro or mini columns (Qiagen). cDNA was synthesised using oligo(dT) primers and either Superscript III reverse transcriptase (Invitrogen) or Sensiscript reverse transcriptase (Qiagen). qPCR reactions were set up with duplicate technical replicates, and the expression of mouse genes determined using Taqman gene expression assays, as detailed in Supplementary Figure legends.

Microarray hybridization and differential expression
RNA extracted as described above from 40,000-70,000 sorted fetal liver LSK cells, was labelled, amplified and hybridised to Illumina MouseWG-6 V2.0 Expression BeadChips according to Illumina standard protocols at the Australian Genome Research Facility. Differential expression analysis was performed as described in Supplementary Methods. GEO accession number: GSE21404.

Gene set testing
The overlap of the current microarray results with previous experiments was assessed using mean-rank gene set enrichment tests. A Wilcoxon signed-rank test was used to assess whether each external gene set was highly ranked when all genes on the
microarrays were ordered from most to least differentially expressed using the current data.

**Genuine association of expression profiles (GENAS)**

A novel procedure, implemented in the GENAS function of the limma package, was applied to estimate correlations between the expression profiles of different mouse mutants. Each mutant was compared to wild-type, producing a set of log₂-fold changes. The GENAS analysis determines whether these fold changes are correlated between mutants, after allowing for estimation uncertainty. The method is a generalization of the limma empirical Bayes analysis\textsuperscript{32} in which the moderated t-statistics are modelled using a multivariate t distribution. The biological correlation between the mutants was estimated from the prior correlation of the log₂-fold changes in the Bayesian hierarchical model. In this way, the method is able to separate true biological correlation from technical correlation. The statistical significance of the correlation is assessed by a likelihood ratio test.

**Romer gene set enrichment analysis**

Gene set enrichment analysis (GSEA) is an approach which correlates a large database of co-regulated gene sets with respect to a microarray data set.\textsuperscript{33} Romer is a new GSEA method suitable for microarray linear model analyses including array weights and possibly small numbers of replicates. Romer was applied using the curated (c2) gene sets from MSigDB (http://www.broadinstitute.org/gsea/). Each gene set was converted to mouse using the orthology mapping from the Jackson Laboratory. Romer can find gene
sets associated with any contrast in a linear model. We looked for gene sets regulated in the same direction by Bmi1 and Suz12 by comparing the average of Bmi1\textsuperscript{-/-} and Suz12\textsuperscript{Plt8/+} to wild-type. Gene sets regulated in opposite directions by Bmi1 and Suz12 were found by directly contrasting Bmi1\textsuperscript{-/-} vs Suz12\textsuperscript{Plt8/+}. The same steps were undertaken for Bmi1 and Yy1. In each case, sets with p-values < 0.05 for up- or down-regulation were selected.

Results

All core PRC2 components restrict HSC/progenitor population activity

To study the role of PRC2 components (Fig. 1A) in HSC/progenitor populations, we produced Mpl\textsuperscript{-/-} animals heterozygous for null alleles of Eed (Eed\textsuperscript{3354})\textsuperscript{34} or Ezh2 (Ezh2\textsuperscript{Del})\textsuperscript{31}, and compared peripheral blood counts to Mpl\textsuperscript{-/-} animals heterozygous for a loss-of-function point mutant of Suz12 (Suz12\textsuperscript{Plt8})\textsuperscript{26}. Heterozygosity for mutations in Suz12, Eed or Ezh2 significantly ameliorated the Mpl\textsuperscript{-/-} thrombocytopenia and significantly increased peripheral white blood cell counts (Fig. 1B).

We tested the functional activity of long-term repopulating stem cells from Ezh2\textsuperscript{Del/+} Mpl\textsuperscript{-/-} mice by competitive reconstitution of lethally irradiated recipients. Transplanting equal numbers or a 10 to 1 excess of Ezh2\textsuperscript{+/+} bone marrow over Ezh2\textsuperscript{Del/+} bone marrow, resulted in an increased representation of the Ezh2\textsuperscript{Del/+} cells compared with Ezh2\textsuperscript{+/+} across all cell lineages and hematopoietic organs (Fig. 1C). Our results suggest that PRC2 restricts HSC/progenitor activity.
PRC1 and PRC2 have opposing roles in the HSC/progenitor compartment

We tested the interaction between PRC1 and PRC2 in HSC/progenitors by examining the number of platelets in $Mpl^{-/-}$ $Suz12^{Plt8/+}$ heterozygous mice, which were also heterozygous for mutations in the PRC1 genes $Bmi1$, $Mel18$, $Phc1/Rae28$, $Phc2$, $Ring1A$, $Ring1B$ and $Cbx2^{35-40}$. Heterozygosity for $Bmi1$ exacerbated $Mpl^{-/-}$ thrombocytopenia, whereas heterozygosity for $Suz12$ increased peripheral platelet and white blood cell counts ($p < 0.001$, Fig. 2A). Compound heterozygotes for $Bmi1$ and $Suz12$ had peripheral platelet counts intermediate between each of the single mutants, in keeping with opposing functions of PRC1 and PRC2 (Fig. 2A). Consistent results were seen in mice heterozygous or homozygous for each of the other PRC1 mutant alleles; where effects on platelet number were found, they were in the opposite direction to those of the $Suz12^{Plt8}$ allele (Fig. S1).

The long-term repopulating capacity of stem cells from the $Bmi1$ and $Suz12$ compound heterozygous animals was tested using 1:1 competitive transplants of bone marrow cells into lethally irradiated recipients (Fig. 2B). $Bmi1$ and $Suz12$ compound heterozygous animals show a level of contribution to recipients similar to wild-type, and significantly lower than the elevated contribution seen for $Suz12$ single heterozygotes ($p < 0.01$, Fig. 2B). These results show that Bmi1 and Suz12 have opposing activities in HSC/progenitor function.
**Yy1 as part of PhoRC enhances HSC/progenitor activity**

Yy1 may be able to localise PRC1 or PRC2 through its DNA binding capacity, so we investigated whether PhoRC also has a role in HSC/progenitor function by using a null allele of Yy1\(^{41}\). Since Yy1\(^+/\) mice die peri-implantation, we produced Mpl\(^+/\) Yy1\(^{+/}\) mice (Fig. 3A) and found a significantly exacerbated thrombocytopenia and leukopenia (p < 0.001, Fig. 3A). Subtle, but similar, defects were seen in Mpl\(^{+/}\) Yy1\(^{+/}\) mice (Fig. S2). In the same experiment, we tested whether Yy1 and Suz12 co-operate in hematopoiesis. We found that compound heterozygotes for Yy1 and Suz12 displayed intermediate peripheral platelet and white blood cell counts on the Mpl\(^+/\) background (Fig. 3A). Heterozygosity for Yy1 produced a PRC1-like phenotype, rather than a PRC2-like phenotype.

Approximately one in six Yy1\(^+/\) embryos on a mixed genetic background do not survive to weaning, attributable to developmental retardation and neural defects \(^{41}\). On an inbred C57BL/6 background, we found that 30% of Yy1\(^+/\) animals die prior to weaning. The combination of Mpl deficiency and the C57BL/6 inbred background led to an even greater mortality of Yy1\(^+/\) animals, with 60% of Mpl\(^+/\) Yy1\(^{+/}\) animals dying before weaning (p < 0.001, Fig. 3A). Thus, Yy1 and Mpl interact genetically, consistent with a role for both Yy1 and Mpl in HSCs.

As we saw significantly reduced numbers of viable Yy1\(^+/\) adults, we used fetal livers from E14.5 embryos for further studies. We analysed the number of HSC/progenitors using lineage marker negative (Lin-), Sca-1\(^+\), c-Kit\(^+\) (LSK) staining. Heterozygosity for Yy1 in Mpl\(^{+/}\) E14.5 fetal livers resulted in a significant reduction in the proportion and
absolute number of LSK cells, without any decrease in fetal liver cellularity (p < 0.01, Fig. 3B). Competitive transplant of equal numbers of purified Mpl+/+ Yy1+/+ and Mpl+/+ Yy1+/+ fetal liver LSK cells into lethally irradiated recipients (Fig. 3C) suggested that the Yy1+/+ cells are compromised in their ability to contribute to maintenance of the stem cell compartment and to blood cell formation (Fig. 3C and data not shown).

To confirm the role of Yy1 in HSC/progenitors, we used shRNA mediated viral knockdown of Yy1 in fetal liver cells, followed by transplant into lethally irradiated recipients. At 6 to 12 months post-transplantation, the proportion of Yy1 knockdown cells decreased significantly compared with the input cells and the non-silencing control, across all cell lineages including LSK cells, and all hematopoietic organs (Fig. 3D). Consistent with the phenotype of Suz12 mutant mice, Suz12 knockdown cells displayed an increased contribution across all cell types and hematopoietic organs (data not shown), as shown previously\textsuperscript{26}. Splenic B cells were isolated from recipient animals and knockdown of Yy1 and Suz12 confirmed \textit{in vivo} in the shRNA containing (GFP positive) cells (Fig. S3). These results confirm that Yy1 enhances HSC/progenitor activity and number, independent of its role in embryonic development.

\textit{Identification of gene sets differentially regulated by PRC1, PRC2 and PhoRC}

Identification of genes differentially regulated by the PRCs in hematopoietic cells was important to pinpoint the molecular bases for their opposing roles. The \textit{Ink4a/Arf} locus is a recognised target of PRC1 in hematopoietic cells, and repressed by all three PRCs in fibroblasts. Using shRNA mediated viral knockdown of Yy1, Bmi1 and Suz12 we
investigated whether the *Ink4a/Arf* locus is repressed by each complex in *Gata-1* null megakaryocyte erythroid (G1ME) cells. Using an *in vitro* competition assay between infected (GFP+) and uninfected (GFP-) cells, we found a mild selection against *Suz12* shRNA infected cells and a strong selection against cells infected with shRNAs directed against *Bmi1* or *Yy1* (Fig. S4A).

Infected and uninfected cells were sorted on days 4 and 7 post-infection, and analysed by qPCR for knockdown of *Bmi1*, *Yy1* and *Suz12*, and for expression of *Cdkn2a* (p16*) (Fig. S4B). Each hairpin produced greater than 80% knockdown of the target (Fig. S4B). We did not observe any difference in *Cdkn2a* expression between cells uninfected and infected with the *Nonsil* or *Suz12* shRNAs, but found significant up-regulation of *Cdkn2a* expression in cells infected with one *Yy1* shRNA and the *Bmi1* shRNA, and a small but not statistically significant increase in a different *Yy1* shRNA (Fig. S4B). Given the potency of p16, increases in *Cdkn2a* need only be modest to slow growth of the cell and bring about a halt in cell cycle progression. Although *Suz12* knockdown did not alter *Cdkn2a* expression in G1ME cells (Fig. S4B), knockdown was effective since we observe changes in three other genes we previously found to be sensitive to Suz12 dose in LSK or Lin- c-Kit+ cells (Fig. S5). This suggests that the *Ink4a/Arf* locus is differentially sensitive to the amount of Suz12, Bmi1 and Yy1 in the G1ME hematopoietic cell line.

To test whether changes in G1ME cells also occurred in LSK cells, we analysed *Cdkn2a* expression by qPCR in fetal liver LSK cells sorted from individual E14.5 embryos (Fig. S6A). *Cdkn2a* was undetectable in wild-type fetal liver LSKs, more than 1000 times
lower than the house-keeping gene *Hprt1*. *Cdkn2a* was detectable in fetal liver LSK cells from some *Bmi1*+/− embryos, and in all *Bmi1*−/− embryos tested, whereas it was never detected in *Yy1*I+/- or *Suz12*Plt8+/+ embryos (Fig. S6A). We saw up-regulation of *Cdkn2a* in G1ME cells depleted of Yy1, but not in LSK cells heterozygous for *Yy1*, suggesting that although the locus is sensitive to dosage of Yy1, derepression of *Cdkn2a* may not be responsible for the deficiency of *Yy1* heterozygous HSC/progenitors. To confirm we could detect gene expression changes in LSK cells from *Yy1* or *Suz12* mutants, we analysed the expression of *Prr6*, a gene we previously identified to be highly responsive to *Suz12* dosage in adult LSK cells26, and G1ME cells (Fig. S5); *Prr6* was up-regulated in fetal liver LSK cells mutant for PRC1, PRC2 or PhoRC (Fig. S6B).

Since we could not account for the defects in *Suz12*Plt8+/+ or *Yy1*+/− HSCs via altered *Cdkn2a* expression, we performed global gene expression analyses to identify more genes differentially responsive to each PRC in HSC/progenitors. Fetal liver LSK cells from *Bmi1*−/−, *Suz12*Plt8+/+ and *Yy1*I+/- E14.5 embryos and their wild-type littermates (all *Mpl*I+/+, n=3-4 replicates for each mutant, n=8 wild-type) were analysed on Illumina mouse whole-genome expression arrays. The gene expression changes, identified by moderated t-statistics 32, were modest, similar to those reported by us and others for global gene expression analyses in the absence of PcG proteins10,15,26. Nevertheless, the expression signatures found previously tended to be recapitulated; genes up-regulated in our *Suz12*Plt8+/+ fetal liver LSK cells were enriched for genes previously found up-regulated in *Suz12*Plt8+/+ adult LSK cells (p < 10−7, Table S1) or *Suz12* knockdown adult Lin− c-Kit+ progenitors (p < 0.001, Table S1)26. Genes differentially over and under-expressed in our
Suz12Plt8/+ fetal liver LSKs were enriched for genes bound by PRC2 members Suz12 and Ezh2 in ES cells (p < 0.0005, Table S1)\textsuperscript{19}. Genes differentially expressed in our Bmi1\textsuperscript{-/-} fetal liver LSKs were enriched for genes bound by PRC1 member Ring1B in ES cells (p < 0.0005, Table S1)\textsuperscript{19}.

To analyse the multitude of genes regulated by PcG proteins we devised two new statistical methods for data analysis. The first method, GENAS, analyses the correlation between specific comparisons (e.g. Bmi1\textsuperscript{-/-} vs wild-type and Suz12Plt8/+ vs wild-type), to identify classes of genes co-ordinately regulated by more than one PRC. We removed all probes with no evidence of differential expression in any of our three comparisons, and plotted fold change for the remaining 7824 probes (5907 unique genes) for each comparison against the others. The PRC1 vs PRC2 plot (Fig. 4A) shows a clearly visible group of genes (coloured blue) that are repressed by both Suz12 and Bmi1. There are also less obvious clusters of genes with more modest fold changes that exhibit opposing changes in expression (coloured red and yellow). The GENAS analysis showed that the majority of probes follow this opposing trend (p < 0.00001), reminiscent of the opposing phenotypes displayed by these cells, even though the individual genes with largest fold changes are in the concordant group. In contrast, there was a strong positive correlation between genes differentially regulated in Bmi1\textsuperscript{-/-} and Yy1\textsuperscript{+/-} LSKs (p < 0.00001, Fig. 4B) and very few genes with opposing expression changes, consistent with these factors co-operating to regulate gene expression in HSC/progenitors. To select specific genes, we applied a log\textsubscript{2}-fold-change cut off of 0.25. This identified 128 genes that were up-regulated in fetal liver LSK cells depleted for either Bmi1 or Suz12 (Fig. 4A, coloured
blue, Table S2), that is, repressed by both PRC1 and PRC2. A larger set of 172 genes are up-regulated in fetal liver LSK cells depleted for Suz12 but down-regulated in Bmi1 deficient LSK cells (Fig. 4A, coloured red, Table S2). PRC2 represses expression of these genes, whereas PRC1, directly or indirectly, enhances their transcription. Reassuringly, these genes behave similarly in cells depleted for Yy1 as those depleted in Bmi1 (Fig. 4B).

Our second novel statistical method is called Romer; a powerful tool that takes into account the large number of moderate gene expression changes, and performs competitive gene set tests using all of the gene sets from the Broad Institute’s Molecular Signatures database. We used Romer to analyse the classes of genes enriched in those co-ordinately or differentially regulated by PRC1, PRC2 and PhoRC (Table S3). All comparisons had significant enrichment for genes sensitive to histone deacetylase inhibitor treatment, indicating the deregulated genes were sensitive to epigenetic control.

Within the class of genes co-ordinately regulated by PRC1 and PRC2, genes repressed by Bmi1 and Suz12 (blue genes, Fig. 4A) were significantly enriched for E2F target genes, consistent with the known roles for PRCs in this pathway. Genes up-regulated by Bmi1 and repressed by Suz12 (red genes, Fig. 4A) were enriched for Hox genes, specifically genes activated by HoxA9 (Fig. 4C). Genes repressed by Bmi1 and up-regulated by Suz12 (yellow genes, Fig. 4A) were enriched for genes up-regulated in the absence of Dnmt1, along with genes repressed by HoxA9 and C/EBPα (Fig. 4C). Genes responsive to IL-6, a downstream target of C/EBPα, were also enriched within this group of genes. We validated a small selection of the genes identified as co-ordinately or differentially
regulated by PRC1 and PRC2 by qPCR (Fig. S7). There were also gene sets co-ordinately regulated by PRC1 and PhoRC. Genes activated by C/EBP transcription factors were up-regulated in the context of PRC1 or PhoRC depletion, as they were for PRC2 depletion (Fig. 4C), unlike genes repressed by C/EBPα, which appear to be differentially regulated by PRC1 and PRC2. Genes that were co-ordinately down-regulated in the absence of Bmi1 or reduction of Yy1 (Fig. 4B) were enriched for hematopoiesis-related transcription factors and Hox genes (Fig. 4C), in keeping with the defective HSC/progenitor activity of LSK cells depleted in Bmi1 or Yy1. These results confirm that depletion of PhoRC produces a similar molecular phenotype to deficiency in PRC1.

We extended our analyses to include adult LSK cells from Suz12Plt8/+ animals, either replete or deficient for c-Mpl. We found a positive correlation between the gene expression changes identified in fetal and adult LSK cells (genes coloured blue, green, red and yellow in Fig. 4A, shown in Fig. S8 and Table S4), independent of Mpl genotype (correlation of 0.39, p < 9x10^{-12} for Mpl^{-/-} and 0.31, p < 5x10^{-8} for Mpl^{+/+}). These studies confirm the expression changes we observe in the fetal liver LSK compartment, and suggest that similar mechanisms govern the activity of PRCs in the adult stem cell compartment.

Discussion

In recent years, the role of PcG proteins in HSCs has been a subject of interest due to the clinical relevance of understanding the molecular mechanisms underpinning HSC activity, and the relationship between stem cell biology and cancer. One puzzling
finding has been that PRC1 components appear to enhance HSC function, while PRC2 components Suz12 and Eed restrict HSC and progenitor function, respectively\textsuperscript{26,27}; results which are inconsistent with the predominant hierarchical model for PRC function. The molecular basis for the apparently opposing effects of PRC1 and PRC2 has remained elusive. Furthermore, any potential contribution of PhoRC to HSC biology had not been studied.

Here we analysed the role of PRC1, PRC2 and PhoRC in HSCs, and found that all core PRC2 components (Suz12, Ezh2 and Eed) are involved in restricting HSC/progenitor function. Overexpression of Ezh2 was previously shown to enhance HSC activity\textsuperscript{44}. One potential explanation for this discrepancy was that Suz12 has a role outside of PRC2, however careful testing has revealed a similar phenotype in mutants of all three PRC2 components. We have confirmed that PRC1 and 2 have opposing roles in HSC/progenitors and shown that PRC1 and PRC2 do not synergise in their contribution to HSC/progenitor function. This finding is similar to previous observations in myeloid and B-lineage progenitors, using mutant alleles of \textit{Eed} and the same knockout allele of \textit{Bmi1}\textsuperscript{27}. Therefore, at least at some loci critical to the function of HSCs (and progenitors), PRC1 may be targeted to chromatin via PRC2-independent mechanisms, consistent with afore mentioned studies in other cell types\textsuperscript{9,15,23}. Our results suggest the loci where PRC1 and PRC2 act independently are important for HSC/progenitor function, and both the loci themselves and the mechanism of PRC action at these loci are worthy of further consideration.
It is unclear how PRC1 may be targeted by PRC2-independent mechanisms or indeed how PRC2 is directed to specific loci, so we investigated the role of the only DNA-binding PcG protein, Yy1, in HSC function. Yy1 has a role in determining HSC/progenitor number and enhances HSC/progenitor function; PhoRC appears to play a PRC1-like role in HSC biology. Previous studies in other mammalian cell types have shown that Yy1 can associate with PRC1, PRC2 or both. Since Yy1 plays a similar role to PRC1 in HSC/progenitors, perhaps PhoRC directs PRC1 binding.

Given the differing roles of each of the PRCs in HSC/progenitors, we examined the genes controlled by each. We found that the Ink4a/Arf locus (specifically Cdkn2a expression) is differentially sensitive to the activity of PRC1, PRC2 and PhoRC in fetal liver LSK cells. Our genome-wide expression analysis of these cells has led us to propose that PRCs have multiple potential modes of action, which can vary locus by locus (Fig. 4D); some genes are differentially sensitive to PRC dosage (eg. Cdkn2a), some are independently controlled by PRC1 with PhoRC or PRC2 alone (red and yellow genes), and others are co-ordinately controlled by PRC1, PhoRC and PRC2 (cooperative, blue genes). Interestingly, genes that are co-ordinately regulated by all three PRCs show the largest fold changes, which may explain the focus on the hierarchical model. This has been misleading, however, as the opposing phenotypes we have observed, suggest that the more numerous but smaller fold changes seen in the genes independently controlled by each PRC are more important for HSC/progenitor function. Our results highlight the biological importance of small expression changes across gene sets.
Within the genes that are activated in LSK cells depleted of PRC1 or PhoRC but not PRC2, we found enrichment for genes activated in the absence of Dnmt1. A recent study in fibroblasts has identified a link between H2AK119Ub and DNA methylation in regions that are not necessarily enriched in H3K27me3\(^{15}\). Therefore one intriguing possibility is that DNA methylation, along with PhoRC, plays a role in targeting PRC1 in HSC/progenitors.

The genes differentially regulated by PRC1 and PRC2 were enriched for HoxA9 targets. Furthermore, there was good correlation between the behaviour of PRC2 depleted HSCs and HSCs that overexpress HoxA9\(^{47}\), and PRC1 or PhoRC depleted HSCs and those deficient in HoxA9\(^{48}\). Expression changes in C/EBP targets were generally consistent between the mutant genotypes; however, genes repressed by C/EBP\(\alpha\) specifically, responded differentially to inhibition of PRC2 versus PRC1. One possible explanation for this result is that Bmi1 cooperates with C/EBP\(\alpha\) to mediate silencing, and that this function is independent of PRC2. The described role for C/EBP\(\alpha\) in HSCs fits with the differential changes we see in its targets in PRC1 compared with PRC2 mutants\(^{49}\). Therefore, modest changes in the HoxA9 and C/EBP\(\alpha\) transcriptional networks may explain some of the differences in the roles of the three PRCs in HSC/progenitors. One key challenge for the future will be to address the binding patterns of each complex in this rare but important cell type.

In addition to HSCs, PcG proteins have important functions in other stem cell populations, and in cancer. Whether our findings are more broadly applicable to neural or
mammary stem cells, will be important to test. Our findings may also have implications for hematopoietic malignancies. A recent study reports frequent heterozygous somatic mutation of \textit{EZH2} in diffuse large B cell lymphomas\textsuperscript{50}, a class of lymphoma where BMI1 overexpression correlates with poor prognosis\textsuperscript{51}. Both HoxA9 and C/EBP\textgreek{a} are involved in hematopoietic malignancies, and may be differentially regulated by PRC1 and 2 in malignant cells, as they are in HSC/progenitors. Recent work shows that overexpression of PRC1 or PRC2 members inversely correlate with prognosis in breast cancer\textsuperscript{52}. Therefore understanding whether the PRCs have multiple potential modes of action in stem cells and cancer is critical not only to our understanding of stem cell biology, but also for optimal treatment of cancers that overexpress or possess mutated forms of these proteins.

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\textbf{Author Contributions}

IJM and MEB designed research, performed research, analyzed data, and wrote the paper. MER, BP, YH and GSK contributed new analytical tools and analyzed data.
JC and MP performed research

AE, MB, HK contributed reagents

WSA and DJH supervised research and edited the manuscript

Conflict of Interest Disclosure

The authors declare no conflict of interest.

References


**Figure legends**

Figure 1. All core PRC2 components restrict HSC/progenitor activity. A. The three PRCs, displaying mammalian components, *Drosophila* counterparts (in italics), and enzymatic activities. B. Peripheral blood platelet (upper panels) and white blood cell counts (lower panels) at seven weeks of age from mice of the given genotypes. The horizontal line marks the mean for n=20-25 per genotype. Eed<sup>3354</sup> mice were on a mixed genetic background. C. Bone marrow cells from three *CD45<sup>Ly5.2</sup>* donor mice of the given genotype were mixed with *Ezh2<sup>+/+</sup> Mpl<sup>-/-</sup> CD45<sup>Ly5.1</sup>* competitor cells at 1:1 (left panel) or 1:10 (right panel) ratios and used to reconstitute three lethally irradiated recipients per donor. Contribution of test cells is shown at 10-16 weeks post-reconstitution. Blood refers to peripheral white blood cells, Mφ refers to macrophages. Asterisks denote statistical significance (*p < 0.05, **p < 0.01, ***p < 0.001 corrected for multiple testing) and error bars indicate standard error of the mean (SEM).
Figure 2. PRC1 and PRC2 do not synergise in HSC/progenitor function A. Peripheral blood platelet (upper panel) and white blood cell counts (lower panel) at seven weeks of age from mice of the given genotypes, all additionally Mpl\(^{-/-}\). The horizontal line marks the mean for n=40 per genotype. B. Bone marrow cells from six to nine Mpl\(^{-/-}\) CD45\(^{Ly5.2}\) donor mice of the given genotype were mixed 1:1 with wild-type Mpl\(^{-/-}\) CD45\(^{Ly5.1}\) competitor cells and used to reconstitute three lethally irradiated recipients per donor. Contribution of test cells to each hematopoietic organ is shown 10-16 weeks post-reconstitution. Blood refers to peripheral white blood cells. Asterisks denote statistical significance (*p < 0.05, **p < 0.01, ***p <0.001 corrected for multiple testing) and error bars indicate SEM.

Figure 3. Yy1 does not synergise with PRC2 and has a role enhancing HSC/progenitor activity A. Peripheral blood platelet (upper panel) and white blood cell counts (lower panel) at seven weeks of age from Mpl\(^{-/-}\) mice of the given genotypes. The horizontal line shows the mean, for n=11-40. B. Fetal liver cellularity (left panel), proportion (middle panel) and number (right panel) of LSK cells per E14.5 fetal liver of the given genotype. Data is from one litter of 4 Yy1\(^{+/+}\) and 5 Yy1\(^{+-}\) embryos, representative of data from three independent litters. C. Fetal liver LSK cells from 4 to 5 individual CD45\(^{Ly5.2}\) donor embryos of the given genotype were mixed with an equal number of wild-type CD45\(^{Ly5.1}\) competitor fetal liver LSK cells (n=2 samples) and used to reconstitute four lethally irradiated recipients per donor sample. Contribution of test cells to each hematopoietic organ and LSK cells is shown for 12-16 weeks post-reconstitution. Blood refers to
peripheral white blood cells. D. E14.5 fetal liver cells were infected with retrovirus carrying shRNAs against Yy1 or a nonsilencing control (Nonsil) and used to reconstitute lethally irradiated recipients. The proportion of cells infected with each shRNA (GFP+) was compared at input, and at 6 to 12 months post-reconstitution in spleen, thymus, bone marrow and the LSK cell compartment. The effect of the Nonsil shRNA in each of three experiments was normalised to 1. Asterisks denote statistical significance (*p < 0.05, **p < 0.01, ***p <0.001 corrected for multiple testing) and error bars indicate SEM.

Figure 4. PRC1, PRC2 and PhoRC control different gene sets in HSC/progenitors A&B. Log2-fold changes for 7824 differentially expressed Illumina expression array probes in fetal liver LSK cells, comparing Bmi1−/− and Bmi1+/+ (PRC1 vs wild-type), Suz12Plt8/+ and Suz12+/+ (PRC2 vs wild-type) and Yy1+/− and Yy1+/+ (PhoRC vs wild-type). Probes coloured blue in A are the same as those coloured blue in B, similarly for those coloured red, yellow or green. C. Romer analysis of gene expression comparisons shown in part A. Broad Institute gene sets that are enriched in particular comparisons are listed, see Supplemental Material for full analysis. D. Picture showing the multi-faceted regulation of gene expression by PRCs. PRC1 and PRC2 may bind some targets cooperatively to repress expression; these genes are named blue genes from Fig. 4A. PRC2 and PRC1 may act independently at some loci, and other loci may be differentially sensitive to depletion of PRC1 (for example Bmi1) or PRC2 (for example Suz12).
Figure 2

A

Platelet count x10^3/μL

Bmi1^+/+ Suz12^+/+  Bmi1^+/+ Suz12^+/+  Bmi1^-/- Suz12^+/+  Bmi1^-/- Suz12^Plt8/-

WBC count x10^3/μL

Bmi1^+/+ Suz12^+/+  Bmi1^+/+ Suz12^+/+  Bmi1^-/- Suz12^+/+  Bmi1^-/- Suz12^Plt8/-

B

TEST CONTRIBUTION (% of Ly5 positive cells)

Blood  Spleen  Thymus  Marrow

Bmi1^+/+ Suz12^+/+  Bmi1^-/- Suz12^+/+  Bmi1^-/- Suz12^Plt8/-

**  ***  ***  **  ***  **  **  **
Figure 3

A

Platelet count x 10^3/μL

***

Yy1^+/+ Suz12^+/+

Yy1^+/+ Suz12^+/+

Yy1^+/+ Suz12^+/+

Yy1^+/+ Suz12^+/+

Yy1^+/+ Suz12^+/+

WBC count x 10^3/μL

***

Yy1^+/+ Suz12^+/+

Yy1^+/+ Suz12^+/+

Yy1^+/+ Suz12^+/+

Yy1^+/+ Suz12^+/+

Yy1^+/+ Suz12^+/+

B

Cells per fetal liver (x10^7)

Mpl^+/+ Yy1^+/+

Mpl^+/+ Yy1^+/+

Mpl^+/+ Yy1^+/+

Mpl^+/+ Yy1^+/+

Mpl^+/+ Yy1^+/+

LSK proportion of live cells (%)

***

Mpl^+/+ Yy1^+/+

Mpl^+/+ Yy1^+/+

Mpl^+/+ Yy1^+/+

Mpl^+/+ Yy1^+/+

Mpl^+/+ Yy1^+/+

LSK number per fetal liver

Mpl^+/+ Yy1^+/+

Mpl^+/+ Yy1^+/+

Mpl^+/+ Yy1^+/+

Mpl^+/+ Yy1^+/+

Mpl^+/+ Yy1^+/+

C

TEST CONTRIBUTION

(% of Ly5 positive cells)

Mpl^+/+ Yy1^+/+

Mpl^+/+ Yy1^+/+

Mpl^+/+ Yy1^+/+

Mpl^+/+ Yy1^+/+

Mpl^+/+ Yy1^+/+

D

Normalised GFP+

Spleen

Thymus

Marrow

LSK

Nons shRNA

Yy1.1 shRNA

Yy1.6 shRNA

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C \textbf{Romer analysis of Broad Institute Genesets}

\textbf{PRC1 vs PRC2}
\textbf{Positive correlation, up-regulated in both Bmi1 and Suz12 depleted LSK cells (blue)}
E2F target genes (5 gene sets, 0.005 < p < 0.05)
Genes activated by C/EBP transcription factors (1 gene set, 69 genes, p < 0.05)

\textbf{Negative correlation, up-regulated in Suz12 depleted, downregulated in Bmi1 deficient LSK cells (red)}
Hox genes (1 gene set, 25 genes, p < 0.05)
Genes activated by HoxA9 (1 gene set, 69 genes, p < 0.05)

\textbf{Negative correlation, down-regulated in Suz12 depleted, upregulated in Bmi1 deficient LSK cells (yellow)}
Genes up-regulated in Dnmt1 knockout cells (1 gene set, 49 genes, p < 0.0001)
Genes repressed by C/EBPα (1 gene set, 24 genes, p < 0.05)
IL6 responsive genes (2 gene sets, p < 0.05)
Genes repressed by HoxA9 (1 gene set, 24 genes, p < 0.05)

\textbf{PhoRC vs PRC1}
\textbf{Positive correlation, up-regulated in both Bmi1 and Yy1 depleted LSK cells}
Genes activated by C/EBP transcription factors (1 gene set, 69 genes, p < 0.05)
Genes up-regulated in Dnmt1 knockout cells (1 gene set, 49 genes, p < 0.05)
Genes involved in oxidative phosphorylation or induced by hypoxia (2 gene sets, p < 0.05)

\textbf{Positive correlation, down-regulated in both Bmi1 and Yy1 depleted LSK cells}
Hematopoiesis related transcription factors (3 gene sets, 0.01 < p < 0.05)
Hox genes (1 gene set, 25 genes, p < 0.05)
Opposing roles of polycomb repressive complexes in hematopoietic stem and progenitor cells