

Functional antagonism of the *Polycomb*-Group genes *eed* and *Bmi1* in hemopoietic cell proliferation

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The murine *Polycomb*-Group (PcG) proteins Eed and Bmi1 govern axial patterning during embryonic development by segment-specific repression of *Hox* gene expression. The two proteins engage in distinct multimeric complexes that are thought to use a common molecular mechanism to render the regulatory regions of *Hox* and other downstream target genes inaccessible to transcriptional activators. Beyond axial patterning, *Bmi1* is also involved in hemopoiesis because a loss-of-function allele causes a profound decrease in bone marrow progenitor cells. Here, evidence is presented that is consistent with an antagonistic function of *eed* and *Bmi1* in hemopoietic cell proliferation. Heterozygosity for an *eed* null allele causes marked myelo- and lymphoproliferative defects, indicating that *eed* is involved in the negative regulation of the pool size of lymphoid and myeloid progenitor cells. This antiproliferative function of *eed* does not appear to be mediated by *Hox* genes or the tumor suppressor locus *p16^{INK4a}/p19^{ARF}* because expression of these genes was not altered in *eed* mutants. Intercross experiments between *eed* and *Bmi1* mutant mice revealed that *Bmi1* is epistatic to *eed* in the control of primitive bone marrow cell proliferation. However, the genetic interaction between the two genes is cell-type specific as the presence of one or two mutant alleles of *eed* *trans*-complements the *Bmi1*-deficiency in pre-B bone marrow cells. These studies thus suggest that hemopoietic cell proliferation is regulated by the relative contribution of repressive (Eed-containing) and enhancing (Bmi1-containing) PcG gene complexes.

[Key Words: *eed*; *Bmi1*; *Polycomb*-Group (PcG) genes; hemopoiesis; cellular proliferation]

Received August 4, 1999; revised version accepted August 25, 1999.

The murine *Polycomb* (PcG) and *trithorax* (*trxG*) group of genes are constituents of evolutionary highly conserved epigenetic pathways governing metamer patterning of the axial skeleton by regulation of *Hox* gene expression (for review, see Gould 1997; Schumacher and Magnuson 1997; van Lohuizen 1998). PcG and *trxG* gene products are thought to modify higher order chromatin structures to maintain a repressed and derepressed state of *Hox* gene expression, respectively. Consistent with their role as upstream regulators of *Hox* genes, loss of function of PcG and *trxG* genes in mice alters *Hox* gene expression causing skeletal transformations. For example, dosage-sensitive posterior homeotic transforma-

tions have been observed in mutant alleles of the murine PcG genes *Bmi1* (*B* cell-specific *M*o-*M*LV *i*ntegration site *1*) and *eed* (*e*mbryonic *e*ctoderm *d*evelopment) (van der Lugt et al. 1994; Schumacher et al. 1996).

Beyond axial patterning by control of *Hox* gene expression, several studies also support a role for PcG genes in regulating hemopoiesis. For example, differentiation of primary bone marrow cells is generally accompanied by an up-regulation of PcG gene expression levels (Lessard et al. 1998). Only *Bmi1* exhibits a different pattern of expression with high levels in primitive CD34⁺ cells and very low levels in mature CD34⁻ cells (Lessard et al. 1998). Likewise, the *Hox* gene expression levels are highest in the most primitive bone marrow cells (Sauvageau et al. 1994). These data are consistent with a down-regulation of *Hox* gene expression by PcG proteins as primary

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bone marrow cells differentiate. Furthermore, by virtue of their transcriptional activation upon stimulation of lymphoid B cells, *PcG* genes also act as immediate early genes (Hasegawa et al. 1998).

Further insight into the function of *PcG* genes in hemopoiesis can be derived from mutant analysis. Mice lacking *Bmi1* display a progressive replacement of bone marrow hemopoietic cells by adipocytes along with an impaired proliferative response of the bone marrow progenitors to mitogens (van der Lugt et al. 1994). Similarly, targeted disruption of *mel18*, *Mph1/Rae28*, and *M33* in mice leads to hypoproliferation and/or atrophy of various hemopoietic organs (Akasaka et al. 1996; Core et al. 1997; Takihara et al. 1997). Therefore, in all *PcG* mutants analyzed thus far, the hemopoietic compartment mostly affected correlates with the preferential expression domain, that is, stem/progenitor cells in case of *Bmi1* and mature cells in case of *mel18*, *M33*, and *Mph1/Rae28* (Lessard et al. 1998). Recently, the first downstream mediator of *PcG*-mediated cell proliferation was identified. Removal of the *ink4a* locus, which encodes the tumor suppressors p16^{INK4a} and p19^{ARF}, alleviated the proliferative defects caused by loss of function of *Bmi1* (Jacobs et al. 1999).

Murine *PcG* proteins engage in two distinct multimeric complexes: One complex includes Eed, Enx1/EzH2, and Enx2/EzH1 (Denisenko et al. 1998; Sewalt et al. 1998; van Lohuizen et al. 1998) and the other *Bmi1*, *Mel18*, *Mph1/Rae28*, and *M33* (Alkema et al. 1997; Gunster et al. 1997; Satijn et al. 1997; Satijn and Otte 1999). For simplicity, the *Bmi1*- and Eed-containing complex is herein referred to as 'complex A' and 'complex B,' respectively. Based on the phenotypic similarities among complex A and complex B *PcG* mutants, axial patterning of the primary body axis is likely to involve a common regulatory effect on *Hox* gene expression. Consistent with this hypothesis, *Bmi1/M33* double mutants show synergistic interactions resulting in enhanced axial phenotypes (Bel et al. 1998). Likewise, the penetrance of homeotic transformations in *eed/Bmi1* double mutant animals is significantly increased as compared with the single mutant phenotypes (A. Schumacher and T. Magnuson, unpubl.). In the hemopoietic lineages, complex A *PcG* genes exert a positive regulatory effect on cell proliferation. The function of complex B genes in hemopoiesis is unknown. However, by analogy to the rather uniform *PcG* function in axial patterning, complex B genes are predicted to act as positive regulators of hemopoietic cell proliferation with synergizing effects in double mutant combinations with complex A alleles.

Here, the hemopoietic phenotype of mutant alleles of the complex B gene *eed* is described. Surprisingly, *eed* acts as a negative regulator of myeloid and lymphoid progenitor cell proliferation in the bone marrow. This reflects a functional antagonism between the complex A gene *Bmi1* and the complex B gene *eed*, which act as positive and negative regulators of bone marrow progenitor cell proliferation, respectively. Hyperproliferation of *eed* mutant cells is not associated with altered expression of *Hox* genes and the *ink4a* locus and, therefore,

involves as yet unknown downstream mediators. Inter-cross experiments revealed an epistasis of *Bmi1* to *eed* in the control of bone marrow stem cell proliferation because the *eed/Bmi1* double mutant phenotype is identical to the *Bmi1* single mutant phenotype. However, genetic interaction between the two genes is cell-type specific as the presence of one or two mutant *eed* alleles *trans*-complements the *Bmi1* deficiency in pre-B bone marrow cells. These results reveal an unexpected complexity in *PcG* function in murine hemopoiesis.

Results

Expression of eed and Bmi1 in bone marrow cells

Bmi1 is preferentially expressed in primitive subpopulations of human bone marrow cells (Lessard et al. 1998). In contrast, *eed* is expressed at about equal levels in both primitive and mature human hemopoietic cells (J. Lessard and G. Sauvageau, unpubl.). The expression of these two genes was similarly determined in mouse primary bone marrow cells representing functionally distinct stem/progenitor (*Sca*⁺ *Lin*⁻) and mature (*Sca*⁻ *Lin*⁺) cell compartments (Fig. 1A). In agreement with the human data, only the *Sca*⁺ *Lin*⁻ subpopulation displayed an elevated expression level of mouse *Bmi1* (Fig. 1A, upper panel), whereas *eed* expression was consistently high in all subpopulations (Fig. 1A, middle panel).

eed is a negative regulator of bone marrow progenitor cell proliferation

The ubiquitous expression of human and mouse *eed* in bone marrow cells suggests that this gene may function in both primitive and mature hemopoietic cells. To test this hypothesis, the proliferation and differentiation of primitive and mature bone marrow cells was assessed in *eed* mutants. Hemopoietic cells were derived from animals carrying one or two copies of a viable hypomorphic allele of *eed*, *l7Rn5*^{1989SB} (herein referred to as *eed*^{1989/+} and *eed*^{1989/1989}), or one copy of an *eed* null allele, *l7Rn5*^{3354SB} (herein referred to as *eed*^{3354/+}) (Schumacher et al. 1996). Homozygosity for the *eed*³³⁵⁴ allele is lethal at gastrulation (Faust et al. 1995) and, thus, excludes analysis of hemopoietic cells.

When compared with control littermates, the bone marrow, spleen, thymus, and peripheral blood counts were normal in all young *eed* mutants analyzed, that is, animals of <4 months of age (*n* > 5 per group). Moreover, cytological and cytofluorometric analyses showed that myeloid (*Mac*⁺, *Gr-1*⁺), B- (*B220*⁺*CD43*⁺; *B220*⁺*IgM*⁺), and T- (*CD4*⁺*CD8*⁺; *CD4*⁻*CD8*⁺; *CD4*⁺ and *CD8*⁺) cell populations derived from *eed*^{1989/+}, *eed*^{1989/1989}, and *eed*^{3354/+} mutant mice were similar to that of sibling control animals (data not shown). However, there was a 2- and 3.5-fold increase in the frequency of mature myeloid and pre-B lymphoid progenitors, respectively, in the bone marrow of *eed*^{3354/+} animals (Fig. 2B) when compared with control littermates. Moreover, *eed*^{3354/+}

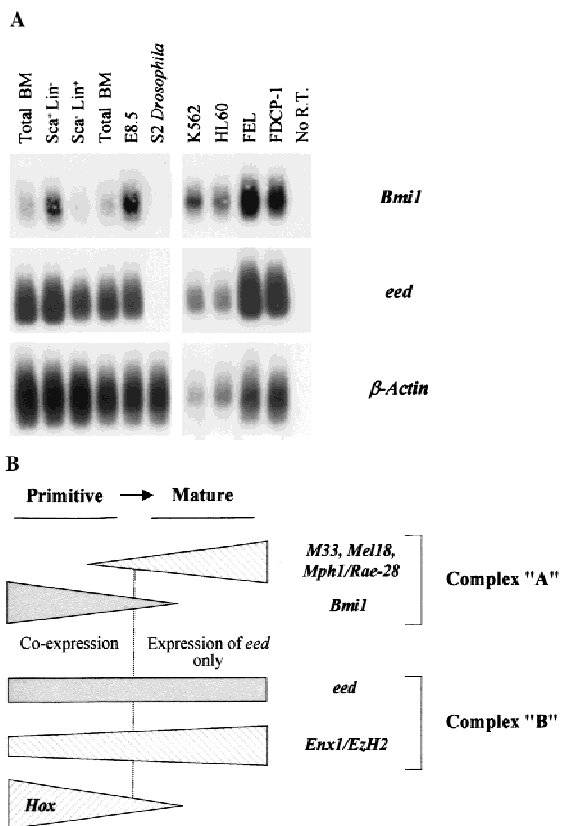


Figure 1. Expression of *eed* and *Bmi1* PcG genes in purified subpopulations of murine bone marrow cells and hemopoietic cell lines. (A) Five to ten thousand cells were isolated from each subpopulation (>98% purity upon reanalysis), and their total RNA was reverse-transcribed and PCR-amplified as described in Material and Methods. Cocktail of lineage-specific (Lin) monoclonal antibodies (MoAbs) contained B220 (B lymphocytes), Gr-1 (granulocytes), and Mac-1 (granulocyte-macrophages). From primitive to mature subpopulations: Sca⁺ Lin⁻ subpopulation (lane 2); Sca⁻ Lin⁺ subpopulation (lane 3). Exposure times (all at -70°C) are as follows: *Bmi1*, 83 hr (except all hemopoietic cell lines, 20-hr exposure); *eed*, 4.5 hr, and β -Actin, 25 min (except S2 *Drosophila* cell line, 75-min exposure). (B) Schematic representation of complexes A and B PcG gene expression patterns in purified subpopulations of primary bone marrow cells (this paper; Sauvageau et al. 1994; Lessard et al. 1998).

pre-B progenitor-derived colonies were on average four times larger than those derived from control animals (data not shown). Morphological analysis and immunophenotyping confirmed that these colonies contained mostly B lymphocytes, indicating that *eed* mutant cells undergo increased proliferation prior to becoming terminally differentiated (data not shown).

To ascertain whether the precursors of the mature myeloid and lymphoid colony-forming cells (CFCs) were affected in *eed* mutant animals, the frequency of the mostly quiescent primitive bone marrow myeloid long-term culture-initiating cells (LTC-ICs) and lymphoid Whitlock-Witte-initiating cells (WW-ICs) was measured by limiting dilution analysis. The results presented in

Figure 2B indicate that LTC-IC frequencies are comparable between the controls ($n = 3$) and the *eed* mutant mice ($n = 6$ total for *eed*^{1989/+} and *eed*^{1989/1989} and $n = 2$ for *eed*^{3354/+}). The WW-IC, which was only evaluated in the *eed*¹⁹⁸⁹ mice, was also not significantly different from the controls.

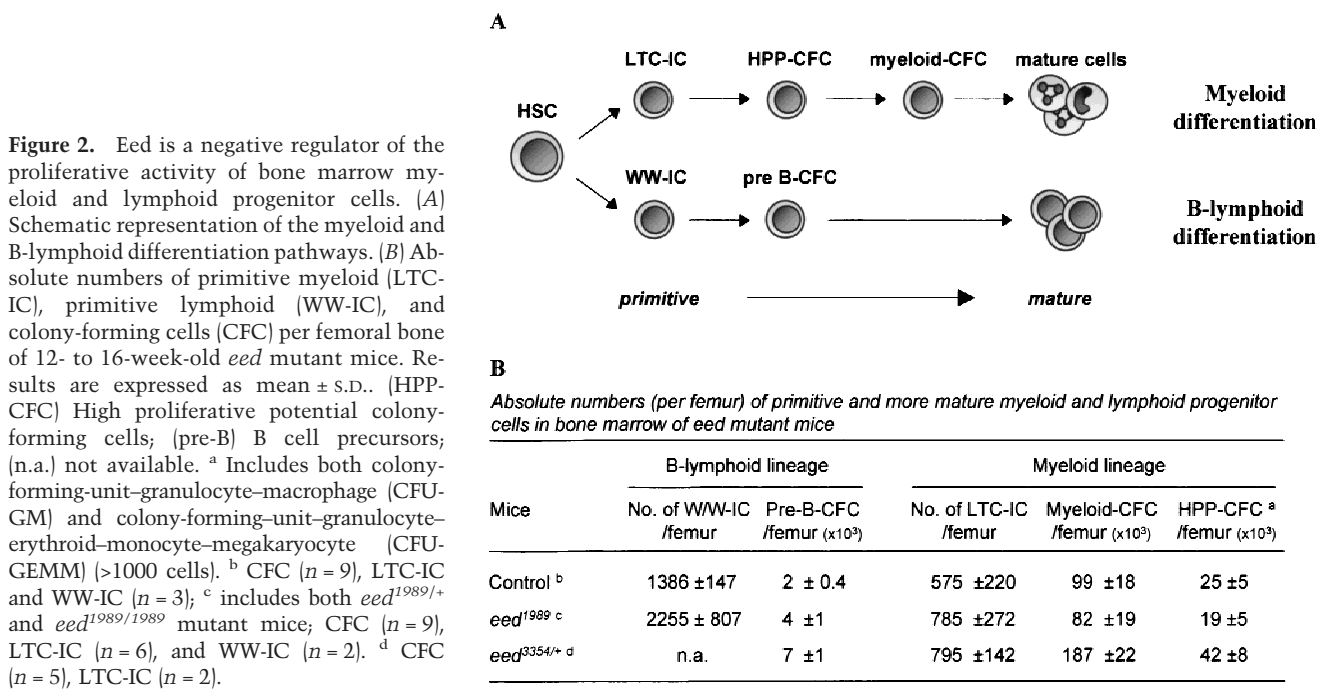
Thus, the significant increase in the number of the more mature myeloid and pre-B bone marrow progenitors in young *eed*^{3354/+} mutant mice is consistent with *eed* acting as a negative regulator of cell proliferation in mature bone marrow progenitors.

Lymphoid and myeloid hyperproliferation in older *eed* mutant mice

To investigate the long-term effect of decreased Eed activity in hemopoietic cells, nine *eed*^{3354/+} mice were analyzed between 7 and 18 months of age. Of these, five had developed lymphoproliferation (LP) (group B; Figs. 3 and 4), three mice suffered from a severe myeloproliferative disease (MPD) (group C; Figs. 3 and 4), and one was still relatively normal (group D; Fig. 3) although it had a noticeable increase in bone marrow pre-B progenitors (Fig. 4A).

The lymphoproliferative defect observed in *eed*^{3354/+} mice (group B) was characterized by a 35-fold increase in the frequency of lymphoid (pre-B) bone marrow progenitor cells with highly increased proliferative activity when compared with control littermates (group A; Fig. 4A). Morphological and cytofluorometric analyses confirmed that these cells were of B lineage origin (data not shown). A smaller but significant increase in the frequency of immature lymphoid (WW-IC) and myeloid (LTC-IC) bone marrow progenitor cells was also found in *eed*^{3354/+} mice when compared with control littermates (3.5-fold and 2-fold increase, respectively; Fig. 4B). Furthermore, as evaluated by the number of B cells produced per WW-IC at limiting dilution, the proliferative potential of individual *eed*^{3354/+} group B immature WW-IC lymphoid progenitors was increased by 12-fold as compared with control animals (Fig. 4C). This provides a possible explanation for the increase in pre-B CFC in these mice.

Despite as much as a 35-fold increase in the frequency of pre-B-cell population in *eed*^{3354/+} mice, there were no significant changes in the absolute numbers of more mature B cells in the bone marrow and peripheral hemopoietic organs of these mice when compared with controls (Fig. 3A,B). These observations suggest that a compensatory mechanism must operate to maintain normal numbers of mature cells in *eed*^{3354/+} animals. One possibility is that pre-B CFC detected *in vitro* would not survive *in vivo* under limiting cytokine (e.g., IL-7) conditions. Therefore, survival assays were performed in which IL-7 was added following an initial culture period of 0, 24, 48, or 72 hr. Such delayed addition of IL-7 did not result in a significant decrease in colony formation in *eed*^{3354/+} cultures with respect to that of control littermates, indicating that *eed*^{3354/+} group B lymphoid precursors (pre-B)



are not more sensitive to cytokine deprivation than control cells (data not shown). This suggests that B-cell homeostasis in these mice might be dependent on 'peripheral regulators' of B-cell numbers.

A severe MPD also developed in some of the older *eed*^{3354/+} mice (group C, 7–18 months of age; Fig. 3), which showed general signs of illness and severe weight loss. When sacrificed, they were athymic and showed splenomegaly and lymphadenopathy. Bacteriology, parasitology, and virology testing of plasma and several tissues isolated from these animals excluded pathogen infections (data not shown). These mice displayed high peripheral white blood cell counts, representing almost exclusively segmented neutrophils (Fig. 3A). Cytofluorometric analysis of cells derived from the bone marrow, spleen, thymus, and lymph nodes of these mice showed a profound increase in the frequency of a myeloid population of cells coexpressing the Mac-1⁺ and Gr-1⁺ surface markers (up to 91% and 57% of total bone marrow cells and splenocytes coexpressed Mac-1⁺ and Gr-1⁺, respectively; Fig. 3B). These mice also exhibited a marked decrease in the number of bone marrow B220⁺IgM⁺ immature B cells (<1% of wild-type levels) and mature IgM⁺IgD⁺ B cells (44% of wild-type levels; Fig. 3B) in secondary lymphatic organs, likely reflecting infiltration and displacement by the expanding myeloid population. In the only mouse presenting an involuted thymus, the ratio of CD4⁺ to CD8⁺ T cells was normal (data not shown). Despite a 2-fold reduction in bone marrow cellularity, myeloid bone marrow progenitors were increased 2.5-fold and consisted predominantly of granulocyte and/or macrophage colony-forming cells (G and/or M-CFCs) (Fig. 4A; data not shown). Consistent with the cytofluorometric studies, the absolute numbers of

pre-B- and immature lymphoid B-(WW-IC) cell populations in these mice were reduced to 1.6% and 37% of wild-type levels, respectively (Fig. 4A,B).

Importantly, the myeloproliferative disorders were observed only in *eed*^{3354/+} mice that derived from the double mutant crosses with *Bmi1*, suggesting that this phenotype may be modulated by the presence of modifier genes in the FVB/N background (C3Hf/101 vs. C3Hf/101 \times FVB/N; Fig. 3).

The hyperproliferative effects observed mostly in the lymphoid B-cell lineage of C3Hf/101 *eed* mutant mice would suggest that *eed* may be another member of the growing family of tumor suppressor genes. To test for this possibility, a larger cohort of *eed* mutant mice (including 12 homozygous *eed*^{1989/1989} and 28 heterozygous *eed*^{1989/+} mutant mice) were monitored for tumor formation for up to 2 years, of which a single case was observed in a 3-month-old *eed*^{1989/1989} mouse. Morphological, cytological, and DNA analyses confirmed that the tumor was a monoclonal B-cell lymphoma [see histology and B-cell receptor (BCR) rearrangement in Fig. 5]. Interestingly, the exposure of *eed*¹⁹⁸⁹ and *eed*^{3354/+} mice to a genotoxic agent, methylnitrosourea (MNU), led to lymphoid tumor formation in 100% of these mice in a time frame in which no tumors would develop in control animals (E. Richie, A. Schumacher, and T. Magnuson, unpubl.).

Together, these data indicate that *eed* performs important antiproliferative activity on a subset of early (WW-IC) and late (myeloid and pre-B CFC) bone marrow progenitors and that ultimately, its absence may lead to tumor formation. These data contrast with previous studies of mutant complex A *PcG* genes (including *Bmi1*) in which hypoproliferation of hemopoietic precur-

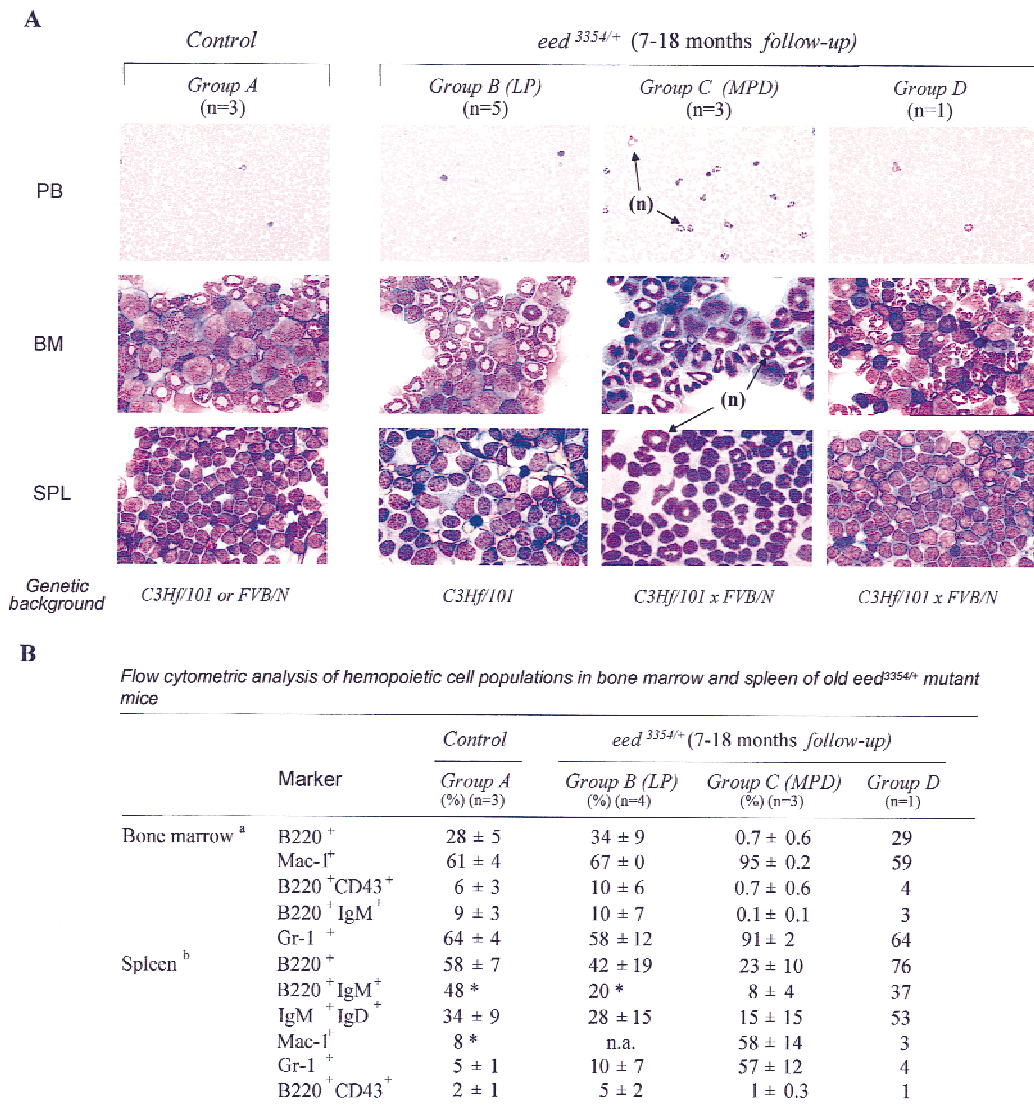
Opposite functions of *eed* and *Bmi1* in hemopoiesis

Figure 3. Cytopathological (A) and cytofluorometric (B) analyses of hematopoietic cells isolated from older control and *eed*^{3354/+} mutant mice. (A) Wright staining of peripheral blood smears (PB), bone marrow (BM) cytopins, and 'touch preparations' of spleen (SPL) from representative control, *eed*^{3354/+} group B (LP), *eed*^{3354/+} group C (MPD) and *eed*^{3354/+} group D mutant mice (also described in Fig. 4). Note the infiltration by neutrophils (n) in all tissues of *eed*^{3354/+} group C (MPD) mutant mice. Magnification, 100× for all, except for peripheral blood, 40×. Note that absolute number of bone marrow cells per femur is reduced about twofold and absolute number of nucleated cells per spleen is increased about fourfold in *eed*^{3354/+} group C (MPD) mutant mice. For B, results are expressed as mean ± S.D. (LP) Lymphoproliferation; (MPD) myeloproliferative disease. ^aAbsolute numbers of bone marrow cells per femur: Control group A $3.2 \times 10^7 \pm 0.2$; *eed*^{3354/+} group B (LP) $2.9 \times 10^7 \pm 0.3$; *eed*^{3354/+} group C (MPD) $1.5 \times 10^7 \pm 0.5$; *eed*^{3354/+} group D 2.1×10^7 . ^bAbsolute numbers of nucleated cells per spleen: Control group A $1.8 \times 10^8 \pm 0.5$; *eed*^{3354/+} group B (LP) $1.4 \times 10^8 \pm 0.1$; *eed*^{3354/+} group C (MPD) $7.8 \times 10^8 \pm 1.6$; *eed*^{3354/+} group D 2.4×10^8 . (*) Only one mouse analyzed.

sors was observed (van der Lugt et al. 1994; Akasaka et al. 1996; Core et al. 1997; Takihara et al. 1997).

eed and *Bmi1* have opposite functions in regulating hemopoietic cell proliferation

Bmi1 null homozygous mice display a severe and progressive reduction in their number and proliferative potential of (mature) bone marrow myeloid and pre-B progenitors (van der Lugt et al. 1994). Interestingly, the *eed*

mutant mice described herein have the opposite hemopoietic phenotype (i.e., a progressive increase in the number and proliferative potential of several types of bone marrow CFCs). The coexpression of *Bmi1* and *eed* in every hemopoietic cell line tested to date together with their expression in primitive Sca⁺ Lin⁻ bone marrow cells (Fig. 1) suggests that *eed* and *Bmi1* may functionally interact to regulate hemopoietic cell proliferation. To obtain genetic evidence for a functional collaboration between these two PcG proteins, *eed* and *Bmi1*

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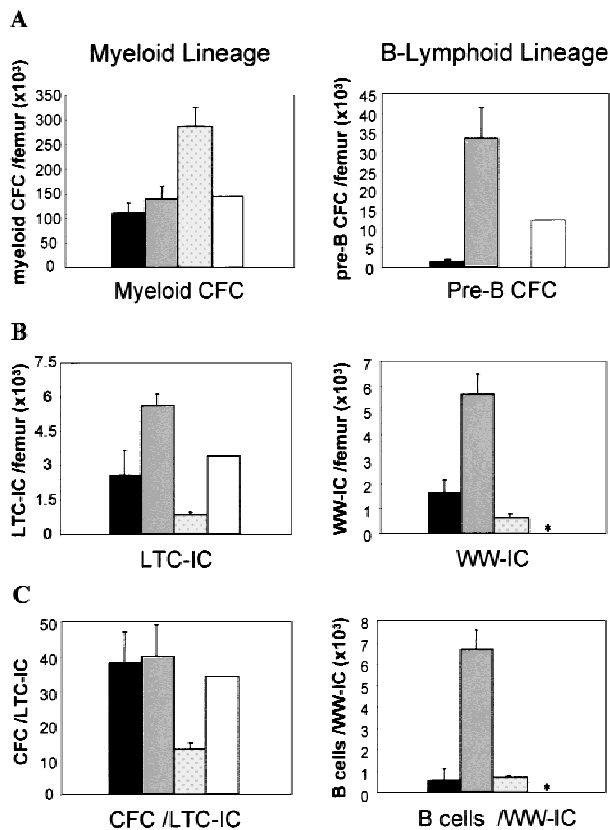


Figure 4. Myelo- and lymphoproliferation occur in old *eed*^{3354/+} mutant mice. (A) Femur-derived bone marrow cells were assayed in methylcellulose for myeloid and pre-B lymphoid CFCs. (B) In vitro long-term culture assays of primitive myeloid (LTC-IC) and lymphoid (WW-IC) bone marrow cells. The results, mean \pm s.d., are given as absolute numbers per femur. (C) CFC generated per LTC-IC at limiting dilution after 28 days of in vitro culture; includes colony-forming-unit-granulocyte-macrophage (CFU-GM) and burst-forming-unit-erythroid (BFU-E). WW-IC-derived B220⁺ B cells (at limiting dilution) were analyzed by FACS at day 21 of culture. (*) Not determined. (Solid bar) Control group A ($n = 4$); (shaded bar) *eed*^{3354/+} group B (LP) ($n = 5$); (dotted bar) *eed*^{3354/+} group C (MPD) ($n = 3$); (open bar) *eed*^{3354/+} group D ($n = 1$).

double mutant mice were generated and their hemopoietic system analyzed.

*eed*¹⁹⁸⁹*Bmi1*^{-/-} and *eed*^{3354/+}*Bmi1*^{-/-} double mutant mice were severely anemic, and in vitro colony formation assays of bone marrow progenitor cells isolated from these mice gave identical results to those observed with *Bmi1*^{-/-} mice (Fig. 6A). Therefore, the loss of one or two functional alleles of *eed* did not alter the hemopoietic defects observed at the mature CFC level in *Bmi1*^{-/-} mice. As reported previously, the loss of one allele of *Bmi1* resulted in a small reduction in mature myeloid CFC and a significant threefold reduction in pre-B progenitors (Fig. 6A; van der Lugt et al. 1994). In contrast, the number of pre-B CFC in the bone marrow of *eed*/*Bmi1*^{+/-} double mutant mice was close to that found in control littermates. This indicates that the loss of one or

two alleles of *eed* trans-complemented the deficit at the pre-B CFC level found in *Bmi1*^{+/-} mutant mice ($n = 7$ vs. 13 *Bmi1*^{+/-} and *eed*/*Bmi1*^{+/-} mice analyzed, respectively; Fig. 6A). These data are consistent with a cell type-specific interaction between the two genes and reveal an unexpected complexity in *PcG* function in hemopoiesis.

The capacity for *eed* and *Bmi1* to functionally interact in the more primitive bone marrow LTC-ICs, and WW-ICs was also evaluated. Since LTC-IC and WW-IC numbers and proliferative capacity had not been determined previously in *Bmi1* mutant mice, we first completed this evaluation. The results presented in Figure 6B indicate that in *Bmi1*^{-/-} mice, the absolute numbers of both primitive myeloid (LTC-IC) and lymphoid (WW-IC) cell populations were profoundly reduced to 4% and 1% of wild-type levels, respectively. Moreover, the proliferative potential of these primitive progenitor cells was severely affected. Absolute numbers of LTC-IC-derived CFCs and WW-IC-derived B220⁺ B cells analyzed at limiting dilution were reduced 17-fold and 34-fold, respectively, in *Bmi1*^{-/-} mice as compared with littermate control mice. The pool and proliferative activity of *Bmi1*^{-/-} bone marrow primitive precursors progressively decreased with age (data not shown), and, as reported previously, *Bmi1*^{-/-} mice did not survive beyond 20–25 weeks of age. Importantly, the numbers and proliferative potential of primitive myeloid (LTC-IC) and lymphoid (WW-IC) cells were not different between *eed*/*Bmi1*^{-/-} double mutant mice and the single *Bmi1*^{-/-} mice (Fig. 6B).

In *Bmi1* heterozygous mice, reduction of *Bmi1* expression levels neither altered the number nor the proliferative potential of primitive myeloid cells (LTC-ICs) but clearly reduced the proliferative capacity of primitive lymphoid (WW-IC) progenitors (control: 2295 B cells/WW-IC; *Bmi1*^{+/-}: 275 B cells/WW-IC; Fig. 6B). This indicates that heterozygosity for *Bmi1* limits the proliferative capacity of these progenitor cells. The proliferative potential of *Bmi1*^{+/-} WW-ICs was partially rescued by the loss of one or two alleles of *eed* (from 275 \pm 43 to 919 \pm 341, for *Bmi1*^{+/-} and *eed*/*Bmi1*^{+/-} double mutant mice, respectively).

Taken together, these results indicate that the marked reduction of mature bone marrow progenitors and differentiated blood cells previously reported in *Bmi1*-deficient mice may in fact originate from a major proliferative defect in the pool of primitive myeloid and lymphoid progenitors. In addition, these studies demonstrated that, although one allele of *Bmi1* is sufficient for the generation of primitive bone marrow cells, both alleles are required for the proliferation of a group of B-cell precursors. Furthermore, a reduction in functional *eed* trans-complements the loss of one allele of *Bmi1* in this subset of precursors.

ink4a and *Hox* gene expression is not altered in *eed* mutant mice

Recent studies revealed a marked increase in the expres-

Opposite functions of *eed* and *Bmi1* in hemopoiesis

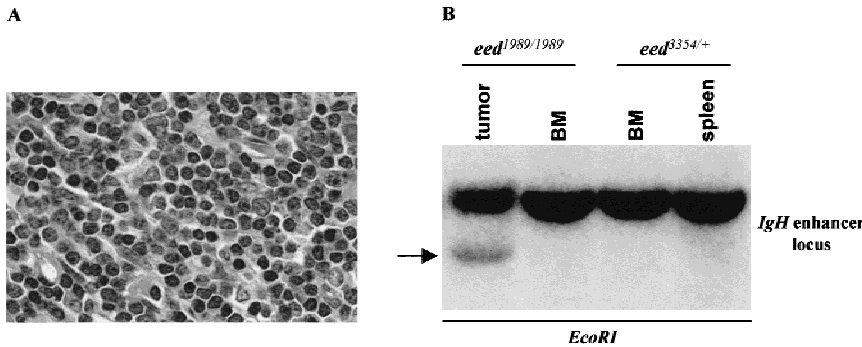


Figure 5. Monoclonal B-cell tumor in a 3-month-old *eed*^{1989/1989} mutant mouse. (A) Histological analysis of a hematoxylin and eosin section of a thoracic tumor (1.5 grams). Note the infiltration by mature darkly stained lymphocytes. Magnification, 100×. (B) Southern blot analysis of genomic DNA isolated from the tumor showing clonal (see arrow) BCR rearrangement. Note the absence of clonal BCR rearrangement in bone marrow cells of this same mouse (BM) and in the spleen and bone marrow of a healthy control mouse. Membrane was hybridized to a probe corresponding to the *IgH* intronic enhancer locus capable of detecting both *V(D)J* and *DJ* joints. Exposure time was 84 hr. (*IgH*) Immunoglobulin heavy chain.

sion levels of the tumor suppressor genes *p16*^{INK4a} and *p19*^{ARF} along with concomitant down-regulation of cyclin A and E activity in *Bmi1*-deficient mouse embryonic

fibroblasts and lymphocytes (Jacobs et al. 1999). To identify candidate downstream mediators of *eed* function, *p16*^{INK4a} and *p19*^{ARF} expression was analyzed in bone

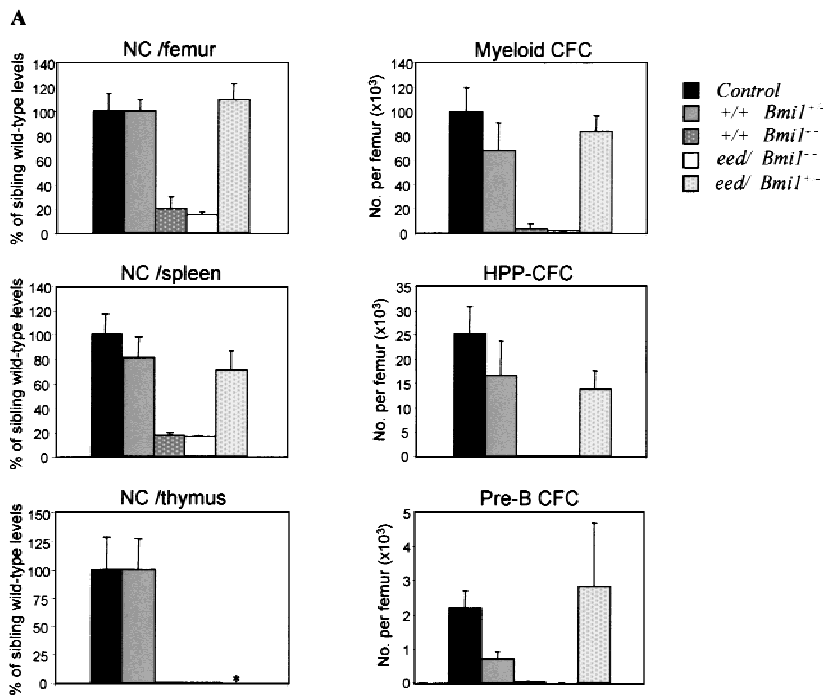


Figure 6. Hemopoietic parameters of *Bmi1* mutant and *eed/Bmi1* double mutant mice analyzed at 12–16 weeks of age. (A) Bone marrow, spleen, and thymic cellularity (left) and bone marrow CFC numbers (right) in control ($n = 9$), *Bmi1*^{+/-} ($n = 7$), *Bmi1*^{-/-} ($n = 3$), *eed*^{+/-} *Bmi1*^{-/-} (either *eed*^{3354/+} or *eed*^{1989/+}; $n = 3$), and *eed/Bmi1*^{+/-} (either *eed*^{1989/+} or *eed*^{1989/1989}; $n = 13$) mutant mice. For clarity, the results for single C3Hf/101 × FVB/N *eed* mutant mice were excluded from this table because the data are superimposable to that of the C3Hf/101 *eed* mutant animals presented in Fig. 2B. (B) Frequency and proliferative potential of primitive myeloid (LTC-IC) and primitive lymphoid (WW-IC) bone marrow cells in *Bmi1* vs. *eed/Bmi1* double mutant mice. All results shown in A and B represent mean values ± SD. (*) Not determined. (NC) Nucleated cells; (HPP-CFC) high proliferative potential-CFC. ^aAbsolute number of B220⁺ cells analyzed by FACS at day 21 of in vitro culture; reflects the proliferative potential of individual WW-IC. ^b Absolute number of CFC per LTC-IC; reflects the proliferative potential of individual LTC-IC. ^c One *eed*^{1989/+} *Bmi1*^{-/-} and two *eed*^{3354/+} *Bmi1*^{-/-} mutant mice were included in this calculation because they had very similar phenotypes. ^d Includes both *eed*^{1989/+} *Bmi1*^{+/-} and *eed*^{1989/1989} *Bmi1*^{+/-} double mutant mice because they had very similar phenotypes. ^e The values corresponding to the *Bmi1*^{+/-} and *eed/Bmi1*^{+/-} groups of mice are not overlapping, but because of an extreme value obtained in one *eed/Bmi1*^{+/-} mouse (1980 B cells/WW-IC), they are not statistically different ($P < 0.07$, one-tailed Student's *t*-test with unequal variance).

B Absolute number per femur and proliferative potential of primitive myeloid (LTC-IC) and primitive lymphoid (WW-IC) bone marrow cells in *Bmi1* versus *eed/Bmi1* double mutant mice

Mice	B-lymphoid lineage		Myeloid lineage	
	No. of WW-IC /femur	No. of B cells /WW-IC ^a	No. of LTC-IC /femur	No. of CFC /LTC-IC ^b
Control ($n=3$)	1428 ± 181	2295 ± 880	797 ± 469	17 ± 9
<i>Bmi1</i> ^{+/-} ($n=5$)	868 ± 326	275 ± 43	3139 ± 2270	16 ± 12
<i>Bmi1</i> ^{-/-} ($n=2$)	15 ± 15	67 ± 16	34 ± 7	1 ± 0.1
<i>eed/Bmi1</i> ^{+/-} ($n=3$) ^c	22 ± 9	73 ± 11	24 ± 8	1 ± 0.3
<i>eed/Bmi1</i> ^{+/-} ($n=4$) ^d	1032 ± 128	919 ± 341 ^e	996 ± 130	17 ± 7

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marrow (mostly myeloid), spleen (mostly B and T cells), and thymus (T cells), of old *eed*^{3354/+} mice and in the B-cell tumor that developed in the *eed*^{1989/1989} 3-month-old mouse. The results presented in Figure 7 (top) do not show any difference in the expression level of *p16*^{INK4a}/*p19*^{ARF} between the various mice analyzed (i.e., old *eed*^{3354/+} vs. littermate controls vs. young *eed*^{1989/1989}). This suggests that these two genes do not mediate the hyperproliferative activity of mutant Eed. Interestingly however, *p16*^{INK4a} and *p19*^{ARF} expression was undetect-

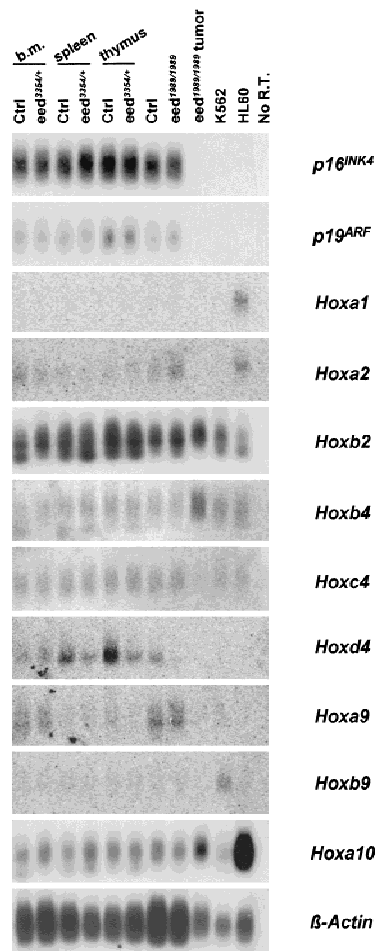


Figure 7. Expression of *p16*^{INK4a}, *p19*^{ARF}, and selected *Hox* genes in various hemopoietic organs of *eed* mutant mice together with an *eed*^{1989/1989} mutant mouse presenting a monoclonal B-cell lymphoma. Isolation of total RNA from *eed* mutant hemopoietic cells is described in Material and Methods. Control (lanes 1–3, 5) and *eed*^{3354/+} (lanes 2–4, 6) mutant mice were all 15 months old, and control (lane 7) and *eed*^{1989/1989} (lanes 8,9) mutant mice were 3 months old. Exposure time for *Hoxc4*, *Hoxb4*, *Hoxa2*, *Hoxb9*, and *Hoxa1* was 20 hr; for *Hoxd4* and *Hoxa9*, 26 hr (all on a phosphorImager cassette). Exposure time for *p16*^{INK4a} was 3.5 hr; for *p19*^{ARF} and *Hoxa10*, 77 hr; for *Hoxb2*, 20 hr; and for β -Actin, 2 hr (all at -70°C). *eed*^{1989/1989} tumor is described in Fig. 5. (K562 and HL60) Human myeloid cell lines used as positive controls for *Hox* and negative controls for *p16*^{INK4a}/*p19*^{ARF} expression. (No R.T.) No reverse transcription.

able in the *eed*^{1989/1989} mutant mouse B-cell tumor as well as in the human leukemic cell line controls, likely reflecting inactivation of the *ink4a* locus, an event observed in several hemopoietic malignancies (Pinyol et al. 1998; Maloney et al. 1999).

Based on the observation that *Hox* gene overexpression causes myelo- and lymphoproliferation (Sauvageau et al. 1997; Thorsteinsdottir et al. 1997; Kroon et al. 1998), this group of genes represents another potential mediator of *eed* function. However, as shown in Figure 7, the expression levels of *Hoxa1*, *Hoxa2*, *Hoxa6* (not shown), *Hoxb2*, *Hoxb3* (not shown), *Hoxb4*, *Hoxc4*, *Hoxd4*, *Hoxa9*, *Hoxb9*, and *Hoxa10* in hemopoietic tissues of *eed* mutant mice (see above) were similar to those of control sibling littermates. This indicates that the increase in the proliferative activity of hemopoietic cells in *eed* mutant mice does not involve major changes in *Hox* gene expression. Therefore, the hyperproliferative phenotype in *eed* mutant mice involves a set of unknown downstream mediators.

Discussion

This study presents evidence for several critical differences in the function of the murine PcG genes *eed* and *Bmi1* in hemopoiesis. (1) *eed* and *Bmi1* represent functional antagonists in hemopoietic cell proliferation. Whereas Eed acts as a negative regulator of hemopoietic progenitor cell proliferation, *Bmi1* enhances the proliferative activity of primitive (this study) and more mature (van der Lugt et al. 1994) bone marrow progenitor cells. (2) The antiproliferative function of Eed does not seem to involve *Hox* genes or the tumor suppressor locus *p16*^{INK4a}/*p19*^{ARF}. (3) Double mutant analysis reveals that *Bmi1* is epistatic to *eed* in the control of bone marrow stem cell proliferation. (4) The genetic interaction between *eed* and *Bmi1* is cell-type specific because the presence of one or two mutant *eed* alleles *trans*-complements the *Bmi1* deficiency in pre-B bone marrow cells.

Functional antagonism between *eed* and *Bmi1*

Based on hyperproliferation of primitive and mature bone marrow progenitors in *eed* mutant mice, wild-type *eed* functions as an inhibitor of hemopoietic progenitor cell proliferation. This stands in sharp contrast to the role of *Bmi1* as a positive regulator of the proliferative activity of progenitor cells. These opposite effects of *eed* and *Bmi1* may be attributed to their involvement in distinct protein complexes. Biochemical studies indicate that the PcG proteins Bmi1, Mel18, M33, and Mph1/Rae28 are constituents of a multimeric protein complex A, which localizes to discrete nuclear foci in U-2 OS osteosarcoma cells (Alkema et al. 1997; Gunster et al. 1997). Importantly, Eed neither interacts physically with Bmi1 nor engages in this protein complex (Sewalt et al. 1998; van Lohuizen et al. 1998). Instead, Eed forms a complex B with the PcG proteins Enx1/EzH2 and Enx2/EzH1, which lacks signs of a discrete subnuclear distri-

bution and is found rather uniformly throughout the nucleoplasm of U-2 OS osteosarcoma cells (van Lohuizen et al. 1998).

In all cases, the protein interaction domains have been delineated. For example, the central helix–turn–helix domain of the complex A protein *Bmi1* is required for interaction with other PcG proteins, such as *Mel18*, *Mph1/Rae28*, and *M33* (Alkema et al. 1997; Gunster et al. 1997; Satijn et al. 1997; Satijn and Otte 1999). The complex B protein *Eed* contains several WD domains, whose integrity is essential for interaction with the amino termini of the PcG proteins *Enx1/EzH2* and *Enx2/EzH1* (Denisenko et al. 1998; Sewalt et al. 1998; van Lohuizen et al. 1998b). Accordingly, the proline substitution in the second WD motif of the *eed*³³⁵⁴ null allele provides a maximally disruptive effect on protein folding (Schumacher et al. 1996) and ablates interaction with *Enx1/EzH2* and *Enx2/EzH1* (Denisenko et al. 1998; van Lohuizen et al. 1998b). In contrast, the relative conformational tolerance of an asparagine substitution in the second WD motif of the *eed*¹⁹⁸⁹ hypomorphic allele (Schumacher et al. 1998) permits interaction with *Enx1/EzH2*, albeit at a reduced efficiency (van Lohuizen et al. 1998). Similarly, unlike the *eed*³³⁵⁴ null allele, the *eed*¹⁹⁸⁹ hypomorphic allele does not cause major defects in B-cell proliferation.

These results are consistent with a distinct function of the two murine PcG complexes, which, similar to *Drosophila melanogaster*, may constitute heterogeneous regulatory complexes at various target loci (Strutt and Paro 1997; Sinclair et al. 1998; DeCamillis et al. 1992). Gene dosage effects and haploinsufficiency were reported in mice bearing one inactivated allele of the complex A *Bmi1*, *mel18*, *M33*, *Mph1/Rae28*, and complex B *eed* PcG genes (van der Lugt et al. 1994; Akasaka et al. 1996; Core et al. 1997; Takihara et al. 1997), suggesting that a change in the concentration of one component of either of these PcG complexes affects the activity of critical regulators of cellular proliferation in mice. Therefore, proliferation of hemopoietic cells appears to be controlled by a stoichiometric relationship between complex A-mediated stimulation and complex B-mediated inhibition of cell proliferation. Disturbances in this equilibrium, for example, over-representation of the *Bmi1*-containing stimulatory complex A in *eed* mutant cells, may lead to important proliferative abnormalities. The loss of a single *Bmi1* or *eed* allele inhibits and stimulates the proliferative activity of B-cell progenitors, respectively. By virtue of their coexpression during hemopoiesis and physical interaction, loss of function of *Enx1/EzH2* and/or *Enx2/EzH1* should therefore result in dosage-sensitive proliferation defects in bone marrow cells that are reminiscent of the *eed* phenotype.

Eed, a PcG protein with tumor suppressing activity

The hyperproliferative defects observed in *eed* mutant animals together with the susceptibility of these mice to develop hemopoietic tumors would suggest that *Eed* has tumor suppressing activity. Because point mutations at

the *eed* gene locus that account for the *eed*¹⁹⁸⁹ and *eed*³³⁵⁴ alleles were shown to disrupt its interaction with *Enx* (see above), it will be interesting to determine whether *Enx* mutant animals also develop tumors, thereby ascribing the tumor-suppressor function to the *Eed*–*Enx* PcG ‘B’ complex. The requirement for exposure to genotoxic agents and the long latency before tumor development indicate that additional genetic events are required for ‘full transformation’ of hemopoietic cells lacking a functional *eed* gene.

A genetic hierarchy of PcG function in hemopoiesis

eed and *Bmi1* function in axial patterning is likely to be governed by a common regulatory effect on *Hox* gene expression. For example, a null allele of *Bmi1* displays posterior homeotic transformations along the anteroposterior (A/P) axis (van der Lugt et al. 1994). Several of these skeletal transformations are phenotypically identical to those observed in a hypomorphic allele of *eed* (Schumacher et al. 1996). Furthermore, consistent with the regulation of a common set of *Hox* genes, the penetrance of homeotic transformations in *eed/Bmi1* double mutants is significantly increased as compared with the single mutant phenotypes (A. Schumacher and T. Magnuson, unpubl.). This is similar to *Drosophila melanogaster*, wherein axial homeotic phenotypes are enhanced in various double and triple PcG mutant combinations (Soto et al. 1995). Importantly, this includes the interaction between *esc* (*extra sex combs*) and *Psc* (*Posterior sex combs*), the fly homologs of *eed* and *Bmi1* (Campbell et al. 1995).

In contrast, genetic analysis of *eed* and *Bmi1* function in hemopoiesis reveals an unexpected complexity because the *eed/Bmi1* double mutant phenotype in primitive bone marrow cells was identical to the *Bmi1* single mutant phenotype. This suggests that *Bmi1* is epistatic to *eed* in the control of hemopoietic stem cell proliferation. Alternatively, the two genes may not be coexpressed in the relevant subtypes of bone marrow progenitors, and hence, epistasis is not tested in the double mutants. WW-ICs and pre-B lymphoid CFCs as the primary target cells of *eed* and *Bmi1* function are certainly rare and unlikely to be isolated to absolute purity. However, several observations argue for the coexpression of *Bmi1* and *eed* in these bone marrow progenitor cells. First, *eed* is expressed in every tissue analyzed thus far (Schumacher et al. 1996; Denisenko et al. 1998). Second, all 10 hemopoietic cell lines analyzed thus far reveal coexpression of *eed* and *Bmi1* (Lessard et al. 1998). Finally, the two genes are coexpressed at high levels in *Sca1*⁺ *Lin*[−] bone marrow cells, which are highly enriched for stem cells and primitive progenitors (Morrison and Weissman 1994). Therefore, these data provide strong support for the epistasis of *Bmi1* to *eed*.

Remarkably, the genetic interaction between *eed* and *Bmi1* is cell-type specific because the presence of one or two mutant *eed* alleles *trans*-complements the *Bmi1* deficiency in pre-B bone marrow cells. This may reflect differences in genetic interaction between *eed* and *Bmi1*

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as reflected by the lower expression level of *Bmi1* in this cell population. Alternatively, qualitative differences, for example, a divergent composition of complex A and/or B, could also account for this phenomenon. Clearly, these results reveal an unexpected complexity in *PcG* function in hemopoiesis.

Downstream mediators of *eed* and *Bmi1* function

The question arises as to whether, similar to axial patterning, *eed* and *Bmi1* function in hemopoiesis involves a common set of downstream mediators. By virtue of their down-regulation upon differentiation of primary bone marrow cells (Lessard et al. 1998) and their well-documented role as downstream mediators of *PcG* function in axial patterning, *Hox* genes are perhaps the most likely candidates. In particular, they can be considered as primary effectors of the inhibitory function of *eed* in hemopoietic cell proliferation because overexpression of *Hox* genes causes hyperproliferative defects in mouse bone marrow cells (Sauvageau et al. 1997; Thorsteinsdottir et al. 1997; Kroon et al. 1998). Surprisingly, the expression levels of 11 different *Hox* genes (including *Hoxb4* and *Hoxc4* that show altered anterior expression boundaries in paraxial mesoderm in *eed* mutant mice; A. Schumacher and T. Magnuson, unpubl.), were not detectably altered in hemopoietic tissues of *eed* mutant animals. This indicates that the function of *eed* as an inhibitor of hemopoietic progenitor cell proliferation does not involve *Hox* genes.

Recently, the *ink4a* locus was identified as the first downstream mediator of *PcG*-mediated cell proliferation. Removal of *ink4a*, which encodes the tumor suppressors $p16^{\text{INK4a}}$ and $p19^{\text{ARF}}$, alleviated the lymphoid and neurological defects seen in *Bmi1* null mutant animals (Jacobs et al. 1999). However, given the dramatic differences in hemopoietic defects between *eed* and *Bmi1* mutants, the *ink4a* locus is unlikely to be regulated by Eed. The $p16^{\text{INK4a}}$ and $p19^{\text{ARF}}$ expression levels were not altered in *eed* mutant cells.

These results lead to the model illustrated in Figure 8. Therein, the function of the two *PcG* complexes has evolved from a common repressive effect on *Hox* gene expression in axial patterning to a more divergent activity in hemopoietic cells. The hemopoietic target genes involved in the proliferative activity of complex A *PcG* proteins, including *Bmi1*, seem to include the tumor suppressors $p16^{\text{INK4a}}$ and $p19^{\text{ARF}}$. In contrast, the complex B *PcG* proteins, including Eed, exert their function through the regulation of other, as yet unknown, downstream genes. Thus, an intricate equilibrium between two *PcG* protein complexes governing distinct molecular pathways is critical for hemopoietic cell proliferation in mice.

Materials and methods

Animals

Production of *eed*¹⁹⁸⁹ hypomorphic and *eed*³³⁵⁴ null mutant mice (101/R1 × C3Hf/R1 hybrid background) and *Bmi1*^{-/-} mice

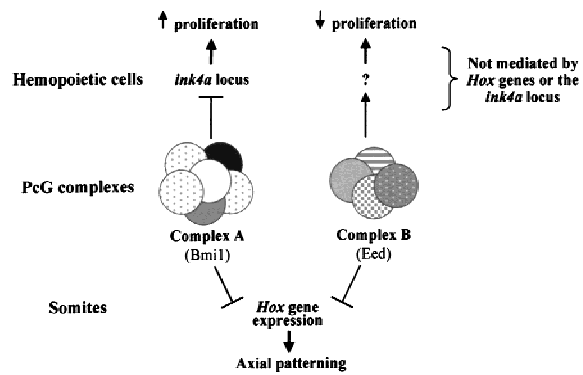


Figure 8. Schematic representation of Eed and *Bmi1* mediated effects in embryonic vs. hemopoietic development in mice. Eed and *Bmi1* *PcG* proteins synergize to regulate differentiation of skeletal/bone precursor cells possibly through a common regulatory effect on *Hox* gene activity. Conversely, Eed and *Bmi1* have opposing functions in regulating hemopoietic cell proliferation in mice that appear to be *Hox* gene independent.

(129Ola/FVB/N hybrid background) have been described before (Schumacher et al. 1996; van der Lugt et al. 1994). Most of the mice were bred at Case Western Reserve University in Cleveland, Ohio and then transported to and maintained in the specific pathogen-free (SPF) animal facility of the Clinical Research Institute of Montreal (IRCM). All animals were housed in ventilated microisolator cages, provided with sterilized food and acidified water, and tested monthly for the presence of viral, bacterial, or parasitic pathogens. Selected animals were sent for complete histopathological studies to rule out the presence of any pathogen.

Generation of double mutant mice

eed^{1989/+} hypomorphic or *eed*^{3354/+} null mutant mice (Schumacher et al. 1996) were interbred with *Bmi1* heterozygous mice (van der Lugt et al. 1994) to generate double homozygous mutant offspring. Newborn mice were genotyped by PCR and/or Southern blot analysis. Only ~2% of littermates were of *eed*^{1989/1989}; *Bmi1*^{-/-} genotype. As expected, none of the *eed*^{3354/3354}; *Bmi1*^{-/-} double mutant mice survived to term. All other genotypes were found at the expected Mendelian frequency.

Mice genotyping

Bmi1 mutant mice were genotyped by Southern blot analysis as described (van der Lugt et al. 1994). The T¹⁰⁴⁰ → C transition at position 1040 in the *eed*³³⁵⁴ null allele destroys an *AluI* restriction site and allows for genotyping of the mice by Southern blot analysis. *eed*¹⁹⁸⁹ hypomorphic mice were genotyped by PCR amplification of D7MIT352. This microsatellite is polymorphic between the 101/R1 × C3Hf/R1 and 129Ola/FVB/N hybrid backgrounds and maps distal to *eed* (Schumacher et al. 1996). In addition, for most of these mice, the presence of the T¹⁰³¹ → A transversion at position 1031 was confirmed by direct sequencing of PCR-amplified genomic DNA.

cDNA generation, amplification, and analysis

Reverse transcription and amplification of total mRNA isolated from purified bone marrow subpopulations were performed as reported previously (Sauvageau et al. 1994). In brief, 10,000 bone

marrow cells purified by cell sorting were pelleted and lysed in 50 μ l of 5 M guanidium isothiocyanate solution. Nucleic acids were precipitated, and cDNA was synthesized with a 60-mer primer containing a 3' polythymidine stretch as described (Brady et al. 1990). A short polyadenosine tail was added to the 3' end of the first strand cDNA using terminal deoxynucleotidyltransferase. Second-strand synthesis and PCR amplification involved the same primer at a higher concentration (Sauvageau et al. 1994). Amplified total cDNA was size fractionated on a 1% agarose gel, transferred to nylon membranes, and hybridized. Probes corresponded to a 329-bp fragment of the human *Bmi1* gene located in the 3' UTR (nucleotides 1938–2248; accession no. L13689, #508) and a 1000-bp 3' fragment of the mouse *eed* coding cDNA (nucleotides 1017–2020; accession no. U78103, #696); probe for *Hoxb9* was a 250-bp *EcoRI*–*PstI* fragment (#639); probe for *Hoxa1* was a *SacI*–*EcoRI* fragment of 150 bp (#633), *Hoxa6* probe was a 250-bp *SacI*–*HindIII* fragment (#634), *Hoxb4* probe was an *EcoRI*–*PmlI* fragment of 485 bp (#405), probe for *Hoxb2* was a 260-bp *HincII*–*PstI* fragment (#419), *Hoxb3* probe was a 380-bp *EcoRI* fragment (#478), probe for *Hoxc4* was a 212-bp *XbaI*–*XhoI* fragment (#214), *Hoxd4* probe was a 765-bp *PvuII*–*HindIII*–*EcoRI* fragment (#770), probes for *Hoxa9* and *Hoxa10* corresponded to full-length cDNAs, and probe for *Hoxa2* was a 320-bp *PstI*–*HindIII* fragment (#650). Probe specific for *p16^{INK4a}* corresponded to exon 1 α (nucleotides 5–197; accession no. AF044335, #775), *p19^{ARF}*-specific probe corresponded to exon 1 β (nucleotides 51–200; accession no. L76092, #776), and probe for β -*Actin* (#212) was isolated as described (Sauvageau et al. 1994).

In vitro clonogenic progenitor assays

For myeloid clonogenic progenitor assays, cells were plated on 35-mm petri dishes (Corning, Fisher) in a 1.1-ml culture mixture containing 0.8% methylcellulose in alpha medium (Sigma) supplemented with 10% fetal calf serum (FCS), 5.7% bovine serum albumin (BSA), 10^{-5} M β -mercaptoethanol (β -ME), 1 U/ml recombinant erythropoietin (Epo), 10% WEHI-conditioned medium (tested to contain 50 ng/ml IL-3), 2 mM glutamine, and 200 mg/ml transferrin. Colonies were scored on day 12 of incubation as derived from CFU-GM, BFU-E, or CFU-GEMM according to standard criteria. For some of the experiments, identification of the colony types was confirmed by Wright staining of cytopins preparations of colonies. For pre-B clonogenic progenitor assays, cells were plated in 0.8% methylcellulose in alpha medium supplemented with 30% preselected FCS (SCT, Vancouver, B.C.), 10^{-4} M β -ME, and 2 ng/ml human IL-7. Pre-B colonies were scored on day 7 of incubation.

In vitro long-term culture assays

Myeloid LTC-IC assays were performed as described (Lemieux and Eaves 1996) by culturing total bone marrow cells at limiting dilution on murine fibroblast S17 feeder cell layers, in 96-well plates, in the presence of alpha medium (Sigma) containing 12.5% FCS, 12.5% horse serum, 10^{-4} M β -ME, 10^{-6} hydrocortisone, 0.016 mM folic acid, 2 mM glutamine, 0.16 mM inositol, and 50 μ g/ml gentamycin. The clonogenic progenitor content of the cultures was assayed 4 weeks after initiation. Similarly, lymphoid (Whitlock–Witte) long-term cultures were performed as described (Whitlock and Witte 1982) by culturing total bone marrow cells at limiting dilution on a S17 feeder cell layer for 3 weeks, in 96-well plates, in RPMI-1640 medium (GIBCO) containing 5% pre-selected FCS, 50 μ M β -ME, and 50 μ g/ml gentamycin.

Flow cytometry

Flow cytometry of hemopoietic cells was performed as described previously (Sauvageau et al. 1997). Briefly, a single cell suspension of bone marrow was prepared by injecting phosphate-buffered salt (PBS) solution containing 2% FCS into femurs to flush out cells, followed by gentle desegregation through a 21-gauge needle. Cells were released from the thymus, spleen, and lymph nodes by disruption through a fine nylon mesh. To lyse erythrocytes, cell suspensions were treated with 0.165 M NH_4Cl and washed once. Cells were stained with primary antibodies in PBS 2% FCS containing blocking solution for Fc receptors on ice for 30 min, washed, and resuspended in PBS 2% FCS containing 1 μ g/ml propidium iodide. Flow cytometric analysis was performed using a Coulter XLTM flow cytometer equipped with a 488-nm laser beam and fluorescence detectors at 525, 575, 620, and 670 nm. Monoclonal antibodies (Mabs) were titrated and used as described previously (Hough et al. 1994, 1996). FITC-labeled B220, CD43 (S7), Gr-1, and Mac-1 and PE-labeled B220 antibodies were purchased from Pharmingen. PE- and FITC-conjugated streptavidin, CD4-FITC, and CD8-PE antibodies were purchased from GIBCO BRL. Biotinylated-IgM antibodies were from Jackson Laboratories and IgD-FITC from Southern Biotech.

Acknowledgments

This work is dedicated to Ms. Mireille Mathieu, a colleague and a friend. This work was supported by grants from the Medical Research Council (MRC) of Canada (MT15064) to G.S. and from the National Institutes of Health (HD24462) to T.M., and Nadine Mayotte for her expert technical assistance. We also acknowledge Marie-Eve Leroux, Christiane Lafleur, and Stéphane Matte for their expertise and help regarding the maintenance and manipulation of the animals kept at the SPF facility of the IRCM. In addition, we are grateful to Louis Lamarre and Caroline Meunier from l'Hôpital Hôtel-Dieu and l'Hôpital Maison-neuve-Rosemont de Montréal, respectively, for their assistance with the analysis of pathological specimens and Dr. Timothy Manser for providing the *IgH* enhancer locus probe. The support of Nathalie Tessier is also acknowledged for FACS analysis. J.L. is a recipient of a Studentship from MRC of Canada, U.T. is a fellow from the Leukemia Research Fund of Canada, and G.S. is an MRC Clinician–Scientist Scholar.

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