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Expression et fonction des gènes du groupe *Polycomb (PcG)* dans l'hématopoïèse normale et leucémique

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RESUME EN FRANÇAIS ET MOTS CLES FRANÇAIS

Des études présentées dans cette thèse ont permis de démontrer que le gène du *Groupe Polycomb (PcG) Bmi-1* est un déterminant génétique essentiel à l'activité proliférative des cellules souches hématopoïétiques (CSHs) foetales et adultes. Appuyant cette observation, l'homozygotie pour un allèle nul du gène *Bmi-1* conduit à une anémie aplasique progressive et létale chez la souris. De plus, ce travail démontre que la fonction du gène *Bmi-1* est maintenue au niveau des cellules souches leucémiques (CSH-Ls). Cette importante observation renforce la notion d'une structure dans la hiérarchie leucémique où *Bmi-1* définit la "primitivité" et suggère que la fonction des cellules souches (qu'elles soient normales ou leucémiques) est régulée par un groupe commun de déterminants géniques. Il devient alors impératif de vérifier si la fonction de *Bmi-1* s'étend aussi aux cellules souches normales et cancéreuses provenant d'autres types tissulaires. Une étude d'expression détaillée de gènes de la famille *Polycomb (PcG)* dans des populations purifiées de cellules de moelles osseuses humaines a révélé une curieuse dissociation entre le patron d'expression du gène *Bmi-1* (restreint aux cellules souches hématopoïétiques) et celui des autres protéines *Polycomb* (PcG) avec lesquelles Bmi-1 interagit physiquement. Dans le but de définir la composition du complexe contenant Bmi-1 au niveau des CSHs, nous avons utilisé une approche de double-hybride chez la levure. Ceci a conduit à l'identification de huit nouveaux co-facteurs de Bmi-1 spécifiques aux CSHs, incluant une molécule à doigts de zinc qui semble représenter un médiateur clé de la fonction pro-proliférative de Bmi-1 au niveau des CSHs. Par ailleurs, nous avons démontré que le gène *Polycomb (PcG) eed* possède une activité antagoniste à *Bmi-1* dans la régulation de la prolifération des CSHs. Les souris hétérozygotes pour un allèle nul du gène *eed* (*eed*$^{3354/+}$) développent des anomalies myelo- et lympho-prolifératives sévères, indiquant qu'Eed est impliqué dans la régulation négative de la prolifération des progéniteurs de la moelle osseuse. En conclusion, le travail présenté dans cette thèse démontre que l'activité proliférative de la cellule souche hématopoïétique est régulée par la contribution relative d'un complexe PcG contenant Bmi-1 stimulant leur prolifération et d'un complexe contenant Eed, réprimant cette activité.

Mots clés: Gènes du *Groupe Polycomb (PcG)*, cellule souche hématopoïétique (CSH), cellule souche leucémique (CSH-L), hématopoïèse, *Bmi-1*, *eed*.

RESUME EN ANGLAIS ET MOTS CLES ANGLAIS

The studies presented in this thesis establish that the *Polycomb Group (PcG)* gene *Bmi-1* is a key genetic determinant of the proliferative capacity of fetal and adult hemopoietic stem cells (HSCs). Consistent with this, homozygosity for a null allele of *Bmi-1* in mice leads to progressive and lethal aplastic anemia by early adulthood. This work also shows that the function of *Bmi-1* is preserved in leukemic stem cells (L-HSCs), providing the first molecular basis for the concept that stem cell function (whether normal or neoplastic) is regulated by common regulatory genes. These findings reinforce the notion of a structure in the leukemic hierarchy where *Bmi-1* defines "stemness". Determining whether this function of *Bmi-1* extends to other types of normal and "neoplastic stem cells" is eagerly awaited. A detailed expression analysis of selected members of the *PcG* gene family in purified subpopulations of human bone marrow cells revealed a curious dissociation of the expression profile of *Bmi-1* (mostly restricted to the HSC compartment) and that of the other known PcG proteins which physically interact with Bmi-1. In an attempt to define the biochemical nature of the Bmi-1-containing complex in HSCs, a yeast-two-hybrid screen was performed using an expression library enriched for primitive hemopoietic cells. This approach led to the identification of eight novel stem cell-specific co-factors of Bmi-1, including a zinc-finger molecule which appears to represent a key mediator of Bmi-1-induced HSC proliferation. Conversely, it was established that the *PcG* gene *eed* performs an antagonistic function to *Bmi-1* in the regulation of HSC proliferation. Heterozygosity for a null allele of *eed* ($eed^{3354/+}$) leads to severe myelo- and lympho-proliferative defects and lymphoid tumor development in mice, indicating that Eed is involved in the negative regulation of the pool size of early bone marrow progenitor cells. Together, the work presented in this thesis reveals that the proliferative tone of the HSC is intrinsically regulated by the relative contribution of a pro-proliferative (Bmi-1-containing) and an anti-proliferative (Eed-containing) PcG gene complex.

Key words: *Polycomb Group (PcG)* genes, hemopoietic stem cell (HSC), leukemic stem cell (L-HSC), hemopoiesis, *Bmi-1*, *Eed*.

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Abréviation	Signification
3-AT	3-amino-1,2,4-triazole
a.a.	Amino acid
AD	Activation domain
Ade	Adenine
A/P	Anterior-posterior
Ag	Antigen
AGM	Aortic-gonadal-mesonephros
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
ANT-C	Antennapedia complex
Arg3	Arginine 3
Asx	Additional sex combs
ATM	Ataxia Telangiectasia Mutated
ATP	Adenosine triphosphate
B-CLL	B-cell chronic lymphocytic leukemia
BCR	B-cell receptor
BFU-E	Burst-forming-unit-erythroid
BHIP-1	Bmi-1 Hemopoietic Interacting Protein-1
BM	Bone marrow
β-ME	*Beta*-mercaptoethanol
Brm	Brahma
BSA	Bovine serum albumin
BX-C	Bithorax complex
C	Cysteine
C_2H_2	Zinc finger domain of the C_2H_2 subtype
Ccf	Centrosomal and chromosomal factor
cDNA	Complementary DNA

CDS	Coding sequence
CFC	Colony-forming cell
CFU-GEMM	Colony-forming-unit-granulocyte-erythroid-monocyte-megakaryocyte
CFU-GM	Colony-forming-unit-granulocyte-macrophage
CFU-S_{12}	Colony-forming-units in spleen day 12
CIHR	Canadian Institute of Health Research
CKI	Cyclin-dependent kinase inhibitor
CtBP	C-terminal binding protein
CTRL	Control
D	Aspartic acid
DN	Double negative
DNA	Deoxyribonucleic acid
DP	Double positive
E	Glutamic acid
E(Pc)	Enhancer of Polycomb
E(z)	Enhancer of Zeste
Eed	Embryonic ectoderm development
EGFP	Enhanced green fluorescent protein
ENU	Ethylnitrosourea
Epo	Erythropoietin
Esc	Extra Sex Combs
EST	Expressed sequence tag
ETP	Enhancers of Trithorax and Polycomb
Eu	Immunoglobin heavy chain enhancer
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FL	Fetal liver
GC	Germinal center
GEF	GTP/GDP exchange factor
GTF	General transcription factor

H	Histidine
H3	Histone 3
HA	Hemagglutin protein
Hb	Hunchback
HDAC	Histone deacetylase
hGM-CSF	Human granulocyte-macrophage colony-stimulating factor
His	Histidine
HMTase	Histone methyltransferase
HOM-C	Homeotic
Hox	Homeobox
HPC	Highly proliferative clone
HPP-CFC	High proliferative potential-CFC
HRS	Hodgkin's disease Reed-Sternberg
HSC	Hemopoietic stem cell
HTHTHT	Helix-turn-helix-turn-helix domain
hVPS11	Human Vacuolar protein sorting 11
i.d.	Identification
IgH	Immunoglobulin heavy chain
IL	Interleukin
IP	Immunoprecipitation
IRCM	Institut de Recherches Cliniques de Montréal
K	Lysine
K4	Lysine 4
K9	Lysine 9
K27	Lysine 27
KRAB	Kruppel-associated box
L	Lymph node
L-blast	Leukemic blast
LCBQ	Leukemic Cell Bank of Quebec
LD	Limiting dilution
Leu	Leucine

L-HSC	Leukemic hemopoietic stem cell
Li	Liver
Lib	Library
Lin	Lineage-specific
LN	Lymph node
LOH	Loss of heterozygosity
LP	Lymphoproliferation
LPP-CFC	Low proliferative potential-CFC
LTC-IC	Long-term culture-initiating cell
LTRC	Long-term repopulating cell
Lys12	Lysine 12
Lys27	Lysine 27
Mabs	Monoclonal antibodies
MBLR	Mel-18 and Bmi-1-like RING finger
MCL	Mantle cell lymphoma
M-CSF	Macrophage colony-stimulating factor
MDa	Mega Dalton
MDR	Multi-Drug Resistance
ME	Maintenance element
MEC	Mammary epithelial cell
MEF	Mouse embryonic fibroblast
MLL	Mixed lineage leukemia
MMLV	Moloney murine leukemia virus
MNU	N-methyl-N-nitrosourea
MoAbs	Monoclonal antibodies
MPD	Myelo-proliferative disease
MRC	Medical Research Council of Canada
MSCV	Murine Stem Cell Virus
Mxc	Multi sex combs
MZ	Mantle zone
N.A.	Not analyzed

NC	Nucleated cell
N.D.	Not determined
NED	No evidence of disease
NHL	Non-Hodgkin's lymphoma
NLS	Nuclear localization signal
No R.T.	No reverse transcription
NSCLC	Non-small cell lung cancer
NuRD	Nucleosome remodeling and histone deacetylation
PBL	Peripheral blood
PBS	Phosphate-buffered salt
Pc	Polycomb
PcG	Polycomb Group
PcGe	PcG complex making the epigenetic mark
PcGm	PcG complex involved in the maintenance
Pcl	Polycomblike
Pco	Polycombeotic
PE	Phycoerythrin
PEV	Position effect variegation
Ph	Polyhomeotic
PI	Propidium iodine
PLZF	Promyelocytic leukemia zinc finger
pRb	Retinoblastoma tumor-suppressor protein
PRC1	Polycomb repressive complex 1
PRE	Polycomb response element
PS	Para-aortic splanchnopleura
Psc	Posterior sex combs
R	Arginine
Rb	Retinoblastoma protein
RNA	Ribonucleic acid
RNAi	RNA interference
RT-PCR	Reverse transcriptase-polymerase chain reaction

S	Spleen
Sce	Sex combs extra
Scm	Sex comb on midleg
SDS	Sodium dodecyl sulfate
Ser	Serine
Shh	Sonic hedgehog
SP	Single positive
SP	Side population
SPL	Spleen
SPF	Specific pathogen-free
Sxc	Super sex combs
T	Thymus
Thy	Thymus
TBP	TATA-binding-protein
TCR	T-cell receptor
TCRβ	T-cell receptor β
TdT	Terminal deoxynucleotidyl transferase
TNT	Transcribed and translated
T-PLL	T-cell prolymphocytic leukemia
TR	Thyroid hormone receptor
Trp	Tryptophane
TrxG	Trithorax Group
TS	Trophoblast stem
Tx	Transplanted
VSV	Vesicular stomatitis virus
WBC	White blood cell
WW-IC	Whitlock-Witte-initiating cell
Xeed	*Xenopus* eed
Y2H	Yeast-two-hybrid
Zn	Zinc finger domain
ZNF3	Zinc finger protein 3

LA DÉDICACE

A ma famille et amis,

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CHAPITRE 1

INTRODUCTION: SECTION 1

Molecular Genetics of Hemopoietic Stem Cells

The present Ph.D. thesis, consisting of 7 chapters, describes the expression, function and mechanisms of action of selected members of the *PcG* gene family in normal and leukemic hemopoiesis.

Chapter 1 is a literature review which is subdivised into two sections. The first section describes the genetic determinants of early hemopoiesis, the intrinsic and extrinsic regulators of hemopoietic stem cell (HSC) self-renewal, and the mechanisms regulating stem cell homeostasis in model systems such as the *Drosophila* germ line. The next section summarizes the role of selected members of the *Polycomb Group (PcG)* gene family in the regulation of normal and leukemic hemopoiesis. A particular emphasis is given to the epigenetic mechanisms underlying *Polycomb Group (PcG)* gene function across species. This last section has been recently published as a review Article. It was written by Julie Lessard under the supervision of Dr Guy Sauvageau.

1.1.1 Genetic determinants of early hemopoiesis

Hematopoiesis is an ordered developmental program of differentiation and proliferation, leading to the generation of mature blood cells of multiple distinctive lineages from totipotent hemopoietic stem cells (HSCs). Regulation of this highly complex process occurs at multiple levels, and can be simplistically viewed as the combined effects of external influences (composed of both humoral factors and cell-cell or cell-matrix interactions) and intracellular signaling events, ensuring transcriptional factor regulation and consequent changes in gene expression programs.

Until recently, very little was known about the genetic mechanisms that bring about the intrinsic processes of lineage commitment and subsequent lineage-specific differentiation of early hemopoietic cells. Accumulating evidence, from a number of recent studies, is now pointing to transcription factors such as *SCL/tal-1* (stem cell leukemia hemopoietic transcription factor), *rbtn2/LMO2* (also known as *ttg-2*), *AML-1* (*also known as RUNX1/CBFA2 and PEBP2B*), *GATA-2* and *Ikaros* as key genetic determinants of early hemopoiesis. Depicted in figure 1 are the positions of essential function for these transcription factors in the hemopoietic hierachy. The b-HLH domain-containing tal-1/SCL and the LIM domain-containing rbtn2/LMO2 proteins (which have been shown to interact *in vivo* and form a heterocomplex) [1,2] act very early in ontogeny, as mice deficient for either of these gene products lack all lineages of both primitive and definitive hemopoiesis [3,4]. A role for the runt homology domain protein AML-1 in the initiation of definitive hemopoiesis has been demonstrated, as *AML-1* null mice present normal primitive hemopoiesis but completely lack definitive hemopoiesis [5]. The *TEL* (*translocaction-Ets-leukemia* or *ETV6*) gene was shown to be required specifically for hemopoiesis of all lineages in the bone marrow [6]. Further studies will be necessary to establish whether this reflects an inability of $TEL^{-/-}$ HSC/progenitors to migrate to the bone marrow or, more likely, to respond appropriately and/or survive within the bone marrow microenvironment. Absence of the zinc finger protein GATA-2 in mice appears to impair the proliferation capacity of early hemopoietic cells [7]. Mice that lack functional c-myb protein have a phenotype similar to that of the *GATA-2* null mice, with

the exception of the megakaryocyte lineage which appears to develop normally in the absence of *c-myb* [8]. Both *GATA-2* and *c-myb* are normally expressed in primitive hemopoietic cells and then down-regulated as these cells differentiate [9,10]. Forced overexpression of *GATA-2* and *c-myb* in progenitor cells promotes their proliferation and blocks differentiation [10,11]. Homozygous null mice for the ets family member *PU.1* lack cells of the granulocytic, monocytic and B cell lineages –the cells in which *PU.1* is normally expressed [12]. Interestingly, a separate line of *PU.1* null mice (derived using a different targeting vector) has a more severe phenotype with additional defects in the T and erythroid lineages [13]. Finally, the *Ikaros* gene encodes six alternative transcripts that are differentially expressed in lymphoid cells and whose products regulate the expression of a number of lineage specific genes [14]. Mice lacking Ikaros function displayed a complete absence of all cells of the lymphoid lineage (T, B and NK), whereas both progenitor and mature cells of the myeloid and erythroid lineage where increased [15].

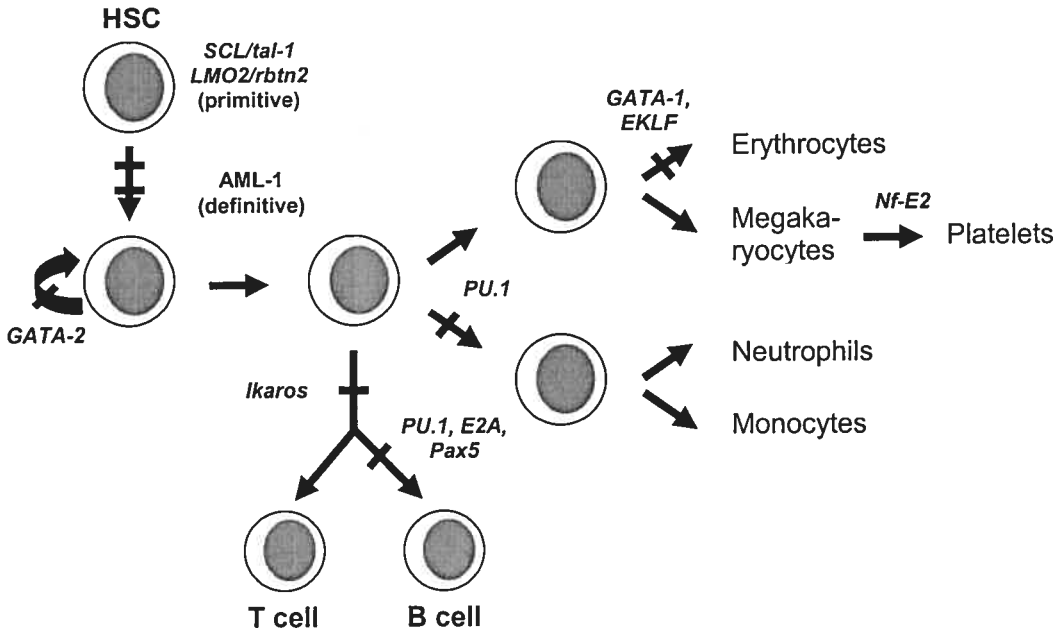

Fig. 1.1 Schematic representation indicating the position of essential function of some transcription factors known to be active in hemopoiesis. Positioning of each gene product is based on the earliest block observed in hemopoiesis resulting from its absence. Adapted from Shivdasani R A. and Orkin S.H. (1996) *Blood* 87: 4025-4039.

Other factors appear to be more lineage specific in action such as *GATA-1*, *EKLF*, *NF-E2*, *Pax5* and *E2A*, as their absence affects only one hemopoietic lineage [16-22]. For example, absence of GATA-1 blocks hemopoietic differentiation at the proerythroblast stage followed by apoptosis of these cells, suggesting that the functional role of GATA-1 is to permit survival and maturation of erythroid progenitor cells by preventing apoptosis [23].

1.1.2 Self-renewal: a key property of the hemopoietic stem cell?

The hemopoietic stem cell (HSC) can be operationally defined as a long-term repopulating cell with both lymphoid (T and B) and myeloid potential [24]. The first evidence for the existence of such a cell-type comes from experiments by Ray Owen and

colleagues, in 1945, which showed that bovine fraternal twins, sharing a single placenta and blood circulation, retained production of blood cells genetically defined to be from both throughout their life [25]. Twenty years later, elegant experiments by Till, McCulloch, Wu, Becker, Simonovitch and colleagues demonstrated that adult bone marrow contained single cells that had the ability to form macroscopic nodules of myeloerythroid cells on the spleen, 8 to 12 days after intravenous injection into myelo-ablated recipients [26-28]. These spleen-colony-forming-units (CFU-S) were shown to be clonal [29] and, in many cases, could generate similar colonies upon transplantation into secondary recipients [28]. As they shared several characteristics attributed to HSCs (including high proliferative potential, multipotentiality and self-renewal ability), CFU-S were initially considered to be HSCs [28]. The validity of the CFU-S assay to detect HSCs with long-term repopulating potential was questioned after the discovery that some of these cells were capable of only unilineage differentiation and/or lacked the ability to self-renew (functional heterogeneity). Although most of the cells possessed the ability to differentiate into the erythrocyte and myeloid lineages, their lymphoid potential remained controversial [30-32]. It is now clear that most CFU-S cells in the adult bone marrow are committed myeloid progenitors [33,34] which can be physically separated from more primitive cells with long-term lympho-myeloid repopulating potential [35-39]. Although the CFU-S assay played a key role in the development of concepts of primitive hemopoietic cell organization and regulation, its inability to analyze pure stem cells meant that most of their functions were implied rather than directly analyzed.

The first attempts at purifying the HSC came from the school of Till and McCullogh [33,40] and Van Bekkum in the Netherlands [41] . From this work, it has become possible to routinely identify and isolate highly purified murine and human HSCs based mainly on characteristic cell surface proteins that are either present (Sca-1 and c-kit) or absent (using markers of lineage committed cells such as CD38, Mac-1 and CD8) (for review [42]).

Despite the progress that has been made in identifying and obtaining enriched HSC populations, analysis of the population dynamics and cell cycle kinetics of HSCs

remains difficult. One of the most intriguing properties of adult HSCs is a robust maintenance of the dynamic equilibrium between self-renewal and differentiation [43]. Under homeostatic conditions *in vivo,* most HSCs are quiescent, as demonstrated by their relative resistance to killing by the cytotoxic drug 5-fluorouracil (5-FU) when compared to committed progenitor cells [44,45]. When they enter cycle, HSCs can divide asymmetrically or symmetrically, resulting in different HSC fates. Stem cell maintenance divisions give rise to one daughter HSC with essentially identical biological properties (a process referred to as self-renewal) and one committed daughter cell. The committed daughter cell enters a transient state of rapid cellular proliferation and, upon exhaustion of its proliferative potential, withdraws from the cell cycle and progressively acquires the specialized characteristics of one of the 10 mature blood cell lineages (a process known as differentiation). Although the relative influence of intrinsic versus extrinsic factors on HSC self-renewal remains to be determined, it has been easier to identify the environmental factors having a negative impact on this process than those that enhance it. Thus, most *in vivo* culture conditions defined to date lead to depletion of the HSC pool by favoring symmetric divisions (generation of two daughter differentiated cells) and concomitant expansion of committed progenitor populations [43].

Several studies using retroviral marking of HSCs have demonstrated the ability of HSCs to undergo self-renewal divisions [46-49]. Although most of these studies failed to accurately quantify the magnitude of self-renewal events, considerable evidence suggests that this property is not unlimited. First, following bone marrow transplantation, the HSC pool is not found to regenerate to levels higher than 10% of normal pre-transplantation values, despite a complete regeneration of bone marrow cellularity and progenitor cell numbers [50-54]. Some investigators have suggested the involvement of negative feedback mechanisms imposed *in vivo* by more mature cells as a possible mechanism that could prematurely inhibit HSC expansion following transplantation [55]. Alternatively, this loss of long-term repopulating ability may result from damage to the recipient's microenvironment inflicted by the conditioning regimen (i.e. irradiation). However, experiments performed in the anemic (WW^V) recipient mouse strain, which possess a normal microenvironment but poorly competitive

hemopoietic cells due to a mutation in the c-kit ligand receptor [56], rather suggest that this defect is intrinsic to the transplanted cells themselves [51,57].

A major concern is that nearly all HSC assays assessing self-renewal rely on the generation of functionally mature cells, and therefore provide a retrospective rather than a current view of potential HSC attributes. In a transplantation setting, the accuracy of the HSC readout relies on the efficiency of the transplanted cells to home and engraft to the specialized niches of the bone marrow microenvironment [58]. The heterogeneity of the HSC compartment further complicates the interpretation of such experimental designs. Age-related and strain-specific [59,60] differences in HSC numbers and/or competitive abilities have been reported (for review [61]). Moreover, a functional decline in the proliferative potential of HSCs derived from the fetal liver, umbilical cord (at birth) and adult bone marrow indicates ontogeny-related differences in HSC function [54,62-64]. Whether this heterogeneity represents true intrinsic quantitative and/or qualitative differences in HSC properties, or in the expression of this potential due to stochastic events, remains unclear. So, a question remains: can HSCs truly self-renew or does cell division impair their qualitative (biological) properties?

1.1.3 Intrinsic regulators of HSC self-renewal

While the concept of "true HSC self-renewal" remains controversial, genetic programmes regulating the self-renewal and differentiation outcomes of early hemopoietic cell divisions have been described. Recent attention has focused on hemopoietic cytokines (see next section) [65-69] and cell intrinsic pathways whose activation has caused some HSC expansion *ex vivo,* with the ultimate goal of durable *in vivo* engraftment. Enforced expression of the P glycoprotein pump genes *MDR1* and *ABCG2* in murine bone marrow cells led to the expansion of side population cells with retained *in vivo* repopulation ability [70]. Activation of retinoic acid receptor signaling by addition of all-trans retinoic acid resulted in retention of long-term repopulating activity of cultured hemopoietic stem cells [71]. Constitutive Notch signaling in purified Sca-1$^+$ Lin$^-$ c-kit$^+$ bone marrow cells led to the immortalization of blast-like cells that retained

pluripotency and long-term repopulating potential [72]. In particular, retroviral overexpression of *HOXB4* in mouse bone marrow cells significantly enhanced the rate of HSC self-renewal (potentially up to 1000-fold net increase of transduced HSCs) in both primary and secondary recipients [73-75]. Of note, this stem-cell specific proliferative effect of *HOXB4* occurs without impairing normal differentiation or inducing cellular transformation. A role for the Wnt signaling pathway in self-renewal of HSCs has also recently been demonstrated [76]. Overexpression of activated *B-catenin* expands the pool of HSCs in long-term cultures by both phenotype and function. Ectopic expression of *axin* or a *frizzled* ligand-binding domain, inhibitors of the Wnt signaling pathway, leads to inhibition of HSC growth *in vitro* and reduced reconstitution *in vivo*. Furthermore, activation of Wnt signaling in HSCs induces increased expression of *Notch1* and *Hoxb4*, suggesting a molecular hierarchy of regulation of HSC development. Purified Wnt3a protein also induces self-renewal of repopulating cells (HSCs?), signifying its potential role in tissue engineering [77]. Serial transplantation studies demonstrated a critical role for the transcription-regulating/chromatin modifying CREB-binding protein (CBP), but not its paralogous protein p300, in maintaining an adequate pool of murine HSCs through self-renewal [78]. The cyclin-dependent kinase inhibitor (CKI) $p21^{CIP}$ also appears to be an essential component of the molecular switch governing the entry of the hemopoietic stem cells in cycle as in its absence, increased cell cycling leads to stem cell exhaustion [79]. Most importantly, the *Polycomb group* gene *Bmi-1* is absolutely required for the maintenance/self-renewal of both fetal and adult HSCs (see below)[80,81].

It was recently demonstrated that the homeoprotein *Nanog* is required for maintenance of pluripotency in mouse epiblast and embryonic stem (ES) cells [82,83]. Despite very different genetic programs *in vivo*, under normal circumstances, both embryonic (ES) and hemopoietic stem cells share fundamental common properties: multipotency and the ability to self-renew. Insights into the regulation of Nanog- as well as other regulators of self-renewal/multipotency such as Oct4-, FoxD3-, Sox2- and Stat3-directed transcription pathways and the network of crosstalk between these factors might contribute to a better understanding of stem cell behavior in other renewing tissues such as blood. However, an important caveat is that ES cells truly self-renew whereas HSCs

may not. This raises the hypothesis that distinct molecular programmes may be involved in regulating this process.

1.1.4 Extrinsic regulators of HSC self-renewal

1.1.4.1 Regulation of germ line stem cell (GSC) self-renewal in *Drosophila*

Further defining the molecular mechanisms controlling stem cell function is crucial to the future use of stem cells in regenerative medicine as well as understanding the processes of aging, tumor formation and degeneration. As mentioned above, a fundamental characteristic of adult stem cells is their capacity to either divide asymmetrically or symmetrically. Thus, general mechanisms may exist to balance self-renewal capacity with differentiation.

The *Drosophila* germ line represents an excellent system to study adult stem cells at the cellular and molecular level and their relation with their microenvironment. In the female, at the anterior end of each ovariole of an ovary (or germarium) rest two or three germ-line stem cells (GSCs) that originate from primordial germ stem cells (PGCs). These primitive cells are located in a niche which is composed of three differentiated somatic cell types: terminal filament (TF) cells, cap cells and inner germarium sheath (IGS) cells. This microenvironment is instrumental in regulating the expansion of the germ line stem cell pool. Anterior PGCs adjacent to TF/cap cells give rise to two daughters that both contact TF/cap cells and will eventually develop into GSCs (symmetric division). The remaining PGCs (not in physical contact with TF/cap cells) directly differentiate into mature oocytes (asymmetric division) [84-90]. In the male germ line, PGCs are also selected to become GSCs based on their juxtaposition to a cluster of somatic/support cells (called the hub) located at the apical tip of the testis. Upon cell division, the daughter cell maintaining contact with the hub retains stem cell identity, whereas the cell displayed away from the hub initiates differentiation into a gonialblast [91,92]. Thus, the orientation/position of germ line stem cell divisions in the niche seems to be critical in regulating the expansion of the germinal stem cell pool (deterministic fate).

In *Drosophila*, the molecular mechanisms regulating germ line stem cell (GSC) divisions are currently being unraveled. The Janus kinase-signal transducer and activator of transcription (JAK-STAT) signal transduction pathway is an important intrinsic regulator of GSC self-renewal in the male germ line [93,94]. Similarly, *piwi*, the *Drosophila* counterpart of human *HIWI*, is also required for the self-renewal ability of GSCs, but is absolutely dispensable for their differentiation into committed daughter cells [84]. *Decapentaplegic (Dpp)* (the *Drosophila* homolog of human *bone morphogenetic protein 2/4)*, is expressed in anterior somatic cells of the gonad and is essential for PGC proliferation. PGCs mutant for *thick veins,* an essential *dpp* receptor, are impaired in their ability to clonally populate a niche, further suggesting that *dpp* is one of the extrinsic mitotic signals that promote the clonal expansion of GSCs in a niche [90]. In the male germ line, the asymmetric outcome of stem cell divisions is specified, extrinsically, through direct interaction with niche cells expressing the ligand *Unpaired (Upd)* [93,94]. *DE-cadherin*-mediated cell adhesion was also shown to be essential for anchoring ovarian GCSs to their niches and stimulating their proliferation [95]. Loss of function of the *Drosophila Epidermal growth factor receptor (EGFR)* in somatic cells increases the number of GSCs in the male germ line, suggesting that the *EGFR* gene is also an extrinsic (but negative) regulator of GSC proliferation [96].

1.1.4.2 Positional information and the regulation of stem cell self-renewal: a conserved mechanism?

Positional cues from the microenvironment seem to be critical in regulating stem cell-fate decisions in several other organisms and developmental systems. In the *C. elegans* gonadal arm, mitotic germ line stem cells (GSCs) reside distally, while differentiating gametes progressively move towards the proximal end. Somatic distal tip cells (DTCs) located at the extremity of the gonad constitute a niche that is essential for promoting GSC proliferation and preventing meiosis (for review [97]). In the developing brain, mammalian neural stem cells (NSCs) are known to be located in close contact with the ventricular zone surface, whereas differentiated post-mitotic neurons progressively move

towards the dorsal zone (for review, [98]). Interestingly, it has been suggested that NSCs dividing along the ventricular surface give rise to two stem cells, while the perpendicular division generates one stem cell and one differentiated neuronal cell [99]. The small intestine is composed of ciliated villi, each surrounded by crypts, embedded in the intestinal wall for protection. Each crypt is composed of about 250 simple epithelial cells that include the stem cell compartment for replenishing the villi. The multipotent stem cells are located near or at the base of each crypt [100]. To maintain homeostasis, slow cycling stem cells are converted to rapidly but transiently proliferating cells that move to the midsegment and subsequently differentiate into functionally mature and specialized cells (i.e. either absorptive enterocytes, mucus-secreting goblet or enteroendocrine cells). Similarly, the epidermis is composed of a single inner (basal) layer of dividing stem/progenitor cells, which periodically withdraw from the cell cycle, commit to differentiate terminally, and move outward toward the skin surface [101].

The bone marrow also provides adult HSCs with a rich, but complex, milieu (or niche) composed of many cell types including macrophages, adipocytes, fibroblasts and mesenchymal cells. Bone marrow stromal factors that positively impact on HSC maintenance, propagation and homing include soluble and membrane-bound stem cell factor (SCF), soluble Sonic Hedgehog, the fibroblast growth factors FGF-1 and FGF-2, the α-chemokine stromal-1 cell derived factor-1 (SDF-1) and a slew of extra cellular matrix (ECM) molecules, all having the ability to specifically interact with various receptors on the HSC surface (for review [102]). On the other hand, purified primitive ($Sca\text{-}1^{+}$ Lin^{-} $c\text{-}kit^{+}$) bone marrow cells are severely compromised in their short and long-term multilineage reconstituting ability when activated by TNF-alpha or through Fas [103] or when engineered to overexpress Flt3 [104], providing molecular evidence for extrinsic negative regulators of HSC self-renewal.

Despite major efforts to characterize the bone marrow niche, *in vitro* studies indicate that HSCs still survive better when cultured with bone marrow stroma than when placed in a defined medium supplemented with characterized bone marrow components. This is also true for epidermal keratinocyte progenitors which require coculturing with

fibroblasts to display their optimal survival and proliferative capacity [105]. Thus, the orientation of the stem cell division plane in the niche might represent a general and evolutionarily conserved mechanism involved in regulating both germinal and somatic stem cell homeostasis. Locating and analyzing the stem cell niches and identifying the molecules that orchestrate these environmental positional cues are of major importance in stem cell biology.

1.1.5 Cellular organization of normal and leukemic hemopoiesis: a common function for stem cells

There is strong support for the idea that cancer is a stem-cell disease [106]. The similarity in the hierarchical organization of malignant and normal tissues is best characterized in the hemopoietic system. Human acute myeloid leukemia (AML) originates from a rare population of primitive cells ($CD34^+$ $CD38^-$) highly enriched in hemopoietic stem cells (HSCs) [107]. Most leukemic cells (blasts) are limited in their proliferative capacity and must be constantly replenished by rare, self-renewing "leukemic stem cells" (L-HSCs). So, like the normal hemopoietic system, leukemia seems to be organized as a hierarchy that originates from a stem-cell pool which most likely retains remnants of the normal developmental program.

It has also been proposed that the initial, cancer-causing ("transforming") mutations occur in the self-renewing stem cell pool, rather than in already committed precursors. In this view, fewer mutations would be required to generate fully malignant cells if they were to originate from already self-renewing stem cells, as opposed to committed progenitors with low proliferative potential. Thus, two important findings have recently emerged from studies of stem cell biology and carcinogenesis: 1-) in the process of neoplastic transformation, the genetic events responsible for disease progression must occur in a stem cell, unless one of the mutations would permit self-renewal in a downstream committed progenitor; 2) within the cancer or leukemia, only a subset of the cells that make up the tumor mass are tumorigenic –the "cancer stem cells" [108]. These ideas predict similarities in the molecular programmes of normal and cancer/AML stem cells.

The genetic mechanisms regulating self-renewal of the HSCs may be more generally applicable to other regenerating tissue systems. Recent findings implicated the Notch [72,109,110], Wnt and Shh [111-113] signaling pathways in promoting stem cell self-renewal in a variety of different epithelia in addition to HSCs. Interestingly, mutations of these pathways have been associated with a number of human neoplasia, including colon carcinoma and epidermal tumors [114,115] (Wnt), medulloblastoma and basal call carcinoma [116,117] (Shh), and T-cell leukemias [118] (Notch).

As uncontrolled stem cell self-renewal represents the basis of cancer, the identification of stem-cell specific genes, especially those involved in the deregulation of their self-renewal capacity is critical. The goal of my PhD thesis was specifically to identify such a gene. To achieve this, I used a candidate gene approach and decided to focus my studies on the *Polycomb (PcG)* family of genes because: 1-) the *PcG* genes are upstream transcriptional regulators of the homeotic (*Hox)* genes in skeletal precursor cells 2-) the *Hox* genes are important regulators of hemopoietic development; 3-) *HOXB4* is a critical regulator of HSC self-renewal; its retroviral overexpression in mouse bone marrow cells leads to 1000-fold net increase of transduced HSCs in both primary and secondary recipients [74]; 4-) in the mid-90's, expression of some *PcG* genes in human and murine hemopoietic cell lines had already been reported; 5-) van Lohuizen and colleagues had identified the *Polycomb Group (PcG)* gene *Bmi-1* as an essential regulator of the proliferative activity of bone marrow myeloid and lymphoid progenitors [119], suggesting a putative role for this gene in the regulation of HSC behavior. The work presented in this PhD thesis was intended at verifying whether selected members of the *Polycomb group (PcG)* gene family 1-) were expressed in the hemopoietic tissue; and 2-) might be involved in regulating critical aspects of HSC function.

The next section will provide a detailed overview of the *Polycomb (PcG) group* genes, with special emphasis on their implication in the regulation of hemopoietic cell development.

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CHAPITRE 1

INTRODUCTION: SECTION 2

Polycomb Group (PcG) Genes as Epigenetic Regulators of Normal and Leukemic Hemopoiesis

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Abstract

Epigenetic modification of chromatin structure underlies the differentiation of pluripotent hemopoietic stem cells (HSCs) into their commited/differentiated progeny. Compelling evidence indicates that *Polycomb group (PcG)* genes play a key role in normal and leukemic hemopoiesis through epigenetic regulation of HSC self-renewal/proliferation and commitment. The PcG proteins are constituents of evolutionary highly conserved molecular pathways regulating cell fate in several other tissues through diverse mechanisms including (1) regulation of self-renewal/proliferation; (2) regulation of senescence/immortalization; (3) interaction with the initiation transcription machinery; (4) interaction with chromatin-condensation proteins; (5) modification of histones; (6) inactivation of paternal X chromosome and (7) regulation of cell death. It is therefore not surprising that *PcG* genes lead to pleiotropic phenotypes when mutated and have been associated with malignancies in several systems in both mice and humans. Although much remains to be learned regarding the *PcG* mechanism(s) of action, advances in identifying the functional domains and enzymatic activities of these multimeric protein complexes have provided insights into how PcG proteins accomplish such processes. Some of the new insights into a role for the *PcG* cellular memory system in regulating normal and leukemic hemopoiesis are reviewed here, with special emphasis on their potential involvement in epigenetic regulation of gene expression through modification of chromatin structure.

1.2.1 Definition, evolutionary conservation and organization of PcG proteins into distinct multimeric complexes

In *Drosophila melanogaster*, the homeotic *(HOM-C)* genes are clustered in the *Antennapedia (ANT-C)* and *Bithorax (BX-C)* complexes. Parasegment cell identity in the somatic mesodermal tissue is achieved by differential temporal activation and spatial distribution of the *HOM-C* gene products along the anterior-posterior (A/P) axis of the developing embryo, in a manner that correlates with their position along the chromosome (3' to 5') [1,2]. Loss- and gain-of-function of *ANT-C* and *BX-C* genes result in homeotic transformations manifested by aberrant segmentation of the adult body [3-5]. Genetic screens in *Drosophila* identified two distinct classes of regulatory factors responsible for establishing and maintaining homeotic gene expression patterns. Gap and pair-rule genes encode spatially restricted DNA-interacting factors that establish homeotic gene expression patterns [6-8] by acting through specific *cis* regulatory chromosomal sequences named Maintenance Elements (MEs) [9-14]. However, these genes are transiently expressed during early embryogenesis, and *Trithorax Group (TrxG)* and *Polycomb Group (PcG)* genes are necessary to maintain proper expression throughout the remainder of development. TrxG proteins generally maintain homeobox gene transcription within previously established boundaries, whereas PcG proteins suppress previously silenced homeobox genes [15].

Genetic data in *Drosophila* revealed that the *PcG* genes consist of more than 30-40 members [16] (see table I). The various *PcG* names originate from the ectopic expression of organs called sex combs on the second and third legs of male *PcG* mutants [17]. In recent years, homologues have been found in several species such as *C.elegans, Zebrafish*, *Xenopus*, chicken, mouse, human and plants (see table I). The function of *PcG* genes appears to be well conserved throughout evolution. This is probably best illustrated by the demonstration that *M33*, the mouse homologue of *Drosophila Pc*, can largely rescue the phenotype of *Pc* mutant flies [18]. *Drosophila PcG* genes do not share sequence homology and their classification is based mainly on the presence of evolutionary conserved domains (see table I).

In *Drosophila PcG* mutants, the expression patterns of the *HOM-C* genes are correctly initiated by the transiently expressed gap gene products, but after their disappearance at mid-gestation, *Hox* gene misexpression results in a dose-dependent change in the identity of body segments to that of more posterior ones [19-21]. Similarly, mice mutant for the *Bmi-1, Mel-18, M33, RING1B* and *Mph1/Rae-28 PcG* genes show dose-dependent posterior homeotic transformations that correlate with the anterior shifts in the expression boundaries of overlapping as well as distinct subsets of *Hox* genes [22-26]. For example, in *Bmi-1*$^{-/-}$ mice, the anterior limit of expression of *Hoxc5* is shifted anteriorly by one segment, whereas the expression of this gene is not affected in *Mel-18* null mutants. On the contrary, *Hoxc8* is shifted rostrally in both single mutant mice [22,27]. Interestingly, *Ring1A* mutant mice show anterior homeotic transformations and even though most skeletal defects are posterior in *M33*$^{-/-}$ mice, anteriorisation of cervical vertebrae identities (C2 into C1) have been observed, suggesting an unusual sensitivity to *Ring1A* and *M33* gene dosage in axial skeletal patterning [23,28]. Conversely, transgenic mice overexpressing the *Bmi-1* gene present a dose-dependent anterior skeletal transformation associated with posteriorisation of *Hox* gene expression [29]. Nullizygosity for the *eed*, *Enx-1* and *YY1* alleles result in early embryonic lethality, precluding analyses of any skeletal defects [30-32]. However, highly penetrant posterior homeotic transformations, correlating with rostral shifts in *Hox* gene expression boundaries, were observed in homozygous mice for a hypomorphic allele of *eed* (*eed*$^{1989/1989}$) [32,33]. The presence of several mammalian homologues for each *Drosophila PcG* couterpart (see table I) likely explains the weaker (and less penetrant) phenotypes of mouse *PcG* mutants when compared to fly mutants (functional redundancy). Accordingly, fly *PcG* mutants affect the regulation of all *HOM-C* genes whereas in mice, only subsets of *Hox* genes are ectopically expressed in a given *PcG* mutant [21].

Evidence for strong dosage interactions between *Drosophila* and mammalian *PcG* genes is based on the synergism and increased penetrance of the homeotic defects observed upon combining different *PcG* mutations [34,35]. In most double *PcG* mutant mice (i.e,

Bmi-1 x eed, *Bmi-1 x Mel-18* and *Bmi-1 x M33*), exacerbation of the homeotic phenotype correlates with enhanced shifts in *Hox* expression boundaries in the somatic mesodermal tissue ([36,37] and Schumacher et al., unpublished). For example, *Bmi-1 and Mel-18* double mutants die as early embryos (at ~day 9.5 of gestation) with severely affected *Hox* gene expression boundaries, whereas *Bmi-1*$^{+/-}$ *Mel-18*$^{+/-}$, *Bmi-1*$^{-/-}$ *Mel-18*$^{+/-}$ and *Bmi-1*$^{+/-}$ *Mel-18*$^{-/-}$ mutants display intermediate phenotypes [36]. The demonstrations that double *PcG* mutants display *Hox* gene expression defects that are not observed in the single mutants (eg. *M33*$^{-/-}$ *Bmi-1*$^{+/-}$) also suggests that PcG proteins can operate in parallel/distinct molecular pathways [37].

The skeletal defects observed in *PcG* gain- and loss-of-function studies could be caused by alterations in proliferation rates of bone precursor cells, a process in which *Hox* genes are clearly implicated. Although no loss-of-function mutations in human *PcG* genes have been reported so far, deregulation of homeobox *(Hox)* gene expression has been observed in human congenital skeletal defects such as synpolydactyly [38]. The fact that only subsets of *Hox* genes are affected in *PcG* mutants might explain why only the axial skeleton is affected in mutant embryos, whereas patterning of the limbs appears normal.

Recent observations by the Shearn laboratory, however, revealed that some members of the *PcG* and *TrxG* groups (identified as *Enhancers of Trithorax and Polycomb (ETP)),* exhibit the dual property of maintaining both activated and inactivated states of homeotic gene expression. E(z), although classified as a *Polycomb Group (PcG)* member, was shown to maintain activated *Hox* gene expression *in vivo* [39]. A screen for enhancers of a *TrxG* member mutant, *Ash1,* identified several members of the *PcG* family [40]. Accordingly, evidence indicates that *TrxG* and *PcG* members interact with one another in the establishment of a functional regulatory complex of gene expression. For example, *Drosophila* TrxG and PcG proteins where shown to colocalize at Maintenance Elements (MEs) sites on polytene chromosomes [12,13,41,42] and genetic interaction between the two groups has been reported in mice [43]. Finally, biochemical studies showed that the PRC1 PcG complex (see below) can block chromatin

remodeling by the TrxG-containing SWI/SNF complex, by occluding its access to the nucleosomal template [44,45].

The function of the *PcG/TrxG* memory system is not limited to regulation of *Hox* gene expression. TrxG and PcG proteins bind to more than 80-100 chromosomal sites in addition to the *ANT-C* and *BX-C* complexes [41,46-50] and several nonhomeotic target genes have been identified including the *engrailed* and *even-skipped* segmentation genes [51-54]. In addition to homeotic transformations, mutations in *PcG* and *TrxG* genes were shown to confer pleiotropic phenotypes including: imaginal discs, hemopoietic, cerebellar, cardiac, neural-crest, sex-reversal, smooth muscle atrophy, and growth retardation. In contrast to the ubiquitous expression of the fly genes, the expression levels of mammalian *PcG* genes differ among different tissues and cell types [55-61]. The heterogeneity in PcG complex composition due to tissue- and time-specific *PcG* expression likely contributes to target gene specificity and explains the diversity of *PcG* mutant phenotypes.

Several models have been proposed regarding the mechanism(s) of *PcG*–mediated stable gene repression. These include: (1) the establishment of a repressive chromatin structure (through histone modifications, alteration of nucleosome position/conformation, interaction with chromatin condensation proteins and/or specific targeting of chromatin-remodeling complexes) [62-66] (2) inhibition of chromatin-remodeling machineries [45] (3) inhibition of the transcriptional initiation machinery [67-69] (4) recruitment of target genes to repressive nuclear domains [70] (5) inhibition of enhancer/promoter interactions due to DNA looping induced by the interaction between PcG complexes bound at discrete chromosomal sites [71].

The frequent co-localization of PcG proteins at distinct chromosomal loci on *Drosophila* polytene chromosomes reinforced the notion that PcG proteins act in concert to repress gene expression [12,14,46,50,72,73]. Direct evidence for the existence of large multimeric PcG protein complexes was obtained from immunoprecipitation, yeast-two-hybrid and size-fractionation experiments in flies and mammals [14,45,46,74-77].

At least two distinct types of PcG complexes with different properties/functions can be distinguished both in *Drosophila* and mammals [45,57,58,73,78-82]. The Extra Sex Combs (Esc) and Enhancer of Zeste (E(z)) PcG proteins form the core of a ~600 kDa complex isolated from *Drosophila* embryo extracts [79,83] that includes the Rpd3 histone deacetylase, the PcG repressor Su(z)12 and p55/RbAp48, a histone binding protein also found in the chromatin remodeling and chromatin assembly complexes NURF and CAF1 [73,82,84]. The human counterpart of the *Drosophila* Esc-E(z) complex was recently purified from HeLa cells and shown to contain EED, EZH2, SU(Z)12, RbAp48 and AEBP2, a zinc finger transcriptional repressor [66]. These complexes appear to be involved in making the "epigenetic mark" necessary for the establishment and memory trace of the silent state and will therefore be referred to as "PcGe" (see below). The Vav protein, a pan-hematopoietic rho family GTP/GDP exchange factor (GEF) involved in lymphocyte selection and Antigen (Ag) receptor-mediated proliferation [85-88] might also be included in the mammalian PcGe complex because of its interaction with Enx-1/Ezh2 [89]. Finally, Esc/EED was shown to physically associate with the DNA-interacting protein Pho/YY1, providing further insights into the molecular mechanisms by which the Esc/EED-containing PcGe complexes might be recruited to specific target genes [90,91].

The second type of PcG complexes appears to be required for stable maintenance (PcGm) of gene silencing and is exemplified by Polycomb repressive complex 1 (PRC1), a ~3 MDa complex purified from *Drosophila* embryos. This complex contains the PcG proteins Pc, Ph and Psc at stoichiometric levels, and substoichiometric amounts of Sex comb on midleg (Scm) [45,92]. The demonstration that these proteins do not obligatory colocalize on *Drosophila* polytene chromosomes [14,72] nor always co-immunoprecipitate [93] argues for the presence of multiple distincts PRC1-like complexes [14]. Biochemical studies indicate that the mammalian PcG proteins M33/HPC1, BMI-1, MEL-18, HPH1, HPH2, HPH3, HPC2, HPC3, SCMH1, RING1A and RING1B are constituents of heterogenous multimeric protein complex(es) (~1 MDa) that colocalize at distinct nuclear domains termed PcG bodies [56,70,77,94-98]. The *Drosophila* sequence-specific DNA-binding factors Pipsqueak, Zeste and GAGA

coprecipitate with Pc-containing complexes and mediate their binding *in vitro* to ME elements, suggesting that they may contribute to their recruitment *in vivo* [67,93,99-101]. No mammalian homologues for these proteins have been identified so far, but RYBP (RING1- and YY1-binding protein), which does not seem to be present in *Drosophila*, binds RING1A, RING1B, M33 and YY1, and represents a potential targeting factor [102]. Similarly, E2F6, a weak transcriptional repressor preferentially expressed in human $CD34^+$ bone marrow progenitor cells [103], interacts with Bmi-1 and may be involved in recruiting the complex to relevant target genes, such as $p19^{ARF}$ [104,105]. The PLZF (promyelocytic leukemia zinc finger) protein was shown to mediate transcriptional repression of *HoxD* cluster genes through the recruitment of the Bmi-1-containing PcGm complex [106]. Also associated with mammalian PcGm complexes through direct interactions with the HPC2 and RING1B subunits are the C-terminal binding protein (CtBP) co-repressor [107] and Mel-18 and Bmi-1-like RING finger (MBLR) proteins, respectively [108].

Evolutionary conservation of PcG complexes

The PcGe complex appears very "ancient" in evolution and is well conserved in lower organisms where it fulfills an important role in maintaining gene silencing in the germline. In *C. elegans*, the Esc- and E(z) related proteins MES-6 and MES-2 form a stable complex of approximately 255 kDa [109] which is required for germ-line development [110,111]. The conserved partnership extends to plants *(Arabidopsis)*, where proteins related to Esc, E(z) and Su(z)12 are cohort regulators in repressing seed proliferation prior to fertilization [112]. The apparent absence of core PcGm homologues in worms and plants implies that the Esc-E(z) PcGe complex can repress gene expression independently of the PcGm complex. Further work in these systems will address whether the Esc-E(z) complex-associated histone methyltransferase (HMTase) activity represents an evolutionarily conserved "ancient" mechanism to mark chromatin for heritable repression during development.

Synergy between ESC-E(Z) and PRC1 complexes

Despite biochemical and evolutionary distinctions between the Esc-E(z)/PcGe and PcGm complexes, it is clear that they work together to repress gene expression *in vivo*. As loss-of-function mutations in *Esc*, *E(z)*, *Pc* or *ph* produce severe posteriorization phenotypes on their own, removal of one PcG component from either complex is sufficient to collapse the entire repression system [16]. Accordingly, E(z) function is required for chromosome binding of Pc, Psc and Su(z)2 in *Drosophila* embryos [72,113] and *in vivo* repression by artificially tethered Pc or Esc depends upon endogenous PcG components of both the PcGe and PcGm complexes [91,114-116]. This functional interdependence between the two PcG complexes could be explained by a direct but transient contact between Pc, Esc and E(z) specifically in extracts from preblastoderm (0-3hrs) fly embryos [91] or, alternatively, reflect an important function of E(z) in modifying the template to allow stable binding or action of the PcGm complex (see Fig.1.1). Further work is needed to clearly establish whether PcG-mediated silencing involves transient physical and/or functional interactions between the two PcG complexes (see Section 5).

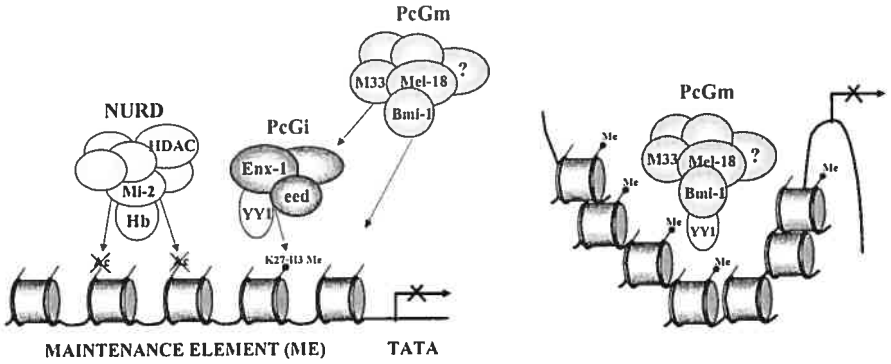

Fig. 1.2 Schematic representation of the functional interaction between the PcGe and PcGm complexes in regulating gene expression through epigenetic modification of chromatin structure. Ac, acetylated group; Me, methylated group; K27-H3, lysine 27 of histone 3.

1.2.2. PcG-mediated regulation of HSC/progenitor self-renewal and proliferation

Hemopoietic stem cells (HSCs) are characterized by their ability to generate, in a controlled fashion, all of the blood cell lineages and maintain their original pool through self-renewal. Prevailing models indicate that HSCs achieve this through a series of binary decisions during which progressively restricted precursors commit to alternative cell fates. These developmental transitions -characterized by the acquisition and loss of expression of lineage-specific genes- correlate with a decrease ability to reverse the lineage choice and terminal differentiation to a specific blood cell type. Although much progress has been made in identifying the cytokines and cell-adhesion molecules controlling HSC lineage decisions/commitment, little is known about the intrinsic genetic/epigenetic mechanisms regulating the self-renewal/proliferation outcomes of the HSCs. Importantly, abnormal regulation of HSC self-renewal and/or proliferation invariably leads to a variety of hemopoietic disorders, ranging from aplastic anemia to leukemia.

Compelling evidence indicates that the *PcG* genes are involved in normal and leukemic hemopoiesis, through epigenetic regulation of HSC/progenitor self-renewal and proliferation. We recently reported that *Bmi-1* expression is restricted to primitive human ($CD34^+$ $CD45^-$ $CD71^-$) and mouse ($Sca\text{-}1^+$ Lin^-) bone marrow cells [59,81]. Nullizygosity for the *Bmi-1* gene in mice leads to severe aplastic anemia due to a progressive impairment of HSC function [25,81,250,251]. Consequently, *Bmi-1* null mutants surviving beyond the first week of birth suffer from pneumonia and infections of the intestinal tract and die within 20 weeks of birth. Bone marrow-derived progenitors lacking *Bmi-1* are severely reduced in numbers and in their proliferative potential [25]. Furthermore, the number and proliferative capacity of primitive myeloid (LTC-IC) and lymphoid (WW-IC) bone marrow progenitors in these mice are severely reduced to 4% and 1% of wild-type levels, respectively [81]. The proliferative defect in progenitors derived from *Bmi-1*$^{-/-}$ E14.5 fetal livers (FLs) is much less pronounced than that observed in the bone marrow, suggesting a progressive impairment of the proliferative potential of hemopoietic cells lacking this gene [25,81,250]. Retroviral expression of

Bmi-1 in *Bmi-1*$^{-/-}$ fetal liver cells completely rescued the absolute numbers of high and low proliferative potential myeloid colony-forming cells (HPP- and LPP-CFC) to wild-type levels, indicating that *Bmi-1* is dispensable for the generation of FL-derived myeloid progenitors, but absolutely essential for their full proliferative activity [250]. Similarly, an experiment performed by our group confirmed the presence of ~normal numbers of long-term repopulating HSCs in *Bmi-1*$^{-/-}$ fetal livers whose detection, at 16 weeks post-transplantation, was strictly dependent on the retroviral expression of *Bmi-1* [250]. Clarke and colleagues recently demonstrated an inability of BM and FL-derived *Bmi-1*$^{-/-}$ cells to contribute to long-term hemopoiesis in reconstitution (FL and BM) as well as competitive (FL) experiments, suggesting a cell autonomous impairment of their self-renewal/proliferation potential [251]. A detailed structure to function study describing the essential domains of *Bmi-1* for its function in FL-derived HSCs is ongoing and should be reported shortly.

The PcGm-containing Mph1/Rae-28 protein also plays a key role in the regulation of fetal liver-derived HSC function. This was first demonstrated by a progressive impairment in the numbers and proliferative potential of E14.5 FL-derived colony-forming-cells (CFCs) in *Mph1/Rae-28* mutant embryos when compared to wild-type littermates. The absolute number and proliferative potential of primitive myeloid long-term-culture–initiating cells (LTC-ICs) and colony-forming units in spleen (CFU-S_{12}) are also severely decreased (up to 20-fold) in *Mph1/Rae-28*$^{-/-}$ fetal livers when compared to controls. Moreover, serial FL cell transplatation experiments performed at limiting dilution (LD) in sublethally irradiated congenic mice demonstrated a 15-20-fold decrease in CRU content/proliferative activity in *Mph1/Rae-28*$^{-/-}$ embryos relative to controls. These studies indicate a crucial role for Mph1/Rae-28 in sustaining the activity of HSCs during fetal hemopoiesis [117] and might explain the severe splenic/thymic hypoplasia and perinatal lethality observed in *Mph1/Rae-28*$^{-/-}$ neonates [24,117].

In contrast to PcGm alleles, heterozygosity for a null allele of the *ectoderm embryonic development* gene (*eed*$^{3354/+}$) in mice causes marked myelo- and lympho-proliferative defects (3-fold increase in primitive (LTC-IC and WW-IC) and 19-fold increase in late

(myeloid and pre-B CFC) bone marrow progenitor cell numbers on average), indicating that the PcGe-containing Eed protein plays a crucial role in the negative regulation of the proliferative capacity of both lymphoid and myeloid progenitor cells. This antiproliferative function of Eed does not appear to be mediated by *Hox* genes or the *INK4a-ARF* tumor suppressor locus, because expression of these genes was not affected in *eed* mutants [81].

The studies described above indicate critical differences in the function of PcG proteins belonging to the PcGe and PcGm complexes and point to an unexpected complexity of PcG function in hemopoiesis. First, Eed (PcGe-containing) and Bmi-1 (PcGm-containing) proteins represent functional antagonists in hemopoietic cell proliferation. Whereas Eed acts as a negative regulator of hemopoietic progenitor cell proliferation, Bmi-1 enhances the proliferative activity of primitive [81] and more mature [25] bone marrow progenitor cells. Intercross experiments between *eed* and *Bmi-1* mutant mice revealed that *Bmi-1* is epistatic to *eed* in the control of primitive bone marrow cell proliferation. However, the genetic interaction between *eed* and *Bmi-1* is cell-type specific, as the presence of one or two mutant alleles of *eed* trans-complements the *Bmi-1* deficiency in pre-B bone marrow cells [81]. Thus, hemopoietic cell proliferation in mice seems to be regulated by an intricate equilibrium between enhancing (Bmi-1-containing) and repressing (Eed-containing) PcG protein complexes.

Several observations also support a role for *PcG* genes in abnormal (leukemic) hemopoiesis through the regulation of HSC self-renewal/proliferation. Hypomorphic alleles of the *multi sex combs (mxc)* gene in *Drosophila* result in premature haemocyte differentiation and tumourous overgrowth of the larval hemopoietic organs [118,119]. Enx-1 directly associates with Vav, the product of a protooncogene that is required for antigen receptor-mediated B- and T-lymphocyte proliferation [87,89]. Overexpression of *Bmi-1* in the lymphocyte compartment in mice (using the *immunoglobin heavy chain* enhancer (*Eu*)) leads to the development of B and T cell lymphoma [120,121]. The human *BMI-1* gene has been located on chromosome 10p13, a region involved in chromosomal translocations in infant leukemias [122], rearranged in adult malignant T

cell lymphomas [123] and amplified in occasional non-Hodgkin's lymphomas (NHLs) [124,125]. In particular, it has been proposed that *BMI-1* gene alterations may contribute to the pathogenesis of malignant lymphomas of the mantle cell type (MCL) [126] and amplification of the *BMI-1* gene has been reported in a specific subset of MCLs with blastoid transformation [124]. The human HPC3 protein interacts with ENL and AF9, two common fusion partners of the human TrxG protein MLL in 11q23 translocations that are associated with highly aggressive acute leukemias in infants, suggesting a direct role for PcG proteins in MLL-associated childhood leukemias [127,128]. Although no direct tumorigenic properties for *EZH2* have been found, its location at 7q35 suggests that it might be involved in 7q35-q36 aberrations in human myeloid leukemia [129]. Finally, *HPH1/Rae-28* is located on chromosome 12p13, a region that is frequently involved in chromosome abnormalities and loss of heterozygosity (LOH) in a variety of malignant hemopoietic diseases, including ALL [130,131].

An emerging concept in the field of cancer biology is that a rare population of "tumor stem cells" exists amongst the heterogeneous group of cells which constitutes a tumor. These "tumor stem cells" have mostly been characterized in human leukemias whose transplantability (self-renewal capacity) depends on the rare "leukemia stem cell" which shares common phenotypical characteristics (e.g., $CD34^{+}CD38^{-}$) with normal hemopoietic stem cells. These biological and phenotypical similarities between normal and leukemic stem cells raise the possibility that the stem cell function (whether normal or neoplastic) is defined by a common set of critical genes. Evidence that *Bmi-1* might be such a common regulator includes: (i) *Bmi-1* expression is restricted to primitive bone marrow cells in both humans and mice [59] but is also expressed in all myeloid leukemias analyzed to date [59,250] and (ii) *Bmi-1* is essential for adult-derived hemopoietic stem cell function (see above). Our recent studies demonstrated that *Bmi-1* is dispensable for the generation of hemopoietic stem cells and for their transformation into leukemic stem cells (L-HSCs). However, *Bmi-1* is essential for the full proliferative activity of both normal and leukemic stem and progenitor cells. In *Bmi-1*$^{-/-}$ leukemias, both groups of cells eventually undergo proliferation arrest and manifest signs of differentiation and apoptosis, leading to transplant failure of the leukemias in syngenic

hosts. Complementation studies indicate that *Bmi-1* can completely rescue the proliferative defect of both normal and leukemic *Bmi-1*$^{-/-}$ stem cells [250]. These findings clearly integrate *Bmi-1* in a growing family of genes (also including *Meis1*, N. Copeland, personal communication) required for fetal hemopoietic stem cell function, but dispensable for their generation. Moreover, they also provided molecular evidence for the emerging concept that the molecular machinery regulating normal hemopoietic stem cell activity is critical for the maintenance of the leukemic phenotype *in vivo*. The apparent difficulties of bypassing the requirement for Bmi-1 in leukemic stem/progenitor cells *in vivo* suggests that adroit molecular targeting of Bmi-1 in leukemic stem/progenitor cells may have potent and specific therapeutic effects.

Although *Hox* genes are best known for their role in the specification of the antero-posterior (A/P) axis, it is now becoming clear that *Hox* genes also play a part in determining cell fate in several other tissues, including blood [132-134]. By analogy to their role in skeletal development, some evidence indicates that *PcG* genes might be involved in hemopoiesis through the regulation of *Hox* gene expression. In sharp contrast to the expression of *HoxA* and *B* cluster genes which is highest in the most primitive bone marrow cells [135], differentiation of primary human bone marrow cells is accompanied by a marked up-regulation in *PcG* gene expression levels including *M33, MEL-18, HPH1* and *ENX-1/EZH2* [59]. Only *BMI-1* exhibits a different pattern of expression with high levels in primitive $CD34^+$ cells and very low levels in mature $CD34^-$ cells (see above) [59]. These data are consistent with a role for PcG proteins in regulating differentiation and/or proliferation of human hemopoietic cells by silencing *Hox* gene expression. Similarly, the *Mel-18, Bmi-1, M33 and Mph1/Rae-28 PcG* genes were shown to be rapidly activated upon antigenic stimulation of lymphoid B cells and are considered as immediate early genes [136]. This contrasts with the ubiquitous expression of the *PcG* genes in *Drosophila* syncytial blastoderms [137] and thus point to an important mechanism for regulating *PcG* gene functions in differentiating bone marrow cells [59]. Interestingly however, no evidence for *Hox* gene misexpression in the hemopoietic tissue of *PcG* mutant mice has been reported yet, even though such a role has been suspected [81,117]. Extensive analyses of *Hox* gene expression levels in highly

purified subpopulations of hemopoietic cells isolated from *PcG* mutant mice is awaited in order to resolve this issue.

1.2.3. Regulation of senescence: the PcG and INK4a/ARF connection

The murine *Bmi-1* gene was first isolated as an oncogene that cooperates with c-myc in the generation of mouse B and T cell lymphomas [120,138,139]. The synergism in lymphomagenesis has been confirmed by the generation of *Bmi-1/c-myc* double transgenic mice that die from massive leukemia as newborns [120]. It was later established that the *INK4a-ARF* tumor suppressor locus is a critical *in vivo* target of Bmi-1 [140] and that the ability of Bmi-1 to collaborate with c-myc in lymphomagenesis relies on the inhibition of c-myc-induced apopotosis through the *INK4a-ARF* pathway [141]. This connection is of considerable interest since this locus encodes the main regulators of the immortalization/senescence checkpoint in rodent and human primary cells (reviewed in [142]). The *INK4a-ARF* pathway functions as part of a fail safe mechanism that is triggered by inappropriate mitogenic signaling, stress invoked by unnatural *in vitro* culture conditions or as primary cells approach their maximum number of cell divisions. The early work of Hayflick demonstrated that primary cells in culture have a finite replicative potential [143]. Once such cell populations have progressed through a certain number of doublings, they stop growing, a process termed senescence [144,145]. In cultures of human fibroblasts, the senescence checkpoint can be circumvented by disabling the Rb and p53 tumor suppressor pathways, thus enabling the cells to continue proliferating for additional generations, until they enter a second state termed crisis. The crisis state is characterized by massive cell death, karyotypic disarray associated with end-to-end fusion of chromosomes, and the occasional emergence of variants (1 in 10^7 cells) that have acquired the ability to multiply without limit, a trait termed immortalization [146]. The *INK4a-ARF*-encoded $p16^{INK4a}$ and $p19^{ARF}$ tumor suppressor proteins are known to impair CDK4 and CDK6-induced phosphorylation of Rb and prevent the functional inactivation of p53 by binding to Mdm2, respectively [147-151]. In absence of *Bmi-1*, primary mouse embryonic fibroblasts (MEFs) are impaired in progression into the S phase of the cell cycle and

undergo premature senescence [140]. In these fibroblasts and in *Bmi-1*-deficient lymphocytes, the expression of the tumor suppressors $p16^{INK4a}$ and $p19^{ARF}$ is raised markedly. Conversely, overexpression of *Bmi-1* in MEFs was found to down-regulate the expression of $p16^{INK4a}$ and $p19^{ARF}$, delay replicative senescence and facilitate immortalisation [140]. Similarly, overexpression of *Bmi-1* in primary human fibroblasts was shown to extend their replicative life-span (via its RING finger and helix-turn-helix domains) by suppressing the $p16^{INK4a}$-dependent senescence pathway [152]. Importantly, epigenetic and genetic abrogation of CDKN2A ($p16^{INK4a}$) and CDKN2B ($p19^{ARF}$) are common lesions associated with poor prognosis in several human leukemias and mouse leukemia models [153-157].

Recent evidence indicated that HSC homeostasis/quiescence is maintained by a dominant antiproliferative tone mediated by critical molecular checkpoints in the cell cycle machinery and excessive HSC cycling may lead to stem cell exhaustion [158,159]. The deleterious effects of excessive HSC cycling may result from accelerating the mechanisms that cause normal aging in a manner analogous to the Hayflick ceiling identified for proliferation of untransformed fibroblasts (MEFs) [143]. Although not investigated in HSCs, inactivation of the *INK4a-ARF* pathway dramatically reduced the hemopoietic cell proliferation defects of *Bmi-1* null mutants, indicating that *INK4a/ARF* is a critical *in vivo* downstream target for Bmi-1 in the regulation of hemopoietic cell proliferation [140]. This change in defects was evident in $CD4^+CD8^+$ double positive T cells as well as in mature $B220^+$ $sIgM^+$ B cells [140]. In *Bmi-1*$^{-/-}$ E14.5 total FL cells, $p19^{ARF}$ expression was undetectable but $p16^{INK4a}$ levels were clearly elevated (Clarke et al., submitted). Interestingly, $p16^{INK4a}$ and $p19^{ARF}$ levels appeared normal in total E14.5 fetal liver cells isolated from *Mph1/Rae-28* null mice when compared to that of control littermates [117]. These studies underscore the importance to investigate a putative role for the *INK4a-ARF* pathway (and other CKIs) in the proliferative impairment of the HSCs deficient for the *Bmi-1 and Mph1-Rae28* gene products [81,117,250,251]. Importantly, the aging of primary human fibroblasts is accompanied by decreased *Bmi-1* expression (both at the RNA and protein level) [152,160] and accumulation of $p16^{INK4a}$ and $p19^{ARF}$ [161]. It will thus be of interest to verify whether the Bmi-1-containing

PcGm complex is part of an evolutionary conserved "clock" mechanism that counts primitive HSC divisions.

The observation that *Mel-18*$^{-/-}$ MEFs also express elevated levels of *p16*^{INK4a} raises the possibility that the *INK4a-ARF* locus represents a more general target of *PcG* function. Importantly however, the *INK4a-ARF* locus does not account for all of the hemopoietic and neurological defects observed in *Bmi-1* null mutants [140] and the homeotic phenotype of *Bmi-1* mutants remains unaffected by genetic deletion of the *INK4a-ARF* pathway, indicating that additional cell-context-dependent targets of the Bmi-1-containing PcGm complex remain to be identified. Interestingly, expression of the T-box protein *TBX-2* can bypass the senescence arrest of *Bmi-1*$^{-/-}$ primary fibroblasts (MEFs) by down-regulating p19^{ARF} expression levels [162] and overexpression of the anti-apoptotic *Bcl-2* oncogene in *Bmi-1* deficient mice (using a Eu-*bcl2*-36SV transgene) partially rescued the hemopoietic defects, indicating that increased apoptosis may contribute to the *Bmi-1*$^{-/-}$ phenotype [141].

1.2.4. *PcG*-mediated repression: a link with the basal transcription and chromatin assembly/condensation at mitosis?

Recent evidence suggests that *PcG*-mediated gene silencing is linked to general chromatin condensation during cell cycle progression and that perturbation of this regulatory system leads to genome instability and cancer. Mutations in the *Drosophila centrosomal and chromosomal factor (Ccf)* gene, which encode a protein required for normal chromosome condensation during mitosis, enhance *Pc, ph,* and *E(z)* phenotypes and Ccf colocalizes to some Psc binding sites on polytene chromosomes [163]. Similarly, it has been demonstrated that ME function depends partly on the Barren and Topoisomerase II proteins required for proper chromatin condensation at mitosis [62]. Barren and Topo II colocalize with PcG proteins at MEs in the *bithorax* complex and specifically immunoprecipitate with Ph. Mutations in *barren* lead to de-repression of the *Fab-7* ME *in vivo* and enhancement of a homeotic phenotype [62]. Although the repression mechanism is still not clear, a role for condensins in interphase gene

repression has ample precedent in *C. elegans* dosage compensation (reviewed in [164]). Intriguingly, *ph* and *barr* mutant embryos display similar chromosome segregation defects, suggesting that the Ph-Barren interaction somehow contributes to genome stability [62,165]. Determining if this level of epigenetic regulation is misregulated in leukemia and solid cancer will be a priority of future work.

Recent studies also suggested that *PcG*-mediated silencing might involve inhibition of general transcription factor (GTF)-mediated transcriptional activation. PRC1 was found to associate with stoichiometric amounts of 6 TBP (TATA-binding-protein)- associated factors (dTAFs) that are components of the general transcription factor TFIID in fly embryo extracts [67]. Analysis of cross-linked chromatin detects colocalization of Pc and Ph with TBP, TFIIB and TFIIF on *Hox* promoters in cultured fly cells [69]. Two of the six TAFs found in PRC1 are histone-fold proteins that could also assist PRC1-DNA contact. These results suggest that maintenance of the silenced state by PcGm complexes might involve inhibition of GTF-mediated transcriptional activation.

Similarly, *Xenopus* Eed (Xeed) directly interacts with $TAF_{II}32$ [68], a component of the general transcription factor TFIID complex [166]. It has been proposed that this interaction might impair gene activation by inhibiting the assembly of the transcription initiation complex at promoters or occluding its interaction with co-activator proteins [68]. Intriguinly, a region of MLL (the human homologue of *Drosophila* Trithorax, also referred to as ALL-1 and HRX) that is required for its transcriptional activation function, binds specifically to human $TAF_{II}31$ [167] and the co-activator CBP [168,169]. Therefore, Esc/EED may antagonize MLL function by inhibiting its association with TAF_s and/or CBP proteins bound at promoters. Validation of these possibilities requires further investigation.

Relevance to hemopoiesis and cancer

Some evidence indicates that *PcG* genes are involved in leukemogenesis and the development of solid tumors through an epigenetic silencing mechanism involving interactions with the general transcription machinery and/or co-activator/co-repressor

molecules. First, dE(z) physically interacts with the dSAP18 protein, a component of the Sin3-HDAC co-repressor complex [170]. Furthermore, mice with monoallelic inactivation of the co-activator *CBP* gene develop highly penetrant, multi-lineage defects in hemopoietic differentiation and, with advancing age, an increase incidence of hematologic malignancies. The latter are characterized, at least in some cases, by loss of heterozygosity (LOH) at the *CBP* locus, suggesting that CBP has tumor-suppressing activity [171]. The association of dCBP with TrxG proteins has been implicated in the maintenance of *Hox* gene expression patterns [172,173]. Chromatin-related properties of CBP fused by the t(11;16)(q23;p13) translocation to MLL generate a myelodysplastic-like syndrome that evolves into myeloid leukemia in mice [174] and is associated with cases of therapy-related acute leukemia and myelodysplasia in humans [175-178].

Overexpression of the SET domain-containing EZH2 PcGe-containing protein was recently demonstrated in hormone-refractory, metastatic prostate cancer and EZH2 protein levels correlated with adverse prognosis. Ectopic expression of *EZH2* in prostate cells induces transcriptional repression of a large cohort of genes, (whereas no genes appeared to be activated with the exception of *EZH2* itself), consistent with a functional role for EZH2 as a general transcriptional repressor in tumor cells. Gene silencing by EZH2 seems to involve an epigenetic mechanism since it requires the HMTase SET domain and is attenuated by inhibiting histone deacetylase activity. These observations suggest that epigenetic gene silencing caused by dysregulation of *EZH2* expression may be associated with prostate cancer progression [179].

The myeloid- and lympho-proliferative defects observed in *eed* mutant animals (see above) together with the increased susceptibility of these mice to develop hemopoietic tumors [81] would suggest that the Eed PcGe-containing protein complex has tumor suppressive activity. Accordingly, it has been demonstrated that heterozygous and homozygous mice for a viable hypomorphic $eed^{1989/+}$ allele have an increased incidence and decreased latency of N-methyl-N-nitrosourea (MNU) [180] and γ-rays (J.L and G.S., unpublished results)-induced B and T cell lymphoma when compared to wild-type littermates. The requirement for exposure to genotoxic agents and the relatively long

latency before tumor development indicate that additional genetic events are required for the "full transformation" of lymphoid cells lacking a functional *eed* gene. Because point mutations at the *eed* gene locus that account for the hypomorphic *(eed^{1989})* and null *(eed^{3354})* alleles were shown to disrupt its interaction with Enx, it will be interesting to determine whether *Enx-1/EZH2* and/or *Enx-2/EZH1* mutant animals also develop tumors, thereby ascribing the tumor-suppressor function to the Eed-Enx PcGe complex. Furthermore, the recent finding that the Enx-1/EZH2-histone methyltransferase (HMTase) activity towards lysine 27 (Lys27) of histone 3 (H3) is Esc/Eed-dependent [66] suggests epigenetic chromatin modification as the underlying mechanism responsible for increased tumor susceptibility in *eed* mutant animals (Lessard et al., ongoing studies).

1.2.5. Epigenetic modifications of histone tails by PcG proteins convey stable inheritance of the silenced state: a role in hemopoiesis?

Modification of the histone tails by acetylation, phosphorylation and methylation is believed to generate a histone code, which may regulate gene expression by controlling the organization of the chromatin fiber into higher order structures [181,182]. Histones codes that are frequently found in inactive and silent genetic loci- and that are enriched in heterochromatin- include histone deacetylation, acetylation on lysine 12 (Lys12) of histone H4 and methylation on Lys9 of H3. Histone codes that correlate with gene activity include H3 acetylation on Lys9 and Lys14, H3 (poly)-methylation on Lys4, H4 acetylation on Lys5 and H4 methylation on arginine 3 (Arg3) [182]. There are strong indications that PcG complexes suppress gene activity through such epigenetic modifications. The apparent dissociation of PcG complexes from chromatin during mitosis implies that an epigenetic mechanism is used to "mark" silent loci for reformation of the appropriate PcG complexes after mitosis [183-185]. The demonstration that ectopic expression of homeotic genes caused by removal of Psc and its homologue Su(z)2 can be reversed if the depleted protein is supplied within a few cell generations, provides further evidence for an heritable mark for PcG-mediated

repression that can persist, at least for a few generations, on genes that are de-repressed [186].

Methylation

The SET domain is an evolutionarily conserved sequence motif initially identified in the *Drosophila* position effect variegation (PEV) suppressor Su(var)3-9 [187] (not a *PcG* member), the PcG protein E(z) [188] and the TrxG protein Trithorax [189]. In eukaryotic cells, the histone methylase SuV39H1 and the methyl-lysine binding protein HP1 functionally interact to repress transcription at heterochromatic sites. SuV39H1 methylates Lys9 of histone H3 (through its SET domain and its two adjacent cysteine-rich regions) [190] creating a binding site for the chromodomain of HP1 [191,192]. Although HP1 is not a *PcG* member, the similarity of its chromodomain with that of Polycomb (Pc) itself originally led to the proposal that PcG proteins might alter chromatin structure at target loci [193].

In addition to heterochromatin silencing, recent studies indicate that HP1 is involved in silencing of specific genes in euchromatin. It was found that Rb (through its pocket domain) associates with SuV39H1 and HP1 *in vivo* to regulate expression of the *cyclin E* gene [194]. The observation that histone H3 methylation and HP1 binding is significantly reduced at the *cyclin E* promoter in the absence of Rb suggests that deacetylation of histone H3 at Lys9 by Rb-associated deacetylase activity (HDAC) is required as a preceding step to SuV39H1-mediated methylation at euchromatic sites. Accordingly, methylation by SuV39H1 cannot take place on an already acetylated lysine [190]. It will be interesting to unravel the mechanism(s) by which this Rb-mediated repressive effect at the *cyclin E* locus can be re-established at every cell cycle event. Of interest, *HP1* genes (such as $HP1^{HS\alpha}$, $HP1^{HS\beta}$ and $HP1^{HS\gamma}$), like most *PcG* genes, are also preferentially expressed in mature subpopulations of human bone marrow cells, suggesting that they might be involved in epigenetic regulation of progenitor cell proliferation/differentiation [59].

Furthermore, evidence suggests that histone H3-K9 methylation may also contribute to chromosomal targeting of components of the PcGm complex. HPC2, Bmi-1 and M31/HP1$^{HS\beta}$ (but not M33/mPc1) were shown to physically associate with SuV39H1 *in vivo*. Overexpression *SuV39H1* (but not an enzymatically inactive mutant) induces selective nuclear localization of HPC2, Bmi-1, RING1 and HPH1/2 (but not EED/EZH2 and HPC1) PcG proteins to large pericentromeric heterochromatin domains at position 1q12 on human chromosome 1 that show increased H3-K9 methylation [195]. Interestingly, these domains are also observed in the osteosarcoma cell line U2OS and are remarkably similar to the PcG domains observed in HT-1080 cells [57,70,94,95]. These observations may suggest a role for the SuV39H1 HMTase and histone H3-K9 methylation in the targeting of human PcGm proteins to modified chromatin structures [195].

The presence of a SET domain in the *Drosophila* E(z) and its human counterpart EZH2 [196] raised the possibility that the Esc-E(z) and EED/EZH2 PcGe complexes might also have histone methyltransferase (HMTase) activity. A *Drosophila* Esc-E(z) complex containing the histone binding proteins RbAp46/RbAp48 and the PcG repressor Su(z)12 was recently reported to display HMTase activity towards Lys27 (K27) and Lys9 (K9) of histone H3 [78]. Mutations in the SET domain of E(Z) disrupted the HMTase activity of the complex, leading to *Hox* gene misexpression *in vivo*. Importantly, methylation on H3-K27 facilitated the binding of Polycomb (Pc), a component of the PRC1 (PcGm) complex, through its chromodomain [66]. Depletion of Esc (using the RNA interference (RNAi) system) resulted in greatly reduced ME binding by E(z), loss of H3-K27 methylation, and concomitant loss of Pc binding [66]. Recruitment of PRC1, in turn, is known to prevent the access of nucleosome remodeling factors, such as SWI/SNF [44,45], leading to the formation of a repressive chromatin state. A second *Drosophila* Esc-E(z) complex containing the same subunits, in addition to the histone deacetylase Rpd3 protein, was also purified by Pirotta and colleagues and shown to methylate H3-K27 as well as trimethylate H3-K9. Importantly, the methyl H3-K27 moiety was shown to make the major contribution to Pc affinity for histone H3. Since the MTase activity of recombinant E(Z) was strictly dependent on the presence of the Esc and RbAp48

subunits *in vitro*, these two WD40 domain-containing proteins likely mediate the interaction with histone H3, while the E(z) SET domain is responsible for the catalytic activity [84]. Similarly, a human EED/EZH2 complex was recently purified from HeLa cells and shown to specifically methylate nucleosomal histone H3-K27 [66]. A recent report presented evidence that Ezh2-mediated methylation of H3-K27 is required for normal *Igh* rearrangement in pro-B cells and thus terminal B cell differentiation [197]. Although the K27 moiety of histone H3 appears to be the major site of methylation by E(z) [66,82,84], some studies also reported E(z)-mediated H3-K9 methylation [78,84]. One possibility is that methylation of H3-K9 by E(z) is nonspecific due to relaxation of substrate specificity under *in vitro* experimental conditions. Alternatively, E(z)-interacting factors and/or specific histone modifications within the nucleosome (specific histone code) might modulate the affinity of E(z) towards either residue. Nevertheless, these observations identify E(Z) and EZH2 as PcG proteins with HMTase activity and clearly implicate E(Z)\EZH2 methylation of histone H3 in *PcG* silencing.

Although the studies described above indicate that methylation of H3-K9 and K27 functions as a repressive mark, not all lysine methylation appears to be a signal for repression of transcription. It was shown recently that methylation of histones H3 (Lys4 and Lys9) and H4 (Lys20) by the *Drosophila* epigenetic activator Ash1 serves as a binding surface for a chromatin-remodeling complex containing the epigenetic activator Brahma (Brm) [198]. Another study reported that the *S. cerevisiae* Set1 protein catalyses di- and tri- methylation of H3-K4 and activates gene expression [199]. Similarly, *Drosophila* Trx and its mammalian counterpart MLL/ALL-1/HRX were shown to possess HMTase activity toward H3-K4 via their SET domain [84], leading to transcriptional activation at *Hox* gene promoters [200,201]. This antagonism between H3-K9 (negative) and H3-K4 (positive) HMTases on chromatin modulation is beeing unraveled at the biochemical level: whereas H3-K9 methylation generates a binding affinity for "repressive" HP1 proteins (see above), H3-K4 methylation prevents association of the negatively acting nucleosome remodeling and histone deacetylation (NuRD) complex [202,203]. Furthermore, H3-K4 methylation is known to impair H3-K9 substrate recognition by the "repressive" Suv39h1 HMTase [202]. It is thus likely

that PcG and TrxG complexes containing the SET-domain proteins E(z), ASH-1 and MLL/ALL-1/HRX use the methylation H3-K9/H3-K4 antagonism to transduce their opposing epigenetic signals at target loci during embryonic development.

Histone deacetylation

Besides histone methylation, genetic and biochemical tests associate histone deacetylation to *PcG*-mediated repression [63,73,92] and hyperacetylation to *TrxG*-dependent active states [173,204]. First, some evidence link the *Drosophila* Esc-E(z) and mammalian EED/EZH2 complexes to histone deacetylases (HDACs) [63,73,78,84,170]. However, the data gathered to date suggests that the interaction with HDACs may be transient or that biochemically distincts ESC/E(Z) complexes may exist [66,78,82]. Second, *Drosophila* dMi-2 (a Hunchback-(Hb) interacting protein) genetically interacts with *PcG* members in enhancing homeotic transformations [65]. Similarly, mammalian Mi-2 and Hunchback-like proteins are part of a complex with histone H3 and H4 deacetylase and ATP-dependent nucleosome remodeling activities (NuRD) [205-207]. As direct evidence for a physical interaction between Mi-2 and PcG proteins is still lacking, it has been proposed that the NuRD complex may induce local chromatin change (such as histone deacetylation) that allow and facilitate the recruitment of PcG proteins. Third, current evidence for the involvement of histone deacetylation in *PcG* silencing *in vivo* came from the demonstration that HDAC1 is recruited to chromosome sites that harbor ectopic MEs [92]. HDAC1 was shown to cofractionate with the *Drosophila* PRC1 complex [67,92], although further studies are needed to address whether the purified complex truly possesses histone deacetylase activity [67,92]. Interestingly, although *Hox* repression does not appear compromised by *HDAC1* mutations alone, enhanced homeotic phenotypes were observed in *HDAC1/PcG* double mutants [92]. Further evidence of a role for HDACs in PcG function *in vivo* arises from studies of mitotic inheritance of the *FAB-7* ME showing that its activation, by transient expression of GAL4 early in embryogenesis, is accompanied by an increase in histone H4 acetylation [204].

We conclude that a combination of histone marks could be a major factor in the establishment of stable patterns of homeotic gene expression and constitute the molecular basis of the PcG/TrxG cellular memory system. Moreover, as histone modifications can be synergistic or antagonistic to each other, it will be important to test for regulatory interactions, or crosstalk, among the various tail modifications (reviewed in [208]). For example, phosphorylation of serine (Ser) 10 on histone H3 is synergistically coupled to the acetylation of Lys9 and/or Lys14 but antagonizes the methylation of Lys9 on the same histone [209]. Likewise, interplay between phospho-H3-S28 and methyl-H3-K27 could resemble the inhibitory crosstalk between phospho-H3-S10 and methyl-H3-K9 [190]. In this view, kinases and phosphatases would be expected to provide regulatory imputs to the PcG/TrxG system. To date, no histone demethylase has been identified suggesting that histone methylation has a more long-lasting impact on the chromatin structure than acetylation [210]. Interestingly, H3-K9 methylation has been shown to trigger DNA CpG methylation in lower organisms [211,212]. This is consistent with other "chromatin-driven" models of gene regulation where plant mutants for the Swi/Snf-like component *ddm1* [213] or the *E(z)* homologue *curly leaf* [214] were shown to affect DNA CpG methylation. It will also be important to determine whether additional PcG/TrxG proteins possess and/or interact with histone modifying activities and to define the histone modification states on *PcG*-repressed and *TrxG*-activated genes *in vivo*. Finally, determining if this level of epigenetic regulation is misregulated in cancer will be a priority of future work [215]. Unlike mutagenic events, epigenetic events in cancer can be reversed to restore the function of key control pathways in malignant and pre-malignant cells.

1.2.6. Role of Eed/Enx1 in paternal X chromosome inactivation in trophoblast stem cells: an epigenetic phenomenon

In mammals, dosage compensation of X-linked genes is achieved by the transcriptional silencing of one X chromosome in the female [216]. This process, called X inactivation, is usually random in the embryo proper. In marsupials and the extra-embryonic region of the mouse, however, X inactivation is imprinted: the paternal X chromosome is

preferentially inactivated whereas the maternal X is always active. Having more than one active X chromosome is deleterious to extra-embryonic development in the mouse [217]. The *Eed* null mutation was shown to affect the stable maintenance (but not initiation) of imprinted paternal X inactivation in extra-embryonic tissues, resulting in two active X chromosomes in females [218]. A second study showed that Eed and Enx1 are directly localized to the inactive X chromosome in XX trophoblast stem (TS) cells and that this association of Eed/Enx1 is mitotically stable [219]. Several epigenetic modifications are associated with the inactivation of the X chromosome, including coating by the cis-acting Xist mRNA, late replication, histone hypoacetylation and methylation of CpG residues in the promoter regions of inactivated genes [216,220]. Based on the recent finding that Eed interacts with histone deacetylases, the authors propose a model in which the early Xist-induced repressed state of genes on the inactive X chromosome in the extra-embryonic tissues provides a signal for the local assembly of the Eed/HDAC complex, causing deacetylation and the formation of higher chromatin structure to secure gene silencing. Once established, Xist is no longer required for maintaining the repressed state [221] and these complexes can be self-propagated through the cell cycle to maintain long-term inactivation of the inactive X chromosome [222]. Recent evidence has shown that H3-K9 methylation occurs rapidly at the onset of random X inactivation, although the enzyme responsible for this activity is uncharacterized [223-226]. It will be important to verify whether the HMTase activity of EZH2 is responsible for paternal X chromosome inactivation in trophectoderm cells. Moreover, a second murine K9-specific HMT, termed SuV39H2 (not a *PcG* gene), with 59% identity to the murine SuV39H1 was recently identified [227]. Although *Suv39h1* and *Suv39h2* display overlapping expression profiles during mouse embryogenesis, the latter is uniquely expressed in the testis of adult mice. Because SuV39H2 specifically accumulates on the sex chromosomes (XY body) which undergo transcriptional silencing during the first meiotic prophase, the HMT activity of SuV39H2 was proposed to be involved in organizing meiotic heterochromatin and genomic imprinting of the male germ line [227]. In this regard, it would be interesting to investigate whether SuV39H2 is involved in paternal X chromosome inactivation in trophoblast stem cells.

1.2.7. *PcG* genes in regulation of lymphoid cell proliferation and differentiation

In peripheral lymphoid organs such as lymph nodes and tonsils, antigen (Ag)- specific immune responses are generated by a highly dynamic process of lymphoid B cell differentiation and proliferation. Germinal centers (GCs) form after the activation of naïve B cells by antigens (Ag) and serve to amplify the number of Ag-specific B cells. As they consist of B cells at various stages of differentiation and proliferation, GCs represent a unique system to study the expression of *PcG* genes during Ag-specific B cell responses. GCs consist of a follicle containing a dark zone (with centroblasts and centrocytes) and a light zone (that contains mainly centrocytes and a follicular network of Ag-presenting dendritic cells) that is surrounded by a mantle zone (MZ) of resting naïve B cells. During the GC reaction, founder Ag-primed B cells from the mantle zone enter the follicle where they begin to divide rapidly to form clonally related Ig^- centroblasts. These cells alter their Ag-receptor specificity by somatic hypermutation and ultimately give rise to resting Ig^+ centrocytes that are subjected to selection. Meijer and colleagues recently demonstrated that expression of the two PcG complexes, reflected by detection of BMI-1/RING1 (PcGm), and EZH2/EED (PcGe), is mutually exclusive at different B cell differentiation stages in germinal centers (GCs) [60]. Resting mantle cells and follicular centrocytes preferentially express the PcGm-containing BMI-1 and RING proteins. By contrast, rapidly dividing centroblasts are BMI-1 and RING negative and express the PcGe complex-containing EZH2 and EED proteins. Expression of EZH2 in proliferating centroblasts coincides with the detection of Mib-1/Ki-67, a marker for proliferation. By contrast, resting $BMI\text{-}1^+$ mantle cells and centrocytes are $Mib\text{-}1^-$. Together, these studies revealed that the transition of resting mantle B cells to rapidly dividing $Mib\text{-}1^+$ follicular centroblasts coincides with loss of BMI-1 and RING1 Pc-Gm protein detection and appearance of ENX and EED PcGe protein expression. By contrast, differentiation of centroblasts into resting centrocytes correlates with reappearance of BMI-1/RING1 and loss of ENX/EED and Mib-1 expression. These results show that expression of *PcG* genes is strictly regulated during follicular B cell development and suggest a distinct role for the two types of PcG protein complexes in the GC cell reaction.

Similar to follicular B cells, Meijer et al recently demonstrated that expression of BMI-1 and EZH2 in mature T cells is mutually exclusive. The majority of resting Mib-1$^-$ mature peripheral T cells expresses BMI-1, whereas dividing Mib-1$^+$ T blasts are EZH2$^+$. The absence of BMI-1 in cycling cells is probably related to the observation that chromatin association of the BMI-1-containing PcGm complex is regulated through the cell cycle [184]. However, the mutually exclusive expression pattern of BMI-1 and EZH2 in peripheral mature T cells is not immediately established in differentiating T cell precursors. Subcapsular immature double-negative (DN) (CD4$^-$ CD8$^-$) T cells in the thymus only express BMI-1 and are Mib-1$^-$, or coexpress BMI-1 and EZH2 and are Mib-1$^+$. Their descendants, the double-positive (CD4$^+$ CD8$^+$) cortical thymocytes, express only EZH2 and are also Mib-1$^+$. Maturation of DP cortical thymocytes to SP (CD4$^+$ CD8$^-$ or CD4$^-$ CD8$^+$) medullar thymocytes correlates with decreased detectability of EZH2 and continued relative absence of BMI-1. Together, these data show that BMI-1 and EZH2 expression in mature peripheral T cells is mutually exclusive and linked to proliferation status [61]. This may suggest that normal regulation of lymphocytic cell division depends on a balance between the BMI-1- and EZH2-containing PcG complexes. Given the well-established role of PcG proteins in regulating gene expression and cell fate, these mutually exclusive patterns potentially reflect stabilization of cell type-specific gene expression and irreversibility of lineage choice.

Meijer and colleagues recently reported coexpression of BMI-1, EZH2 and the proliferative marker Mib-1/Ki-67 in Reed-Sternberg cells of Hodgkin's disease (HRS). As the expression of these two PcG proteins is mutually exclusive at different stages of B cell differentiation in germinal centers (GCs) from which the HRS cells originate (see above), these observations suggest that Hodgkin's disease is associated with the coexpression of BMI-1 and EZH2 [228]. Similarly, coexpression of BMI-1 and EZH2 is associated with enhanced proliferation and degree of malignancy in B-cell non-Hodgkin lymphoma (i.e., small lymphocytic lymphoma, follicular lymphoma, large B-cell lymphoma, mantle cell lymphoma, and Burkitt lymphoma). In contrast to the mutually exclusive pattern of BMI-1 and EZH2 in reactive follicles, the neoplastic cells in B-

NHLs of intermediate- and high-grade malignancy are Mib-1^+ and show strong coexpression of Bmi-1 and EZH2. Large Mib-1^+ neoplastic cells in low-grade B-NHL show weak coexpression of EZH2 and BMI-1. By contrast, small neoplastic cells in low-grade B-NHL show reduced BMI-1 expression in the absence of EZH2 or Mib-1. Together, these findings suggest that the co-expression of BMI-1 and EZH2 in B lymphocytes is an early event in the development of Hodgkin and non-Hodgkin lymphoma in humans.

Similarly to *Bmi-1* mutants, mice lacking the PcGm-containing Mel-18 protein die at about 4 weeks of age and present splenic and thymic hypoplasia that coincide with severe combined immunodeficiency [22,229]. Nakayama and coll. recently demonstrated decreased antigen and bacterial-induced Th2-dependent immune responses in *Mel-18* deficient mice, suggesting a crucial role for Mel-18 in the Th2 cell differentiation process [230]. In *Mel-18*$^{-/-}$ mature peripheral T cells, the production of the Th2 cytokines IL-4, IL-5 and IL-13 is significantly reduced, which correlates with decreased levels in demethylation of the IL-4 gene and reduced GATA3 induction [230]. Although anti-TCR/CD3-induced proliferative responses appear normal in *Mel-18* null mutants [230], a study recently described Mel-18 as a negative regulator of B cell receptor (BCR)- induced proliferation of mature B cells through the down-regulation a c-myc/cdc25 cascade [231]. This phenotype was rescued in *Mel-18/c-myc* double transgenic mice, indicating that c-Myc locates downstream of Mel-18 in the pathway. In *Mel-18* transgenic mice, the impaired B cell proliferative response observed upon BCR stimulation correlates with downregulation of cyclins D2 and E; CK4, -6 and-7; and CDC25A protein levels and hypophosphorylation of the retinoblastoma (Rb) protein. In contrast, the upregulation of c-Myc, CDC25 and CDC2/CDK2 kinase activities in *mel-18* deficient mature B cells results in acceleration of proliferation, even without any stimulation [231]. These results contrast with previous studies showing an impaired mitotic response of *Mel-18*$^{-/-}$ immature B and T cells upon IL-7 stimulation [229] and suggest that Mel-18 differentially regulates distinct signal transduction pathways involved in controlling lymphocyte proliferation. In this view, Mel-18 would function as a positive regulator of proliferation mediated by cytokine receptors (IL-7R) and their

downstream molecules such as JAK3/STAT5. However, Mel-18 would negatively regulate proliferation triggered by antigen receptors (BCR/TCR) and their downstream molecules such as lyn, fyn, lck/ras, MAPK/Rel and NF-kB. In conclusion, the impaired Th2 cell differentiation [230] and the BCR- and IL-7-dependent B and T cell proliferation defects [229] observed in *Mel-18* deficient mice suggest a clear involvement for the Mel-18 gene product in critical developmental aspects of the immune system. However, further studies are clearly needed to unravel the precise molecular mechanism(s) by which Mel-18 controls lymphocyte proliferation.

1.2.8. Deregulated expression of *PcG* genes in solid tumors

SCMH1, the human homologue of *Drosophila* sex combs on midleg (scm) that shares domains with the tumor suppressor lethal(3)malignant brain tumor (l(3)mbt) protein, maps to chromosome 1p34, a region of LOH in cases of well-differentiated gastric cancer and colon cancer [97,232,233]. *BMI-1* overexpression was recently reported in several cases of human high-grade osteosarcomas and 58% of resectable non-small cell lung cancer (NSCLC) [234]. Although *BMI-1* overexpression (and not amplification) in NSCLC could not be associated with tumor characteristics and patient outcomes, a significant inverse correlation between BMI-1 and INK4a expression levels was found. Several breast cancer cell lines as well as immortalized mammary epithelial cells (MECs) were also shown to overexpress *BMI-1* [160]. DNA amplifications and gains of the region encompassing the human *BMI-1* gene locus (10p13) have also been observed in several cases of head and neck carcinomas and other solid tumors [125]. The synovial-sarcoma-specific chimaeric protein SYT-SSX, the product of the chromosomal translocation t(X;18)(p11.2;q11.2), was shown to co-localize with the human PcG proteins BMI-1 and RING1 at specific nuclear domains, suggesting a possible involvement of *PcG* function in synovial cancer [235].

Several observations support a role for Mel-18 as having tumor suppressive activity. Diminution of the Mel-18 protein in NIH 3T3 fibroblasts by expression of antisense RNA leads to the acquisition of tumorigenic activity in nude mice [236]. Interestingly,

the human *MEL-18* gene is located at 17q, a region with putative candidate tumor suppressor genes for breast cancer. Mutations in the *MEL-18* gene are very rare in human sporadic and familial breast cancers. However, decreased *MEL-18* expression has been observed in several human breast cancer cell lines, supporting a role for *MEL-18* haploinsufficiency in breast carcinogenesis. Accordingly, mice haplo-insufficient for *mel-18* are susceptible for the development of mammary gland tumors [237].

Overexpression of *HPC2* (encoding a protein belonging to the PcGm complex) in mouse mammary epithelial (MEC) and human cell lines (U2OS and C57MG) caused decrease c-myc expression whereas interference with HPC2 function (using a dominant-negative HPC2 mutant lacking the conserved C-terminal transcriptional repression domain) in these cells resulted in enhanced expression of c-myc and c-fos and cellular transformation. Similarly, the overexpression of *RING1A* in these cells (encoding another PcGm-containing protein), caused anchorage-independent growth, cellular transformation and metastatic activity in nude mice [76]. This was accompanied by strong enhancement of the expression levels of the oncogenes c-jun and c-fos but not of c-myc. Together, these results argue that HPC2 acts as a tumor suppressor gene whereas RING1 behaves as an oncogene. How these observations can be mechanistically explained at the molecular level is unclear and the *in vivo* relevance of these findings remains to be shown.

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Table I

Polycomb (PcG) and Enhancers of Trithorax and Polycomb (ETP) Groups of Genes

Drosophila	**Mouse (Human)**	**Domain(s); Complex**
***Polycomb Group (PcG)* Genes**		
Polycomb (Pc) [193]	*M33(M33/HPC1), mPc2(HPC2), (HPC3)*	Carboxy-terminal domain, chromodomain; PRC1
Polyhomeotic (ph) [238]	*Mph1/rae28(HPH1), Mph2(HPH2), (HPH3)*	SPM domain*, zinc finger; PRC1
dRING	*Ring/Ring1a (RING1) Ding/Ring1b (RING2)*	*RING finger; PRC1*
Extra sex combs (esc) [239]	*eed(EED)*	WD40 repeats †; PRC2
Super sex combs (sxc) [240]		
Multi sex combs (mxc) [118]		
Polycomb-like (Pcl) [241]	*M96*	PHD fingers
Cramped (cpt) [242]		
Pleihomeotic (pho) [243]	*YY1(YY1)*	*Zinc fingers*
Sex comb extra (Sce) [244]		
Suppressor of zeste 12 (Su(z)12) [245]	*SU(Z)12 or JJAZ1*	Zinc finger;VEFS-box ‡
Enhancers of Trithorax and Polycomb (ETP)		
Additional sex Combs (Asx) [246]	*Asxl1(ASXL1), Asxl2(ASXL2)*	
Enhancer of Polycomb (E(Pc)) [247]	*Epc1(EPC1), Epc2(EPC2)*	Yeast homolog present in NuA4 HAT complex
Enhancer of zeste (E(z)) [196]	*Enx1/Ezh2(EZH2), Enx2/Ezh1(EZH1)*	*SET domain* § *; PRC2*
Posterior sex combs (Psc) [248]	*bmi-1(BMI-1), mel-18(MEL-18)*	RING (zinc finger), PRC1 helix-turn-helix; PRC1
Sex comb on midleg (Scm) [233]	*Scmh1(SCMH1),(SCMH2), Scml1(SCML1), Scml2(SCML2)*	SPM domain *; PRC1 (substoichiometric amounts)
Suppressor of zeste 2 (Su(z)2) [249]	*bmi-1(BMI-1), mel-18(MEL-18)*	HR region (shared with Psc)

* SPM domain refers to the presence of this motif in Scm, Ph and MBT proteins; † WD40 repeat is a conserved domain that usually ends with TrpAsp (WD); ‡ VEFS-box is a conserved motif found in the VRN2-EMF2-FIS2-Su(z)12 proteins; § SETdomain refers to its presence in the Su(var)3-9, E(z) and Trx proteins.

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CHAPITRE 2

ARTICLE

Stage-Specific Expression of *Polycomb Group* Genes in Human Bone Marrow Cells

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Chapter 2 is a co-authored work which describes the expression pattern of selected members of the *Polycomb Group (PcG)* gene family in purified subpopulations of human bone marrow cells. This work was the first to demonstrate the expression of the *PcG* genes in primary human bone marrow cells and lead to the identification of several novel members of the *Polycomb (Pc)* subfamily of *PcG* genes in hemopoietic cells. Most importantly, this study provided to first insights for a role of the *PcG* genes in regulating normal hemopoiesis through the transcriptional repression of the *homeotic (Hox)* genes.

Soheyl Baban, a technical assistant in the laboratory at that time, contributed to this work by providing technical support with the sequencing of the chromoboxes of genes of the *Pc* subfamily of *PcG* genes presented in Table III. The paper was written by Julie Lessard under the supervision of Dr Guy Sauvageau.

Abstract

Mammalian *Polycomb group (Pc-G)* genes, constituting some 5 subfamilies based on their identity to the *Drosophila* genes *Pc*, *Psc*, *ph*, *esc*, and *E(z),* appear to play critical roles in maintaining the transcriptional repression state of *Hox/HOM-C* genes during development. Despite increasing evidence of the important role of *Hox* genes in both normal hematopoiesis and leukemic transformation, little is known about the expression and possible function played by *Pc-G* genes in hematopoietic cells. To address this, we first examined the expression of *Pc* genes in purified $CD34^+$ human bone marrow cells by reverse transcriptase-polymerase chain reaction (RT-PCR), using degenerate primers that specifically amplify the majority of *Pc* genes. This analysis showed the expression of 8 different *Pc* genes in $CD34^+$ bone marrow cells, including $HP1^{Hs\alpha}$, $HP1^{Hs\gamma}$, the heterochromatin p25 protein, the human homologue of the murine *M32* gene, and 4 novel members of this family. To assess whether *Pc-G* mRNA levels change during differentiation of bone marrow cells, a quantitative RT-PCR method was used to amplify the total cDNA originating from three purified subpopulations of $CD34^+$ bone marrow cells known to differ in their ability to grow in long-term or semisolid cultures. In sharp contrast to *Hox* gene expression, which is highest in the most primitive bone marrow cells, these studies show that the expression level of 8 of the 9 *Pc-G* genes studied (ie, $HP1^{Hs\alpha}$, $HP1^{Hs\gamma}$, *M31*, *M32*, *M33*, *Mel-18*, *Mph1/Rae-28*, and *ENX-1*) markedly increases with differentiation of bone marrow cells. Interestingly, *BMI-1* exhibits a strikingly different pattern of expression, with high expression levels in primitive cells and very little expression in mature $CD34^-$ cells. Together, these results document for the first time that differentiation of human bone marrow cells is accompanied by profound changes in *Pc-G* gene expression levels.

Introduction

In *Drosophila*, the homeotic *(HOM-C)* genes of the *ANT-C* and *BX-C* complexes encode highly conserved transcription factors involved in cell fate determination.[1] During embryogenesis, the spatial and temporal expression of these genes is collinear relative to their 3'→5' position on the chromosome. Although this unique expression pattern is established by segmentation genes of the pair-rule (activators) and gap (repressors) families, the maintenance of *HOM-C* gene expression during later stages of development is dependent on the *trithorax (trx-G)* and *Polycomb group (Pc-G)* gene products (reviewed in Simon et al[2]). For the *Pc-G* genes, this repressive function appears to be achieved initially by direct interaction with the transiently expressed gap proteins and, later, by contributing to the formation and stable transmission of heterochromatin.

The *Drosophila Polycomb (Pc)* gene was one of the first members of the *Pc-G* family to be identified based on its ability to maintain segment-specific expression of the *HOM-C* genes.[3] At least 12 other mutations leading to a phenotype similar to *Pc* (or to its enhancement) have been described. These include: *Posterior sex combs (Psc)* [4]; *polyhomeotic (ph)* [5]; *Polycomblike (Pcl)* [6]; *extra sex combs (esc)* [7]; *Additional sex combs (Asx)* [4]; *Enhancer of zeste [E(z)],* [8] also known as *polycombeotic (pco)* [9]; *l(4)102EFc,*[10] recently renamed *pleihomeotic*[11]*; Sex combs extra (Sce)* [12]; *Sex combs on midleg (Scm)* [4]; *super sex combs (sxc)* [13]; *multi sex combs (mxc)* [14]; and *Enhancer of Polycomb [E(Pc)].* [15] These various *Pc-G* names originate from the ectopic expression of organs called sex combs on the second and third legs of male mutants.[16]

Recent studies have provided evidence for the existence of mammalian *Pc-G* genes and their involvement in repressing the transcription of homeotic *(Hox)* genes.[17] *M31*, *M32*, *M33* (murine), the heterochromatin p25 protein (the human homologue of *M31*), $HP1^{Hs\alpha}$, and $HP1^{Hs\gamma}$ (human) are all homologues of the *Drosophila Pc* gene[18]; *bmi-1* and *mel-18* are both murine and human homologues of the *Drosophila Posterior sex combs (Psc)* gene[19]; *Enx-1* (human and mouse), *eed* and *Mph1/Rae-28* (mouse), and *HPH1* and

HPH2 (human) likely represent the mammalian counterparts of the *Drosophila E(z), esc*, and *ph* genes, respectively.20,21

An important clue to *Pc-G* gene function in contributing to the formation of heterochromatin came from the finding that a conserved region called the chromodomain (for chromatin organizer domain) is shared between Pc and the heterochromatin-associated protein HP1.[22] Furthermore, it has been recently shown that both *Drosophila* and mammalian Pc-G proteins interact with chromatin as heterogeneous multimeric complexes.[20,23,24] The exact mechanisms by which these complexes repress transcription of their target genes are not yet fully understood. The chromatin accessibility model predicts that the cooperative interaction of Pc-G protein complexes interacting with *Pc-G* response elements (PREs) regionally compacts the chromatin structure, thus eliminating the accessibility of DNA to transcriptional regulators.[25-29] However, several pieces of evidence suggest that the mechanism of *Pc-G* gene-mediated silencing may not be solely achieved by the general inaccessibility of regulatory sequences. For example, it was shown that Pc proteins can inhibit Gal-4- but not T7-dependent transcription in *Drosophila* embryos.[30] Thus, other models by which Pc-G genes might be acting have been proposed. For example, *Pc-G* genes could act as transcriptional repressors [6,31-35] , they could interact with specific molecules required for pol II transcription, or, alternatively, they could block looping interactions between promoters and enhancers.[36]

The pleiotropic phenotypes observed in many *Pc-G* mutants indicate their participation in numerous cellular processes such as anterio-posterior segmentation, dorso-ventral patterning, neural development, oogenesis, and hematopoiesis.[11-13,37-40] In support of the importance of *Pc-G* genes in hematopoiesis, severe hypomorphic alleles of the *multi sex comb (mxc)* gene in *Drosophila* were shown to result in premature hemocyte differentiation and tumorous overgrowth of the larval hematopoietic organs. [14,41,42]

Recent studies in mice also support a critical role for *Pc-G* genes in hematopoiesis. B and T cell populations in *M33*$^{-/-}$ mice exhibit a decreased proliferative response to plant

agglutinin.[23] Mice lacking *bmi-1* display a progressive aplastic disease characterized by replacement of bone marrow space by adipocytes, as well as a smaller spleen and thymus than control littermates. Although all thymocyte populations are initially normal in newborn *bmi-1*$^{-/-}$ mice, a progressive loss of $CD4^+CD8^+$ cells is observed such that adult thymi contain more than 90% $CD4^-CD8^-$ cells. B-cell development is also abnormal, with bone marrow pro-B and pre-B cells being most affected. In addition, bone marrow macrophage colony-stimulating factor (M-CSF) and interleukin-7 (IL-7), but not IL-3, responsive clonogenic progenitors are decreased in numbers. Interestingly, erythropoiesis does not appear to be altered in these mice. Together, these data suggest that *bmi-1* function in hematopoietic cells is lineage- and stage-specific, displaying a redundant role during embryogenesis but being essential for proliferation of certain adult hematopoietic lineages.[43] Similarly, targeted disruption of the other mammalian *Psc* gene, *mel-18*, leads to B- and T-cell developmental defects caused by an insufficient response to IL-7 stimulation of the lymphoid precursors.[20] An additional line of evidence further suggesting a role for Pc-G proteins in hematopoietic cell function is provided by the finding of a direct interaction between the Pc-G protein Enx-1 and Vav, a proto-oncogene expressed predominantly in hematopoietic cells.[44]

Interestingly, perturbation in expression levels of certain *Pc-G* and *Hox* genes produce comparable phenotypes in hematopoietic cells. Similarly to what has been observed in *bmi-1* and *mel-18*$^{-/-}$ mice, retroviral overexpression of *HOXA10* or *HOXB3* in murine bone marrow cells causes a block in differentiation of early B and T cells, respectively.[45,46] These observations underscore the importance of preserving the downregulation of *Hox* gene expression that occurs during normal differentiation of primitive hematopoietic cells[47] and suggest that Pc-G gene products play a key role in assuming this function. Furthermore, inactivation of *Pc-G* genes may lead to leukemic growth as a consequence of *Hox* gene overexpression.[45,48-51]

In this work, we have investigated the expression of mammalian Pc-G genes in purified subpopulations of human bone marrow cells and in leukemic cell lines. We document the existence of a highly regulated program of Pc-G gene expression with mature bone

marrow subpopulations showing much higher Pc-G gene mRNA levels relative to less differentiated precursors. These results contrast with previously documented Hox gene expression profiles and thus suggest a role for Pc-G proteins in regulating differentiation and/or proliferation of human hematopoietic cells by silencing *Hox* gene expression.

Materials and methods

PCR amplification of the chromodomains of the *Polycomb (Pc)* subfamily of *Pc-G* genes

A set of degenerate oligonucleotides was designed to match all the different sequences of the conserved 5'-end [5'-CAT-GAA-TTC-(GATC)GA-(GA)AA-(GA)AA-(GA)G-T(GATC) (TC)-T(GATC)GA-(TC)(AC)G-3'] and 3'-end [5-TCT-AGA-TCT-(TC)T-C(GATC)G-G(TC)T-CCC-A(GATC)GT-(GA)T-T-3'] of the chromodomains of most members (from *Drosophila* to mammals; see Table II) of the *Polycomb (Pc)* subfamily of *Pc-G* genes. These primers were used to polymerase chain reaction (PCR)-amplify the conserved chromodomain of *Pc* genes from a phage cDNA library made from purified $CD34^+$ human bone marrow cells originating from a single donor.[46] Briefly, phage DNA was obtained by 2 successive phenol-chloroform extractions and approximately 0.1 μg of this DNA introduced in a PCR mixture containing 200 pmol of each degenerate primer (see above), 250 μmol of each four deoxyribonucleotides (dNTP; Pharmacia, Uppsala, Sweden), 1.5 mmol of MgCl2, 10 mmol of Tris-HCl, pH 8.9, 50 mmol of KCl, 5 U of Taq polymerase (Life Technology, Burlington, Ontario, Canada), and water to 50 μL. Parameters for PCR amplification were 30 seconds at 94°C, 2 minutes at 50°C, and 2 minutes at 72°C for 35 cycles. A unique 115-bp fragment was obtained and subcloned into the EcoRV site of Bluescript KS (Stratagene, La Jolla, CA) as described.[47]

DNA sequencing and sequence analysis

DNA sequencing was performed by the dideoxy chain termination method using [^{35}S] dATP and a T7 sequencing kit (Pharmacia) according to the manufacturer's

recommendations. Either the universal, reverse, T3, or T7 primers complementary to sequences within the cloning vector were used. Nonredundant nucleotide sequence databases (GenBank, EMBL) were screened for homologous sequences using the search algorithms BLAST and FastA of the GCG program (Genetic Computer Group, Madison, WI). The BestFit program was used to obtain the similarities and identities of the *Pc-G* genes of the *Pc* subfamily.

Purification of $CD34^+$ human bone marrow cell subpopulations

Low-density cells (<1.077 g/mL) obtained from 3 different healthy bone marrow (BM no. 1, 2, and 3) donors were isolated by centrifugation on Ficoll-Paque (Pharmacia LKB, Uppsala, Sweden) and kept frozen in Iscove's medium containing 10% fetal calf serum (FCS) and 7.5% dimethyl sulfoxide. For fluorescence-activated cell sorting (FACS) experiments, cells were thawed in the presence of DNase I (Sigma, St Louis, MO) to avoid clogging; stained with a series of directly conjugated fluorescent antibodies to CD34 (8G12-Cy5), CD45RA (8d2-R-phycoerythrin), and CD71 (OKT9-fluorescein isothiocyanate); and washed twice (propidium iodide at 1 μg/mL was included in the last wash [Sigma]) before sorting on a FACStarplus (Becton Dickinson Immunocytometry, San Jose, CA) as described.[47] Cells were sorted in three phenotypically and functionally distinct subpopulations: subpopulation I ($CD34^+CD45RA^-CD71^-$), IIM ($CD34^+CD45^+CD71^{lo}$), and IIIE ($CD34^+CD45RA^-CD71^{hi}$), as characterized before.[47] Cells from bone marrows were also stained with 8G12-Cy5 alone and separated into total $CD34^+$ and $CD34^-$ subpopulations. Aliquots from each subpopulation were analyzed and found to be greater than 98% pure.

cDNA generation and amplification

A previously described method for generating representative amplified total cDNA from small numbers of hematopoietic cells using an oligodT-based primer and polyA tailing strategy was used, with modifications designed to improve cDNA yield of even rare transcripts and to provide amplified sequences extending up to 2 kb 5' of the polyadenylation site.[47] In brief, 1,500 to 10,000 cells of each subpopulation were

pelleted and then lysed in a 5 mol/L guanidium isothiocyanate solution containing 20 mmol/L dithiothreitol. Nucleic acids were precipitated by adding 25 μL of 7.5 mol/L ammonium acetate, 20 μg of glycogen as a carrier, and 2 vol of 95% ethanol. The washed pellets were dried at room temperature and resuspended in 9.5 μL of a solution containing 6.1 μL of diethyl pyrocarbonate-treated water, 2 μL of 5× RT buffer (Life Technology), 1 μL of 0.1 mol/L dithiothreitol, 0.2 μL of 25 mmol/L dNTPs (Pharmacia), 0.2 μL of a special 60-mer oligo(dT) primer [1 μg/μL; 5'-CAT-GTC-GTC-CAG-GCC-GCT-CTG-GAC-AAA-ATA-TGA-ATT-$CT_{(24)}$-3'], and 0.5 μL of Moloney murine leukemia virus (MMLV) SuperScript II reverse transcriptase (200 U/μL; Life Technology). The samples were left at 40°C for 1 hour, heated to 75°C for 10 minutes, and ethanol-precipitated with ammonium acetate and a linear polyacrylamide carrier, as described.[47] The pellet was washed once; resuspended in 5 μL of a tailing solution containing 1 μL of 5× terminal deoxynucleotidyl transferase (TdT) buffer (Life Technology), 0.5 μL of 100 mmol/L dATP (Pharmacia), 3.5 μL of water, and 0.5 μL of TdT enzyme (15 U/μL; Life Technology), and incubated for 15 minutes at 37°C. After heat inactivation (75°C for 10 minutes), this solution was directly added to a PCR amplification mixture consisting of 25 μL of a 2× buffer (20 mmol/L Tris, pH 8.8, 100 mmol/L KCl, 9 mmol/L MgCl2), 4 μL of the 60-mer primer (same as described above), 0.5 μL of nuclease-free bovine serum albumin (10 mg/mL; Sigma), 5.25 μL of water, and 2 μL of d(GCT) deoxynucleotides adjusted at 25 mmol/L each. Four micrograms of gene 32 protein (Pharmacia) and 5 U of Taq polymerase (Life Technology) were added to each tube and total cDNA was amplified with an Ericomp thermal cycler (Ericomp, San Diego, CA) using the following parameters: 94°C for 1 minute; 55°C for 2 minutes except for the first cycle, which was performed at 37°C; and 72°C for 10 minutes for 44 cycles.

Southern blot analysis of total amplified cDNA

One-fifth of the total amplified cDNA prepared from each purified subpopulation or cell line was electrophoresed in a 1% agarose gel and transferred to an ionic nylon membrane (Zeta-probe; Bio-Rad, Hercules, CA). Probes were labeled with ^{32}P-dCTP by random priming and purified on Sephadex-G50 columns (Pharmacia). Blots were

prehybridized and hybridized at 65°C in 4.4× SSC, 7.4% formamide, 0.74% sodium dodecyl sulfate (SDS), 1.5 mmol/L EDTA, 0.74% skim milk, 370 μg/mL salmon sperm DNA, and 7.5% dextran sulphate. Membranes were washed three times for 30 minutes at 65°C in 0.3% SSC, 0.1% SDS, and 1 mg/mL sodium pyrophosphate and exposed to Kodak BioMax MS films (Interscience Inc, Markham, Ontario, Canada). Blots were stripped in a 1% SDS solution at 95°C for 30 minutes and tested for the absence of signals by overnight exposure to Kodak BioMax MS films (Eastman Kodak, Rochester, NY) using appropriate intensifying screens (Interscience Inc).

Most probes used in these studies were generated by PCR amplification of cDNA or genomic DNA obtained from various sources and were all sequenced as described above. These probes included (1) a 264-bp fragment of the human *HP1*$^{Hs\alpha}$ gene (nucleotides 416-679; accession no. S62077; 5' primer CTCAAACAGTGCCGATGACA and 3' primer TCCGCATCCTCAGGATATGC) located downstream of the chromodomain; (2) a 222-bp fragment of the human *HP1*$^{Hs\beta}$ gene (or *p25 heterochromatin* gene) also located downstream of the conserved chromodomain (nucleotides 558-779; accession no. U35451; 5' primer GAAAGCTGGCGGGCACTAT and 3' primer GAGCGTTAGTTCTTGTCATC); (3) a 326-bp fragment corresponding to the untranslated exon 7 of the 3' UTR of the murine *M31* gene (nucleotides 68-393; accession no. X95397; 5' primer TGTCTTGACACCATAGAGGT and 3' primer CTACACACATGCTAGGCTGT); (4) a 268-bp fragment of the murine *M32* gene located downstream of the chromodomain (nucleotides 252-519; accession no. X56683; 5' primer ATCTGACAGTGAATCTGAT and 3' primer TTGTGCTTCATCT TCAGGAC); (5) a 322-bp fragment of the murine *M33* gene located downstream of the chromodomain (nucleotides 1346-1667; accession no. X62537; 5' primer AGCTGACTTGCAAGGCAACG and 3' primer GACTCCTTCACGGTGACAGT); (6) a 329-bp fragment of the human *BMI-1* gene located in the 3' UTR (nucleotides 1938-2248; accession no. L13689; 5' primer GATGAATTCGTCACTGTGAATAACGATTT and 3' primer TCTAGATCTACAATCATTTCTGAATGCAT); (7) a 287-bp fragment of the human *Mel-18* gene located downstream of the RING finger domain (nucleotides 876-1168;

accession no. D13969; 5' primer CAAGTACCGTGTCCAGCCAG and 3' primer TCTGCAGGCAGTTCAAGCTA); (8) a 228-bp fragment of the human *ENX-1* gene located downstream to the SET domain (nucleotides 2338-2565; accession no. U52965; 5' primer CTGAAGTATGTCGGCATCGA and 3' primer ACACTTTGCAGCTGGTGAGA); and (9) a 251-bp fragment of the murine *Mph1/Rae-28* gene located in the 3' UTR (nucleotides 3190-3440; accession no. U63386; 5' primer GTGCTACATGGTGACAGCTT and 3' primer AGCTAGGAAAGCTGACCTCT). Probes for $HP1^{Hs\alpha}$, $HP1^{Hs\beta}$, and *ENX-1* were isolated from cDNA obtained from $CD34^+$ human bone marrow cells; *M31* and *M32* were obtained from a pool of total RNA extracted from the Ba/F3, 32D, FEL, and FDC-P1 murine hematopoietic cell lines; *M33* and *Mph1/Rae-28* were obtained from murine genomic DNA; and *BMI-1* and *Mel-18* were obtained from human genomic DNA isolated from K562 cells. Probe for the full-length human $HP1^{Hs\gamma}$ coding cDNA (519 bp) was isolated as a *Bam*HI/*Eco*RI fragment of pGEX-2T (kindly provided by Dr H.J. Worman, College of Physicians & Surgeons of Columbia University, New York, NY). Probes for *β-actin*, human *CD34*, *Multi-Drug Resistance (MDR),* and *β-globin* were isolated as described.[47]

After database searches, probes were designed to minimize any cross-hybridization between the various *Pc-G* genes and other related sequences. To determine whether these probes recognized single copy gene, Southern blot analysis was performed on genomic DNA isolated from mouse thymus and human leukemic cell lines (K562, HL-60) using the same hybridization conditions as described above. A single band was detected with the following probes: *M31, M33, Mel-18, BMI-1, Mph1/Rae-28*, and *ENX-1*. However, *M32* and $HP1^{Hs\gamma}$ hybridized to 5 to 10 different DNA fragments digested with either of the following restriction enzymes: *Eco*RI, *Hind*III, *Bgl*II, *Kpn*I, *Bam*HI, and *Xho*I. The probe used to detect $HP1^{Hs\alpha}$ also showed a single band. However, by using a different probe, it has been shown that this gene is part of a larger family[18] and highly similar sequences to $HP1^{Hs\alpha}$ are found in different EST databases.

Cell lines

Hematopoietic cell lines used in this study were obtained from the American Type Culture Collection (ATCC; Rockville, MD), unless specified otherwise. They included the HL-60 cells derived from a patient suffering from acute myeloid leukemia, the K562 cells obtained from pleural effusion of a patient suffering from blast phase chronic myelogeneous leukemia, the MOLT-4 cells established from the peripheral blood of a patient suffering from acute T-cell lymphoblastic leukemia, the KG-1a cells obtained from a patient suffering from acute myeloid leukemia, the TF-1 human erythroleukemic cell line, the 32D murine mast cell line, the FDC-P1 myeloid cell line derived from long-term bone marrow cultures of DBA-2 mice, the Ba/F3 murine cell line (a gift from A. Miyajima, DNAX Research Institute, Palo Alto, CA), the FEL-745 Friend murine erythroleukemic cell line, and the murine Rat-1 fibroblasts. All cell lines were maintained in RPMI 10% FCS (GIBCO/BRL) except for FDC-P1, 32D, and Ba/F3, which grow in the presence of 5 ng/mL of mIL-3 and TF-1 cells that were maintained in the presence of 5 ng/mL of human granulocyte-macrophage colony-stimulating factor (hGM-CSF). All growth factors were used as diluted COS-cell supernatants produced at Institut de Recherches Cliniques de Montréal (IRCM).

Northern blot analysis

Total cellular RNA from 1×10^7 cells of the murine FEL, FDC-P1, 32D, and Ba/F3 and human K562, HL-60, TF-1, MOLT-4, and KG-1a hematopoietic cell lines was isolated with TRIzol (Life Technology). Approximately 5 μg of each sample was size fractionated by electrophoresis on a 1% agarose gel containing 1× Na-MOPS and 5% deionized formaldehyde and transferred to Zeta-probe nylon membranes. Blots were prehybridized and hybridized at 45°C in 48% deionized formamide, 4.8% SDS, 480 mmol/L phosphate buffer (Na_2HPO_4/NaH_2PO_4, pH 7), 960 μg/mL of nuclease-free bovine serum albumin, and 400 μg/mL of salmon sperm DNA. Membranes were washed twice in 0.2× SSC, 0.1% SDS and once in 0.1× SSC, 0.1% SDS for 30 minutes at 55°C. Total RNA loading was verified by hybridization of membranes with radiolabeled

oligonucleotides (5'-ACG-GTA-TCT-GAT-CGT-CTT-CGA-ACC-3') specific to 18S ribosomal RNA.

Results

Expression of known and novel *Pc* genes in $CD34^+$ human bone marrow cells

To obtain an initial indication of the range and expression pattern of known and potentially novel *Pc* genes that might be expressed in human bone marrow cells, a set of degenerate oligonucleotides spanning a region of the conserved chromodomain of most *Pc* genes was synthesized (Table II) and used to PCR-amplify cDNA isolated from a $CD34^+$ human bone marrow cDNA library. A PCR fragment of 115 bp presumably containing several different chromobox sequences was subcloned and the nucleotide sequences of 73 independent clones were compared with known *Pc-G* genes.

The results from this analysis showed that at least 8 different *Pc* genes are expressed in human bone marrow cells (Table III). The $HP1^{Hs\alpha}$ and $HP1^{Hs\gamma}$ genes were most represented, with 31 (42%) and 15 (21%) of the 73 clones corresponding to these 2 genes. Three *Pc* subfamily members having a chromobox highly similar to that of $HP1^{Hs\alpha}$ were identified in 2 independent PCR reactions, suggesting that they represent novel *Pc* genes. They were named $HP1^{Hs\alpha}$-like A, B, and C. DNA and protein sequence comparison of these clones to $HP1^{Hs\alpha}$ is shown in Table III.

Three additional sequences were identical to the human *heterochromatin p25* protein and one clone, referred to as *p25-like*, was most similar to *p25* but contained 3 mismatches (Table III). Two clones, each obtained from independent PCR reactions, had a single mismatch to the murine *M32* gene and likely represent its human homologue. Finally, seven additional sequences containing a single nucleotide mismatch to either $HP1^{Hs\alpha}$ or $HP1^{Hs\gamma}$ were identified. It is impossible at this point to ascertain whether any of these represent distinct genes or reflect PCR artifacts.

Together, the data obtained with this approach show the presence of at least 8 different *Pc* genes in human bone marrow cells, 4 of which are potentially novel genes, and one is the human homologue of the murine *M32* gene.

Table II

Alignment of the chromodomains of the *Pc* genes and generation of a consensus sequence to design the degenerate oligonucleotides used in these studies.

Pc Genes	Chromodomain Conserved Sequences
HP1 (D)	YVVEKVLDRRVRKGKVEYYLKWKGYPETENTWEPENN*
HP1$^{Hs\alpha}$ (H)	YAVEKIIDRRVVKGQVEYLLKWKGFSEEHNTWEPEKN
HP1$^{Hs\gamma}$ (H)	FVVEKVLDRRVVNGKVEYFLKWKGFTDADNTWEPEEN
M31 (M)	YVVEKVLDRRVVKGKVEYLLKWKGFSDEDNTWEPEEN
M32 (M)	FVVEKVLDRRVVNGKVEYFLKWKGFTDADNTWEPEEN
p25 (H)†	YVVEKYLDRRVVKGKVEYLLKWKGFSDEDNTWEPEEN
Consensus	YVVEKVLDRRVVKGKVEYYLKWKGFPETDNTWEPENN
	FA II RN Q F YSDEH K
	L T AE E

Abbreviation: D, *Drosophila*; H, human; M, mouse.

*Letters in bold represent the amino acid sequence used to construct the 2 degenerate primers used in these studies.

†Human *heterochromatin p25* protein is also referred to as *HSM1* or *HP1*$^{Hs\beta}$ and is the human homologue of the mouse *M31* gene.

Table III

Pc gene sequences obtained from cDNA isolated from purified $CD34^+$ human bone marrow cells with degenerate oligonucleotides designed based on conserved sequences of *Pc* genes.

Pc Genes	cDNA Sequences Between Degenerate Oligos (and Translation)	No. of Clones* Sequenced
	R V V K G Q V E Y L L K W K G F S E E H	
1. HP1^{Hsα}	G CGC GTG GTT AAG GGA CAA GTG GAA TAT CTA CTG AAG TGG AAA GGC TTT TCT GAG GAG CAC	31†
	H	
2. HP1^{Hsα}-like A	. .AT ... ...G ... .G ... ..G	3
	E	
3. HP1^{Hsα}-like B	. ... G..	4†
	F	
4. HP1^{Hsα}-like C	. ... .T.	3†
	R V V N G K V E Y F L K W K G F T D A D	
5. HP1^{Hsγ}	A CGT GTA GTG AAT GGG AAA GTG GAA TAT TTC CTG AAG TGG AAG GGA TTT ACA GAT GCT GAC	15†
	R V V K G K V E Y L L K W K G F S D E D	
6. p25	T CGA GTG GTA AAG GGC AAA GTG GAG TAC CTC CTA AAG TGG AAG GGA TTC TCA GAT GAG GAC	3
	D E	
7. p25-like	. ... .A. ... ... .A. ... ...T	1
	R V V N G K V E Y F L K W K G F T D A D	
8. M32 (homologue)	T CAT GTA GTG AAT GGG AAG GTG GAG TAT TTC CTG AAG TGG AAG GGG TTC ACA GAT GCT GAT	2†
9. Several HP1^{Hsα}-like with a single mutation (considered as potential PCR-induced mutations)		6
10. One HP1^{Hsγ}-like with a single mutation		1

*Four additional clones were unrelated to *Pc-G* genes.
†Sequences obtained from 2 independent PCR reactions.

Quantitative analysis of *Pc-G* gene expression in functionally distinct subpopulations of human bone marrow cells

We next assessed the variation in expression levels of *Pc-G* genes during differentiation of bone marrow cells. This was performed by generating PCR-amplified total cDNA from functionally and phenotypically distinct FACS-purified human bone marrow subpopulations, using antibodies directed against CD34, CD45RA, and CD71 surface antigens (Fig. 2.1). In total, 5 different subpopulations were purified from 3 healthy bone marrow donors: (1) total $CD34^+$ cells, which phenotypically represent 1% to 5% of bone marrow cells and contain all types of progenitors; (2) $CD34^+CD45^-CD71^-$ cells (subpopulation I), which are highly enriched for very primitive long-term culture-initiating cells (LTC-IC); (3) $CD34^+CD45^-CD71^{hi}$, which are enriched in erythroid (ie, burst-forming unit-erythroid [BFU-E]) clonogenic progenitors (subpopulation IIIE); (4)

$CD34^+CD45^+CD71^{lo}$ cells, which are highly enriched in granulocyte-macrophage progenitors (colony-forming unit-granulocyte-macrophage [CFU-GM]; subpopulation IIM); and (5) total $CD34^-$ subpopulation, which contains mature bone marrow cells with no progenitor activity. Detailed functional characterization of each of these purified populations has been described elsewhere.[47,52]

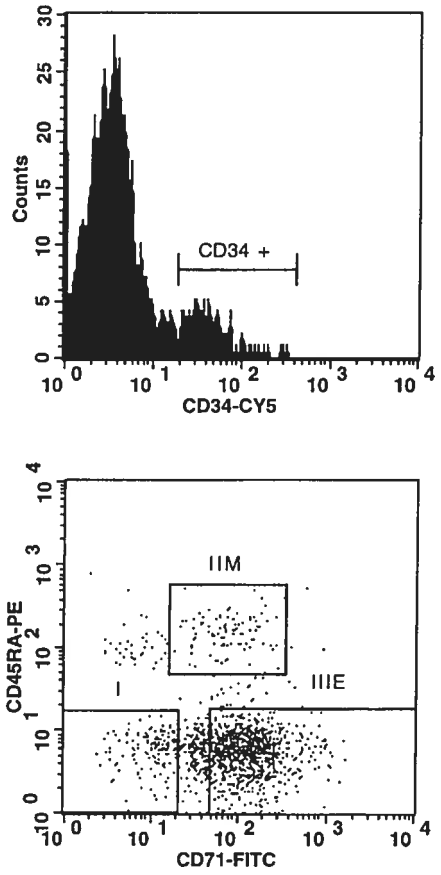

Fig. 2.1 FACS profiles of the $CD34^+$ subpopulations isolated from donor no. 1: subpopulation I, $CD34^+CD45RA^-CD71^-$ (highly enriched in LTC-IC); subpopulation IIM, $CD34^+CD45RA^+CD71^{lo}$ (highly enriched in CFU-GM); subpopulation IIIE, $CD34^+CD45RA^-CD71^{hi}$ (highly enriched in BFU-E). FITC, fluorescein isothiocyanate; PE, phycoerythrin.

Total cDNA isolated from each purified subpopulation was PCR-amplified using a method previously shown to preserve quantitative differences in mRNA abundance using limited cell numbers.[47,53] To assess both the sensitivity and the ability of this procedure to measure quantitative differences of *Pc-G* messages in hematopoietic cells, various numbers of the human K562 cells, which express $HP1^{Hsγ}$, were mixed with Rat-1 fibroblasts that do not express this gene. Total cDNA amplified from each cellular preparation was blotted and hybridized with a probe specific for $HP1^{Hsγ}$ and showed linearity of expression in a range between 40 and 20,000 K562 cells (Fig. 2.2).

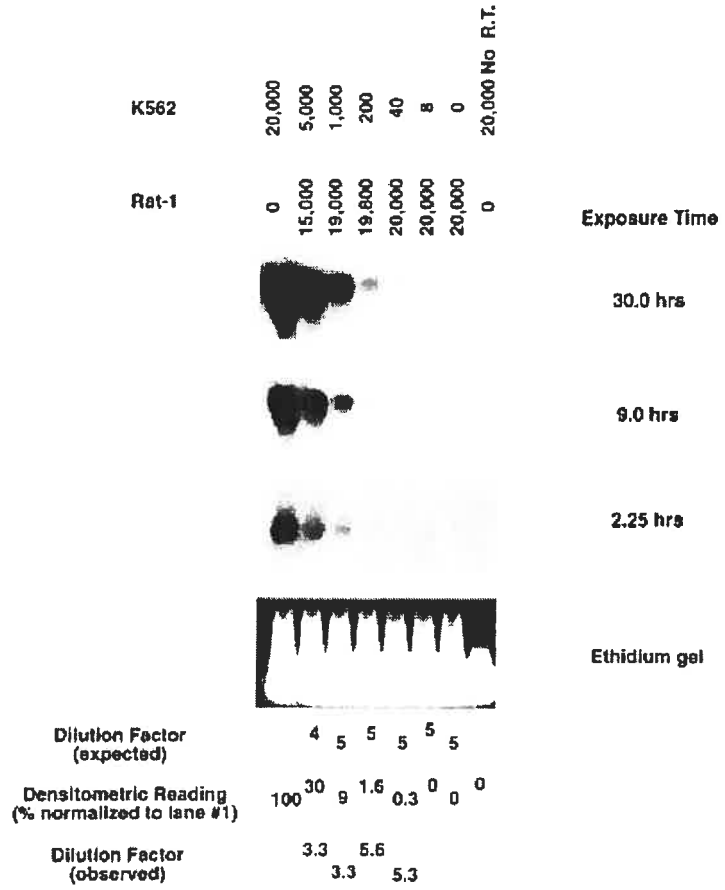

Fig. 2.2 Representative amplification of mRNA by quantitative RT-PCR. Expression of $HP1^{Hsγ}$ in total amplified cDNA obtained from various cellular preparations of K562 (which express $HP1^{Hsγ}$) and Rat-1 cells ($HP1^{Hsγ}$ negative) by Southern blot analysis. Exposure times are as indicated.

Total amplified cDNA from each of the 5 purified subpopulations obtained from one bone marrow donor was analyzed for the expression of the majority of mammalian *Pc, Psc, ph,* and *E(z)* genes characterized to date (Fig. 2.3). Probes were carefully designed to minimize the presence of conserved or repetitive domains within the various *Pc-G* genes. All *Pc-G* genes analyzed to date are expressed at some levels in at least one of the purified subpopulations shown in Fig. 2.3. Interestingly, and in contrast to *Hox* gene expression, which is predominantly observed in primitive subpopulations of $CD34^+$ human bone marrow cells,[47] the expression of most *Pc-G* genes is higher in cells lacking the CD34 surface antigen (compare the signals shown in lane 3 [$CD34^-$ cells] with those in lane 2 [$CD34^+$]; Fig. 2.3). The detailed analysis of these results suggests a progressive increase in *Pc-G* gene expression with bone marrow cell differentiation (Fig. 2.3). Some important variations in expression levels were observed within each subfamily. For the *Pc* subfamily, *M31, M32*, and *M33* had a similar pattern of expression, with very high expression levels in $CD34^-$ cells, moderate levels in subpopulations IIM and IIIE, and little (*M31*) or no detectable expression (*M32, M33*) in the most primitive subpopulation I. The expression pattern of $HP1^{Hs\alpha}$ differed significantly from the other *Pc* genes with similar abundance in $CD34^+$ and $CD34^-$ cells (Fig. 2.3) Expression also differed between the two known members of the *Psc* family *BMI-1* and *Mel-18*. Whereas *Mel-18* expression was most prominent in mature cells ($CD34^-$ and subpopulation IIM), that of *BMI-1* was highest in the most primitive subpopulation and minimal in $CD34^-$ cells. The expression of *ENX-1* [a mammalian *E(z)* gene] was similar to that of $HP1^{HS\alpha}$ and the expression of *Mph1/Rae-28* (a *ph* gene) paralleled that of *Mel-18*. Interestingly, *Mph1/Rae-28* expression was not observed in the 3 cell lines shown in Fig. 2.3, whereas *Mel-18* expression was detected only in human K562 and murine Rat-1 cells.

Several control probes were hybridized to the membranes containing the amplified cDNA obtained from each subpopulation. *Actin* showed comparable loading in each of the 9 lanes, except for lane 4, which was moderately underloaded, and lane 5 (subpopulation IIM), in which a clear distinct signal could be detected only upon prolonged exposure (not shown). Expression of CD34 in these subpopulations correlated with the expression of this antigen as determined by FACS analysis, with a progressive

decrease in expression levels from subpopulation I to IIM and IIIE (data not shown and Sauvageau et al[47]). Expression of CD34 could not be detected in the $CD34^-$ cDNA even upon prolonged exposure (Fig. 2.3). As might be expected, *β-globin* expression was only detected in populations containing mature red blood cell precursors (total unseparated bone marrow and $CD34^-$ cells). Finally, consistent with the findings of our previous studies,[47] the expression of the *multidrug resistance gene (MDR-1)* was highest in subpopulation I and was also abundant in K562 cells (data not shown).

The reproducibility of these data was examined in identical bone marrow subpopulations isolated from 2 additional donors. Probes for $HP1^{Hs\alpha}$, $HP1^{Hs\gamma}$, and *ENX-1* were hybridized to membranes containing total amplified cDNA, and the results from these experiments were superimposable to those shown in Fig. 2.3 (data not shown). Together, these data highlight the complexity of the regulation of *Pc-G* gene expression during differentiation of human bone marrow cells and suggest the existence of diverse Pc-G protein complexes during hematopoietic cell differentiation.

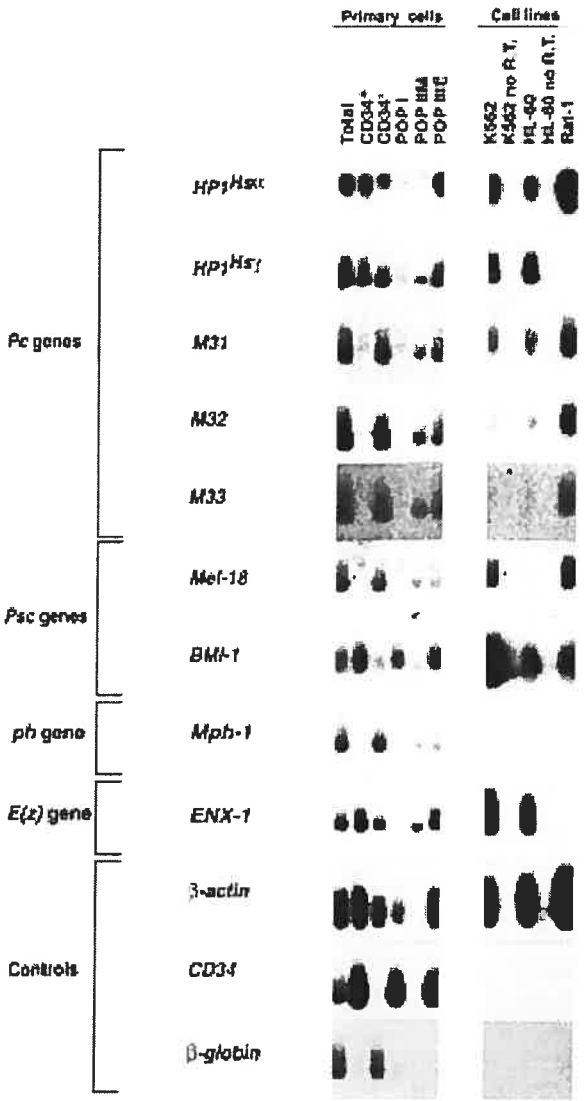

Fig. 2.3 Expression of mammalian *Pc-G* genes in purified bone marrow $CD34^+$ subpopulations. Five to ten thousand cells were isolated from each subpopulation (>98% purity upon reanalysis) and their total RNA was reverse-transcribed and PCR-amplified as described in the Materials and Methods. From primitive to mature subpopulations: subpopulation I, $CD34^+CD45RA^-CD71^-$; subpopulation IIM, $CD34^+CD45RA^+CD71^{lo}$; subpopulation IIIE, $CD34^+CD45RA^-CD71^{hi}$ and $CD34^-$ cells. Exposure times (all at -70°C unless specified) were as follows: $HP1^{Hs\alpha}$, 6.5 hours at room temperature; $HP1^{Hs\gamma}$, 1.2 hours; *M31*, 6 days; *M32*, 27 hours; *M33*, 26 hours; *Mel-18*, 24 hours; *BMI-1*, 8 days; *Mph-1/Rae-28*, 3 days; *ENX-1*, 4.5 hours; *β-globin*, 5 minutes; and *β*-actin and *CD34*, 5 hours each.

Several Pc-G genes are expressed as multiple alternative transcripts in primary cells and in leukemic cell lines.

Previous studies have shown that one of the hallmarks of *Pc-G* genes is their expression as several alternative transcripts. To evaluate whether hematopoietic cells also express multiple *Pc-G* gene transcripts, total RNA isolated from 5 different human (K562, HL-60, MOLT-4, TF-1, and KG1-a) and 4 murine (FEL, FDC-P1, 32D, and Ba/F3) hematopoietic cell lines, representing different lineages and stages of differentiation, was assessed for *Pc-G* gene expression by Northern blot (Fig. 2.4).

This analysis showed the presence of multiple transcripts for 3 of the 5 *Pc-G* genes examined. These include $HP1^{Hsa}$, which had a human and a mouse-specific transcript of 9.9 and 8.7 kb, respectively, and shared a transcript of 1.2 kb in all cell lines examined (Fig. 2.4). Different transcripts of the *M31* gene were detected in human versus murine cell lines, the former expressing transcripts of 10.7 and 2.4 kb and the latter expressing transcripts of 2.4 and 1.1 kb. The probe for $HP1^{Hsγ}$ also detected 2 distinct species (2.1 and 1.1 kb) in human cell lines, whereas murine cells only expressed a transcript of 2.1 kb.

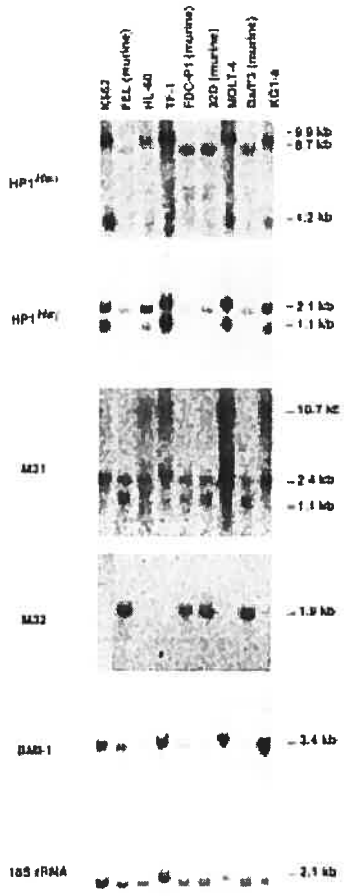

Fig. 2.4 Northern blot analysis showing the expression of selected members of the *Pc-G* family in human and murine hematopoietic cell lines. Five micrograms of total RNA isolated from each cell line was hybridized to probes specific to $HP1^{Hs\alpha}$ (14 hours of exposure), $HP1^{Hs\gamma}$ (5.5 hours), *M31* (96 hours), *M32* (6 days), *BMI-1* (14 hours), and 18S rRNA (4 minutes).

Two of the 5 genes examined expressed a single transcript. These include the *M32* gene, whose expression could only be detected in cell lines of murine origin (Fig. 2.4). The lack of expression of *M32* in human cell lines contrasts with its relative abundance in primary bone marrow cells (Fig. 2.3), suggesting either the presence of different transcripts specifically recognized by our probe in primary cells or its complete absence in the immortalized lines examined here. Similarly, *M33* and *Mph1/Rae-28* expression could not be detected in any hematopoietic cell line examined, although both were easily

detectable in the purified subpopulations shown in Fig. 2.3. Finally, although *Mel-18* expression could not be detected by Northern blot analysis of total RNA (data not shown), RT-PCR analysis showed its presence at low levels in K562 but not in HL-60 cells (Fig. 2.3). These results were confirmed using polyA mRNA isolated from the same cell lines. Two different transcripts for *Mel-18* (1.8 and 3.4 kb) were detected in all the cell lines examined, except in HL-60 cells (data not shown).

To test whether the transcripts identified in the human cell lines reflect those present in primary isolates, mononuclear cells were isolated from 2 different human bone marrow specimens and total RNA hybridized to probes specific for the $HP1^{Hs\alpha}$ and $HP1^{Hs\gamma}$ genes. In both cases, the transcripts observed in primary cells corresponded to those found in human cell lines, except for the 1.2-kb transcript of $HP1^{Hs\alpha}$ whose presence in primary cells remains unclear (data not shown). Together, these data suggest that alternative transcription patterns for the majority of *Pc-G* genes represent an additional level for regulation of the *Pc-G* gene action during hematopoietic cell differentiation.

Discussion

These studies report the expression of at least 13 different *Pc-G* genes in human bone marrow cells, including 4 potentially novel homologues of the *Drosophila Pc* gene. Moreover, our data suggest the existence of a highly defined program of *Pc-G* gene expression in phenotypically distinct subpopulations of human bone marrow cells representing various stages of differentiation. In contrast to the preferential expression of *Hox* genes in the early hematopoietic cells,[47] our study showed that the expression levels of 8 of the 9 *Pc-G* genes studied is much higher in the more mature bone marrow cells than in the primitive subpopulations. For some of the *Pc-G* genes, such as *ENX-1, M31,* $HP1^{Hs\alpha}$, and $HP1^{Hs\gamma}$, this upregulation seems to appear in the earliest stages of hematopoietic differentiation, whereas for others (ie, *M32, M33, Mel-18,* and *Mph1/Rae-28*), increase in their expression levels coincides with later stages of differentiation (summarized in Fig. 2.5). This suggests that Pc-G protein complexes present in primitive hematopoietic cells (ie, population I) differ from those found in mature bone marrow

cells. These results document, for the first time, changes in *Pc-G* gene expression levels with cellular differentiation. This contrasts with their ubiquitous expression in *Drosophila* syncytial blastoderms[24] and thus point to an additional level for regulating *Pc-G* gene functions in mammalian cells.

Using degenerate primers, 4 novel chromobox sequences similar to that of the previously described *Pc* genes (referred to as *HP1$^{Hs\alpha}$-like A, B,* and *C* and *p25-like*) were identified. In most cases, these sequences were obtained in 2 independent PCR reactions, suggesting that they may represent novel human *Pc* members. Because the bone marrow used for this part of the work originated from a single donor, it is impossible to rule out that one of these sequences (eg, clone no. 4, Table III) represents polymorphism. However, this is unlikely, because each novel sequence contains at least one mutation that affects the primary sequence of the highly conserved chromodomain. In support of the existence of several uncharacterized *HP1$^{Hs\alpha}$* members, several *HP1$^{Hs\alpha}$* sequences have been found in EST databases. Hybridization of mouse and human genomic DNA with a probe for *HP1$^{Hs\alpha}$* have also shown several bands.[18] Importantly, the other *HP1$^{Hs\alpha}$*-related sequences identified by Saunders et al[18] were not detected by the probe used in our studies. Together, these data indicate that our knowledge of the full complement of *Pc-G* genes expressed in hematopoietic cells has not been resolved yet.

The ability of each probe to hybridize to specific sequences is shown in Fig. 2.3 by the absence of a signal in at least one of the cell line controls (*HP1$^{Hs\gamma}$, M32, M33, Mel-18, Mph1/Rae-28,* and *ENX-1*) or one of the purified subpopulations analyzed (*HP1$^{Hs\alpha}$* and *M31*). Except for *M32* and *HP1$^{Hs\gamma}$*, all *Pc-G* gene probes used in these studies only detected one DNA fragment, as shown by Southern blot analysis of human and mouse genomic DNA (data not shown). Therefore, *M32* and *HP1$^{Hs\gamma}$* probes possibly cross-hybridized with other *Pc* genes whose expression would also be prominent in mature bone marrow cells (Fig. 2.3), further supporting our observation that *Pc* gene expression is higher in more mature bone marrow cells.

Further insight into *Pc-G* gene expression was provided by Northern blot analysis, which showed multiple *Pc-G* gene signals in several human and murine hematopoietic cells. Because our probe for *M32* and *HP1*^{Hsγ} may recognize more than one gene, some of the signals detected by Northern blot analysis may be derived from related genes. However, the signals observed with the probes specific for *HP1*^{Hsα}, *M31*, and *Mel-18* represent alternative transcripts. It would be interesting to investigate whether some of these transcripts are hematopoietic-specific, lineage-specific, or encode proteins with altered function (eg, dominant negative, etc). Interestingly, the low to undetectable expression levels of the *M32*, *M33*, and *Mph1/Rae-28* genes in all cell lines (Figs. 2.3 and 2.4) contrast with their relatively high expression levels in differentiated primary hematopoietic cells (Fig. 2.3), raising the possibility that these proteins normally perform antiproliferative functions in mature bone marrow cells. Indeed, a correlation can be made between the expression pattern of *BMI-1* and *Mel-18* (the 2 known mammalian *Psc* genes) and their ability to control cellular proliferation. *BMI-1*, a known proto-oncogene, is preferentially expressed in bone marrow cells displaying a high proliferative potential (ie, subpopulation I; Fig. 2.3), whereas *Mel-18*, a gene recently shown to exhibit tumor suppressive activity,[54] is only expressed in mature and nonproliferating CD34$^-$ cells.

Together, our results show a progressive upregulation of most *Pc-G* genes concomitant with differentiation of human bone marrow cells and support a complex-constitution model. In this model, newly expressed *Pc-G* gene products would progressively interact with existing Pc-G protein complexes, favoring novel interactions with target sequences. This, in turn, would allow a progressive packaging of DNA into an heterochromatin-like structure and, for the *Hox* genes, a progressive 3' to 5' closure of the clusters, allowing proper differentiation of the hematopoietic stem cells. This is most interesting in the view that Hox gene expression decreases 3' to 5' during differentiation of hematopoietic cells[47] and overexpression of *Hox* genes in hematopoietic cells profoundly alters their differentiation and proliferation.[45,46,55]

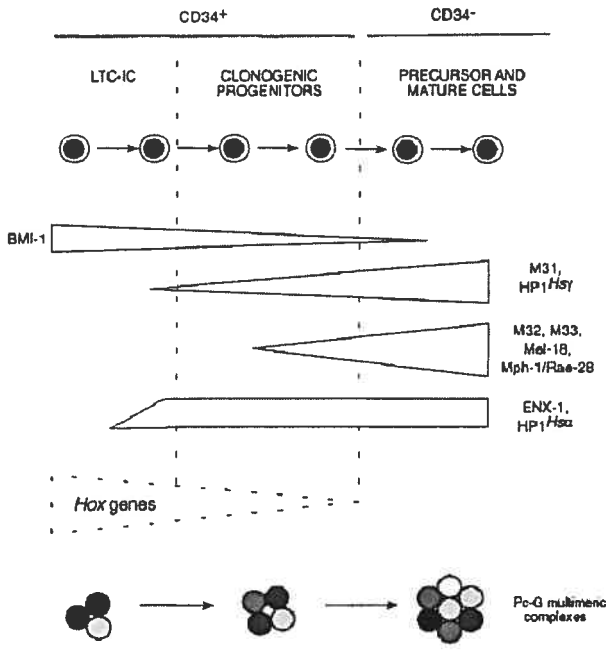

Fig. 2.5 Summary of *Pc-G* gene expression patterns observed in different purified subpopulations of human bone marrow cells.

Acknowledgments

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CHAPITRE 3

ARTICLE

Functional Antagonism of the *Polycomb-Group* Genes *eed* and *Bmi1* in Hemopoietic Cell Proliferation

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Chapter 3 is a multi-authored work which presents a detailed characterization of the hemopoietic phenotype of the loss-of-function of Bmi-1 and eed, two PcG gene products belonging to biochemically distinct PcG complexes. The results presented in this study are consistent with an antagonistic function of *eed* and *Bmi-1* in hemopoietic cell proliferation.

Dr Armin Schumacher contributed to the breeding and genotyping of the *eed* mutant mice used in these studies and to the writing of the manuscript. Dr Unnur Thorsteinsdottir helped with the analysis of some of the *eed* and *eed/bmi-1* double mutant mice presented in Figures 4A and 6A. Drs Maarten van Lohuizen and Terry Magnuson provided us with the *Bmi-1* and *eed* mutant mice used in these studies, respectively. The paper was written by Julie Lessard under the supervision of Dr Guy Sauvageau.

Abstract

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 employ 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 since 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 lympho-proliferative defects, indicating that *eed* is involved in the negative regulation of the pool size of lymphoid and myeloid progenitor cells. This anti-proliferative function of *eed* does not appear to be mediated by *Hox* genes or the tumor suppressor locus $p16^{INK4a}/p19^{ARF}$ since expression of these genes was not altered in *eed* mutants. Inter-cross 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.

Introduction

The murine *Polycomb (PcG)* and *trithorax (trxG)* Group of genes are constituents of an evolutionary highly conserved epigenetic pathway(s) governing metameric patterning of the axial skeleton by regulation of *Hox* gene expression (for review, see Schumacher and Magnuson, 1997; Gould, 1997; van Lohuizen, 1998). PcG and trxG gene products are thought to modify higher-order chromatin structures to maintain a repressed and de-repressed 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 transformations have been observed in mutant alleles of the murine *PcG* genes *Bmi1* (*B cell-specific Mo-MLV integration site 1*) and *eed (embryonic ectoderm development)* (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 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 hypo-proliferation and/or atrophy of various hemopoietic organs (Akasaka et al., 1996; Takihara et al., 1997; Core et al., 1997). Therefore, in all *PcG* mutants analyzed thus far, the hemopoietic compartment mostly affected correlates with the preferential expression domain, *i.e.* 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 (Sewalt et al., 1998; van Lohuizen et al., 1998; Denisenko et al., 1998) and the other Bmi1, Mel18, Mph1/Rae28, and M33 (Gunster et al., 1997; Alkema 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. 3.1A). In agreement with the human data, only the Sca^+ Lin^- subpopulation displayed an elevated expression level of mouse *Bmi1* (Fig. 3.1A, upper panel) whereas *eed* expression was consistently high in all subpopulations (Fig. 3.1A, middle panel).

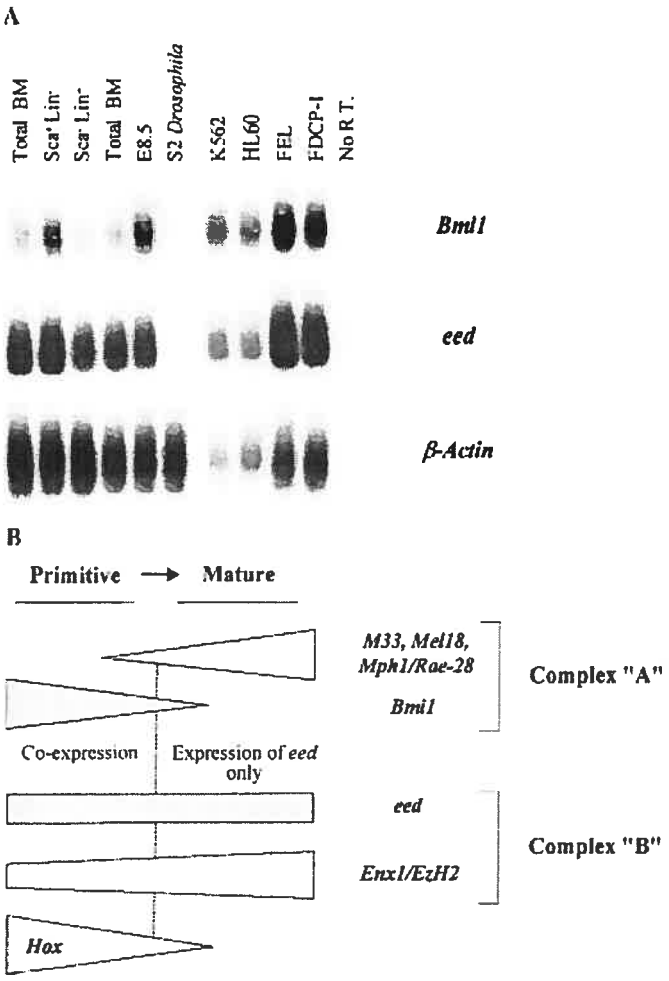


Fig. 3.1 Expression of eed and Bmi1 PcG genes in purified subpopulations of murine bone marrow cells and hemopoietic cell lines. (A) Five to ten thousand cells were isolated from each subpopulation (>98% purity upon reanalysis), and their total RNA was reverse-transcribed and PCR-amplified as described 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; Lessard et al., 1998; Sauvageau et al., 1994).

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.

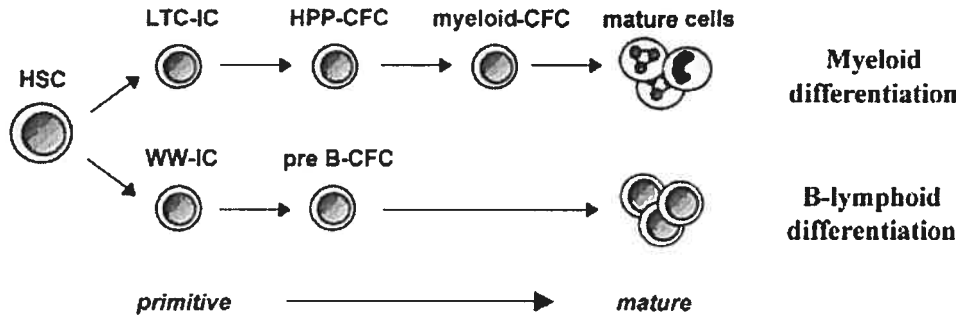
When compared to control littermates, the bone marrow, spleen, thymus and peripheral blood counts were normal in all young eed mutants analyzed, that is, animals of <4 months of age (n > 5 per group). Moreover, cytological and cytofluorometric analysis showed that myeloid (Mac+, Gr-1+), B (B220+CD43+; 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. 3.2B) when compared to control littermates. Moreover, *eed*$^{3354/+}$ 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) were measured by limiting

dilution analysis. The results presented in Fig. 3.2B 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.

A



B

Absolute numbers (per femur) of primitive and more mature myeloid and lymphoid progenitor cells in bone marrow of eed mutant mice

	B-lymphoid lineage		Myeloid lineage		
Mice	No. of WW-IC /femur	Pre-B-CFC /femur (x10³)	No. of LTC-IC /femur	Myeloid-CFC /femur (x10³)	HPP-CFC [a] /femur (x10³)
Control [b]	1386 ±147	2 ± 0.4	575 ±220	99 ±18	25 ±5
eed^{1989} [c]	2255 ± 807	4 ±1	785 ±272	82 ±19	19 ±5
$eed^{3354/+}$ [d]	n.a.	7 ±1	795 ±142	187 ±22	42 ±8

Fig. 3.2 Eed is a negative regulator of the proliferative activity of bone marrow myeloid and lymphoid progenitor cells. (A) Schematic representation of the myeloid and B-lymphoid differentiation pathways. (B) Absolute numbers of primitive myeloid (LTC-IC), primitive lymphoid (WW-IC), and colony-forming cells (CFC) per femoral

bone of 12- to 16-week-old *eed* mutant mice. Results are expressed as mean ± SD. (HPP-CFC) High proliferative potential colony-forming cells; (pre-B) B cell precursors; (n.a.) not available. [a] Includes both colony-forming-unit-granulocyte-macrophage (CFU-GM) and colony-forming-unit-granulocyte-erythroid-monocyte-megakaryocyte (CFU-GEMM) (>1000 cells); [b] CFC (n=9), LTC-IC and WW-IC (n=3); [c] includes both $eed^{1989/+}$ and $eed^{1989/1989}$ mutant mice, CFC (n=9), LTC-IC (n=6), and WW-IC (n=2); [d] CFC (n=5), LTC-IC (n=2).

Lymphoid and myeloid hyper-proliferation in older eed mutant mice

To investigate the long-term effect of decreased Eed activity in hemopoietic cells, nine eed3354/+ mice were analyzed between 7 to 18 months of age. Of these, five had developed lympho-proliferation (LP) (group B; Figs. 3.3 and 3.4), three mice suffered from a severe myelo-proliferative disease (MPD) (group C; Figs. 3.3 and 3.4) and one was still relatively normal (group D; Fig. 3.3) although it had a noticeable increase in bone marrow pre-B progenitors (Fig. 3.4A).

The lympho-proliferative 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 to control littermates (group A, Fig. 3.4A). 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 to control littermates (3.5-fold and 2–fold increase, respectively; Fig. 3.4B). 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. 3.4C). 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. 3.3A,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. 3.3), 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. 3.3A). Cytofluometric 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. 3.3B). 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. 3.3B) 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

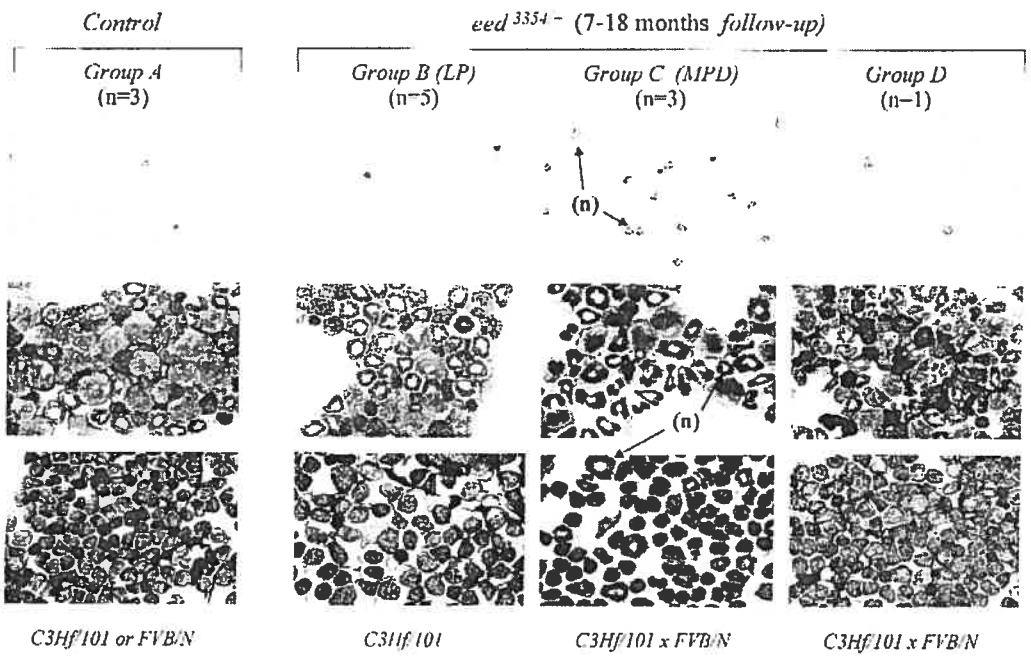
and consisted predominantly of granulocyte and/or macrophage colony-forming cells (G and/or M-CFCs) (Fig. 3.4A; data not shown). Consistent with the cytofluorometric studies, the absolute numbers of pre-B and immature lymphoid B-(WW-IC) cell populations in these mice were reduced to 1.6% and 37% of wild-type levels, respectively (Fig. 3.4A,B).

Importantly, the myelo-proliferative 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 a modifier gene(s) in the FVB/N background (C3Hf/101 vs. C3Hf/101 x FVB/N; Fig. 3.3).

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. 3.5]. 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 timeframe where 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*) where hypoproliferation of hemopoietic precursors was observed (van der Lugt, 1994; Akasaka, 1996; Core, 1997; Takihara, 1997).

A



B

Flow cytometric analysis of hemopoietic cell populations in bone marrow and spleen of old eed3354/+ mutant mice

	Marker	Control Group A (%) (n=3)	eed 3354/+ (7-18 months follow-up) Group B (LP) (%) (n=4)	Group C (MPD) (%) (n=3)	Group D (n=1)
Bone marrow [a]	B220$^+$	28 ± 5	34 ± 9	0.7 ± 0.6	29
	Mac-1$^+$	61 ± 4	67 ± 0	95 ± 0.2	59
	B220$^+$CD43$^-$	6 ± 3	10 ± 6	0.7 ± 0.6	4
	B220$^+$IgM$^-$	9 ± 3	10 ± 7	0.1 ± 0.1	3
	Gr-1$^+$	64 ± 4	58 ± 12	91 ± 2	64
Spleen [b]	B220$^-$	58 ± 7	42 ± 19	23 ± 10	76
	B220$^+$IgM$^+$	48 *	20 *	8 ± 4	37
	IgM$^-$IgD$^-$	34 ± 9	28 ± 15	15 ± 15	53
	Mac-1$^+$	8 *	n.a.	58 ± 14	3
	Gr-1$^+$	5 ± 1	10 ± 7	57 ± 12	4
	B220$^+$CD43$^+$	2 ± 1	5 ± 2	1 ± 0.3	1

Fig. 3.3 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) cytospins, 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. 3.4). Note the infiltration by neutrophils (n) in all tissues of eed$^{3354/+}$ group C (MPD) mutant mice. Magnification, 100x for all, except for peripheral blood, 40x. 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 ± SD. (LP) lymphoproliferation; (MPD) myeloproliferative disease. [a] Absolute numbers of bone marrow cells per femur: Control group A 3.2 x 10^7 ± 0.2, *eed*$^{3354/+}$ group B (LP) 2.9 x 10^7 ±0.3, *eed*$^{3354/+}$ group C (MPD) 1.5 x 10^7 ±0.5., *eed*$^{3354/+}$ group D 2.1 x 10^7; [b] Absolute numbers of nucleated cells per spleen: Control group A 1.8 x 10^8 ±0.5, *eed*$^{3354/+}$ group B (LP) 1.4 x10^8 ±0.1, *eed*$^{3354/+}$ group C (MPD) 7.8 x 10^8 ±1.6., *eed*$^{3354/+}$ group D 2.4 x 10^8 . (*), Only one mouse analyzed.

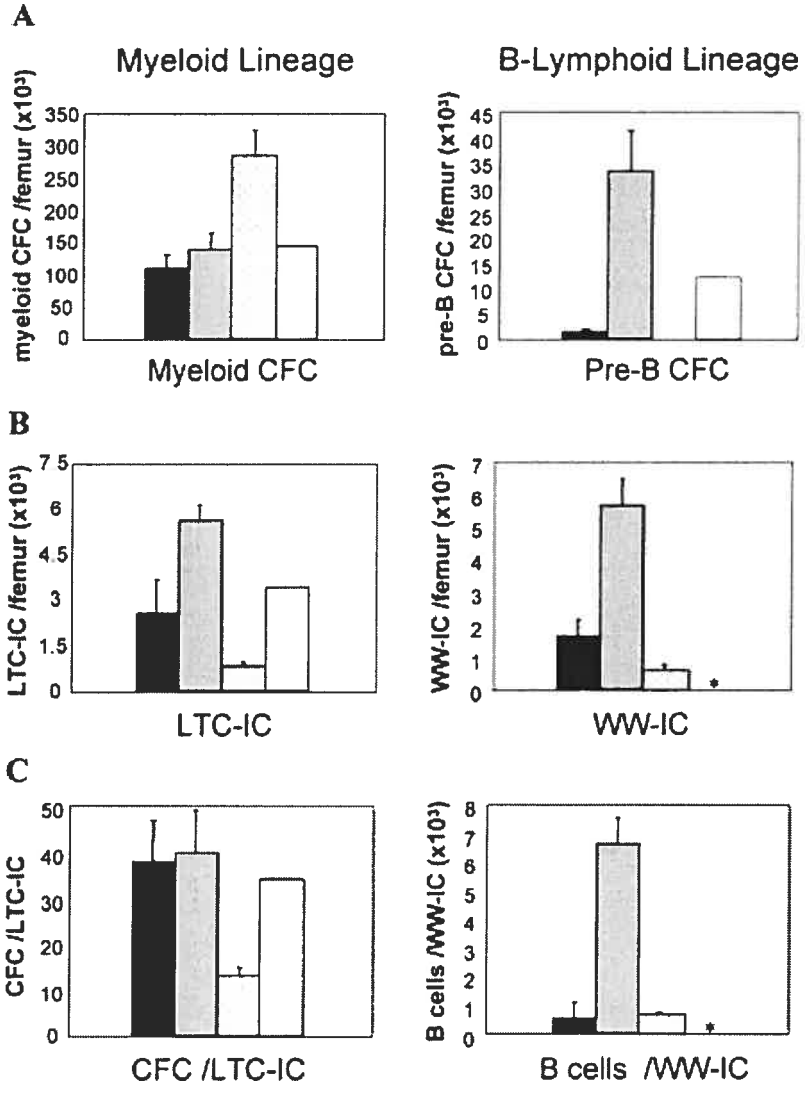


Fig. 3.4 Myelo- and lymphoproliferation occur in old *eed*$^{3354/+}$ mutant mice. (A) Femur-derived bone marrow cells were assayed in methylcellulose for myeloid and pre-B lymphoid CFCs. (B) *In vitro* long-term culture assays of primitive myeloid (LTC-IC) and lymphoid (WW-IC) bone marrow cells. The results, mean ± SD, 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).

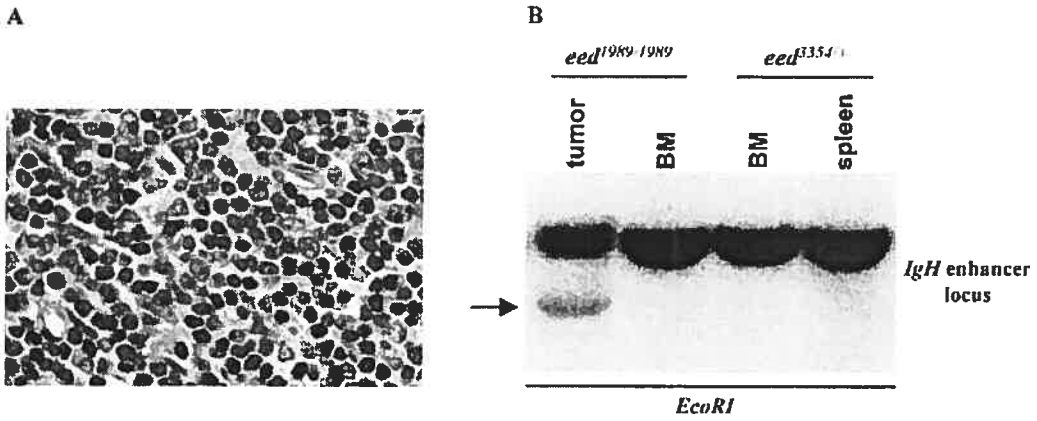


Fig. 3.5 Monoclonal B-cell tumor in a 3-month-old *eed*$^{1989/1989}$ mutant mouse. A) Histological analysis of a hematoxilin and eosin section of a thoracic tumor (1.5 grams). Note the infiltration by mature darkly stained lymphocytes. Magnification 100x. (B) Southern blot analysis of genomic DNA isolated 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.*

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 expresssion in primitive Sca^+ Lin^- bone marrow cells (Fig. 3.1), 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* double mutant mice were generated and their hemopoietic system analyzed.

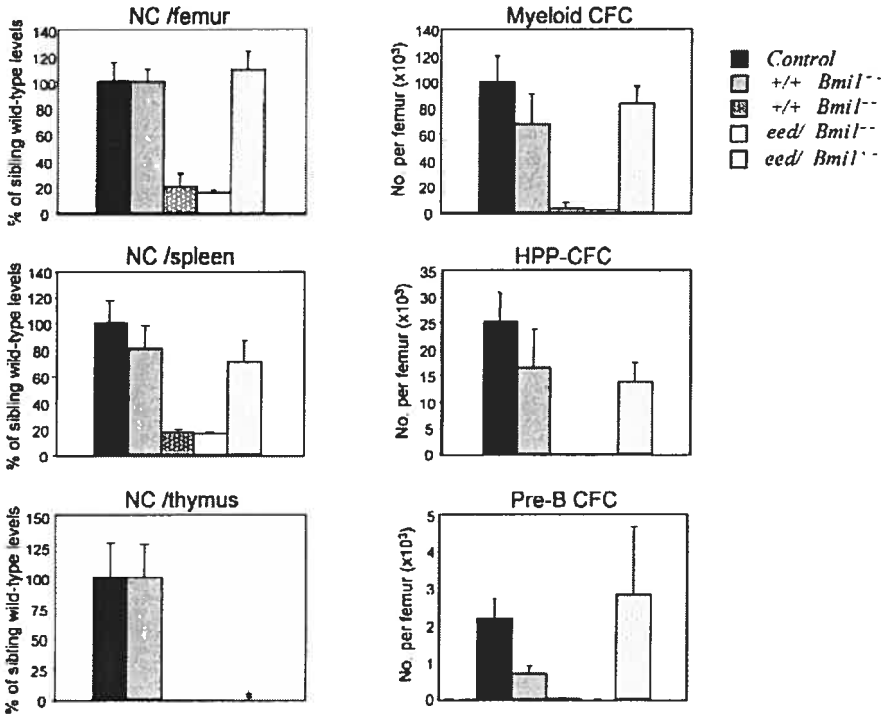
eed^{1989}*Bmi1*$^{-/-}$ and *eed*$^{3354/+}$*Bmi1*$^{-/-}$ double mutant mice were severely anemic, and *in vitro* colony formation assays of bone marrow progenitors cells isolated from these mice gave identical results to those observed with *Bmi1*$^{-/-}$ mice (Fig. 3.6A). Therefore, the loss of one or two functional alleles of *eed* did not alter the hemopoietic defects observed at the mature CFC level in *Bmi1*$^{-/-}$ mice. As previously reported, 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. 3.6A; 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. 3.6A). 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 Fig. 3.6B 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. 3.6B).

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. 3.6B). 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 ± 43 to 919 ± 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.

A



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
Bmi1$^{+/-}$ (n=5)	868 ± 326	275 ± 43	3139 ± 2270	16 ±12
Bmi1$^{-/-}$ (n=2)	15 ± 15	67 ± 16	34 ± 7	1 ± 0.1
eed/ Bmi1$^{+/-}$ (n=3) [c]	22 ± 9	73 ± 11	24 ± 8	1 ± 0.3
eed/ Bmi1$^{+/-}$ (n=4) [d]	1032 ± 128	919 ± 341[e]	996 ± 130	17 ± 7

Fig. 3.6 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 x 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. 3.2B. (B) Frequency and proliferative potential of primitive myeloid (LTC-IC) and primitive lymphoid (WW-IC) bone marrow cells in *Bmi1* versus *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. [a] Absolute 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).

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

Recent studies revealed a marked increase in the expression 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 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 Fig. 3.7 (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 undetectable 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 Fig. 3.7, 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.

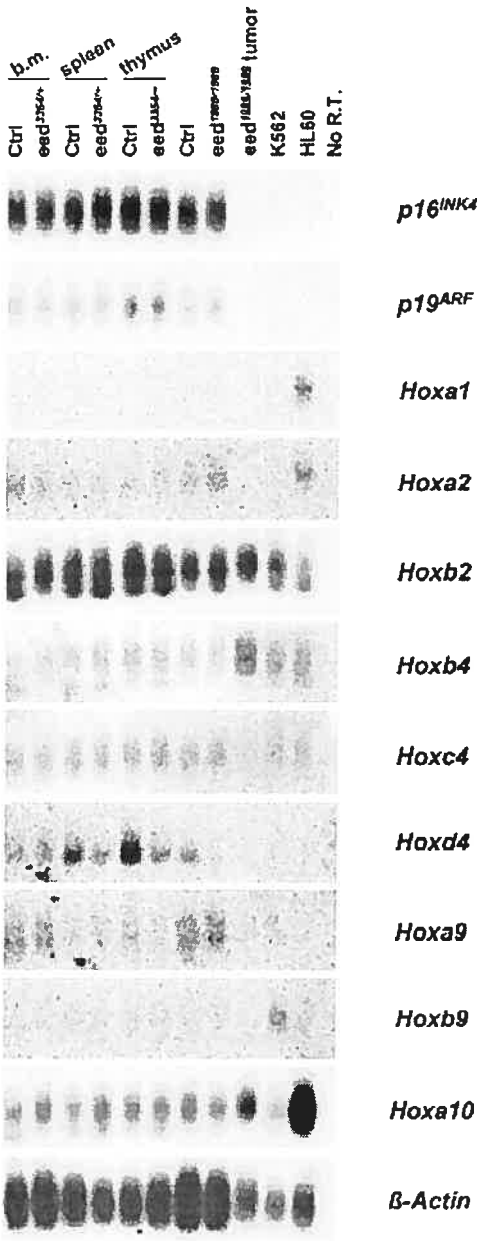


Fig. 3.7 Expression of $p16^{INK4a}$, $p19^{ARF}$, and selected *Hox* genes in various hemopoietic organs of *eed* mutant mice together with an $eed^{1989/1989}$ mutant mice presenting a monoclonal B-cell lymphoma. Isolation of total RNA from eed mutant hemopoietic cells is described in Material and 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 times for *Hoxc4*, *Hoxb4*, *Hoxa2*, *Hoxb9*, and *Hoxa1* was 20 hr; *Hoxd4* and *Hoxa9*, 26 hr (all on a phosphoImager cassette). Exposure

times 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. 3.5. (K562 and HL-60) 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.

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 anti-proliferative 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/Rae-28 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 (van Lohuizen et al., 1998b; Sewalt 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 distribution 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 (Gunster et al., 1997; Alkema et al., 1997; Satijn and Otte, 1999; Satijn et al., 1997). 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 (van Lohuizen et al., 1998; Denisenko et al., 1998; Sewalt et al., 1998). 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 (van Lohuizen et al., 1998; Denisenko et al., 1998). 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 heterogenous regulatory complexes at various target loci (Sinclair et al., 1998; DeCamillis et al., 1992; Strutt and Paro, 1997). Gene dosage effects and haploinsufficiency were reported in mice bearing one inactivated allele of the complex A *Bmi1*, *mel18*, *M33*, *Mph1/Rae-28* and complex B *eed PcG* genes (van der Lugt et al., 1994; Core et al., 1997; Akasaka et al., 1996; 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. In fact, the loss of a single *Bmi1* or *eed* allele inhibits and stimulates the proliferative activity of B-cell progenitors, respectively. By virtue of their co-expression 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. Since point mutations at the *eed* gene locus which 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 anterio-posterior (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 homologues 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 tissues 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* as reflected by the lower expression level of *Bmi1* in this cell population. Alternatively, qualitative differences, *e.g.* 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 downregulation 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* which 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 $p16^{INK4a}$ and $p19^{ARF}$, 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 $p16^{INK4a}$ and $p19^{ARF}$ expression levels were not altered in *eed* mutant cells.

These results lead to the model illustrated in Figure 3.8. 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 $p16^{INK4a}$ and $p19^{ARF}$. 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.

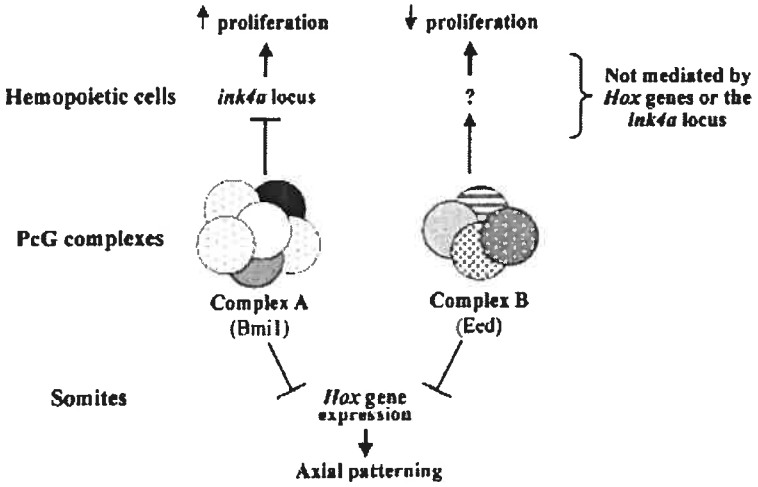


Fig. 3.8 Schematic representation of Eed and Bmi1 mediated effects in embryonic vs. hemopoietic development in mice. Eed and Bmi1 PcG proteins synergize to regulate differentiation of skelettal/bone precursor cells possibly through a common regulatory effect on *Hox* gene activity. Conversely, Eed and Bmi1 have opposing functions in regulating hemopoietic cell proliferation in mice that appear to be *Hox* gene-independent.

Materials and Methods

Animals

Production of eed^{1989} hypomorphic and eed^{3354} null mutant mice (101/R1 x C3Hf/R1 hybrid background) and $Bmi1^{-/-}$ (129Ola/FVB/N hybrid background) mice 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 offsprings. 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 analyses as described (van der Lugt et al., 1994). The $T^{1040}\rightarrow C$ transition at position 1040 in the *eed*^{3354} null allele destroys an *Alu1* 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 x 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 $T^{1031}\rightarrow A$ 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 messenger RNA isolated from purified bone marrow subpopulations was 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 µl of 5 M guanidium isothiocyanate solution. Nucleic acids were precipitated, and cDNA was synthezised with a 60-mer primer containing a 3' poly-thymidine 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); 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 *Eco*RI/*Pst*I fragment (#639), probe for *Hoxa1* was a *Sac*I/*Eco*RI fragment of 150-bp (#633), *Hoxa6* probe was a 250-bp *Sac*I/*Hind*III fragment (#634), *Hoxb4* probe was a *Eco*R1/*Pml*I fragment of 485-bp (#405), probe for *Hoxb2* was a 260-bp *Hinc*II/*Pst*I fragment (#419), *Hoxb3* probe was a 380-bp *Eco*RI fragment (#478), probe for *Hoxc4* was a 212-bp *Xba*I/*Xho*I fragment (#214), *Hoxd4* probe was a 765–bp *Pvu*II/*Hind*III/*Eco*RI fragment (#770), probes for *Hoxa9* and *Hoxa10* corresponded to full-length cDNAs and probe for *Hoxa2* was a 320-bp *Pst*I/*Hind*III fragment (#650). Probe specific for $p16^{INK4a}$ corresponded to exon 1α (nucleotides 5-197; accession no. AF044335, #775), $p19^{ARF}$ specific probe corresponded to exon 1β (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 of 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 cytospins preparations of colonies. For pre-B clonogenic progenitor assays, cells were plated in 0.8% methylcellulose in alpha medium supplemented with 30% pre-selected FCS (SCT, Vancouver B.C.), 10^{-4} M β-ME, and 2 ng/ml of 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 fibroblasts 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 μg/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 μM β-ME and, 50 μg/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 ug/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 titered 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.

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CHAPITRE 4

The *Polycomb Group* (*PcG*) Gene *embryonic ectoderm development (eed)* is a Negative Regulator of Bone Marrow Progenitor Cell Proliferation and Suppresses Radiation-Induced Lymphomagenesis in Mice

Julie Lessard, Amélie Faubert & Guy Sauvageau

(Manuscript in preparation)

Chapter 4 summarizes the experimental data obtained so far describing the tumor suppressive activity of the *eed Polycomb Group (PcG)* gene and its ability to suppress radiation-induced tumorigenesis in mice. Amelie Faubert, a graduate student in the laboratory, helped Julie Lessard with the cytofluometric analyses of the tumors (not shown) and the determination of their clonal composition by Southern blotting (Figures 4.4 and 4.6). This project is now being pursued by Martin Sauvageau, a graduate student in the laboratory.

The mouse Polycomb-group (PcG) gene *embryonic ectoderm development (eed)* is a component of the PcGe multimeric protein complex that governs anterior-posterior patterning of the axial skeleton by regulating *Hox* gene expression 1,2. The mouse gene encodes a 441-amino-acid protein with five WD motifs which, except for the amino terminus, is highly homologous to *Drosophila* ESC (Extra Sex Combs 3 and see Fig. 4.1). Beyond its role in embryonic development, eed is also involved in regulating hemopoiesis. Heterozygosity for a null allele of *eed* ($eed^{3354/+}$) causes marked myelo- and lympho-proliferative defects in mice (3-fold increase in primitive (LTC-IC and WW-IC) and 19-fold increase in late (myeloid and pre-B CFCs) bone marrow progenitor cell numbers on average), indicating that *eed* plays a crucial role in the negative regulation of the proliferative capacity of both lymphoid and myeloid progenitor cells (see Chapter 3). These data indicate that Eed performs important anti-proliferative activity on a subset of early and late bone marrow progenitors and that ultimately, its absence may lead to tumor formation.

The hyperproliferative defects observed mostly in the lymphoid B cell lineage of heterozygous $eed^{3354/+}$ null mice would suggest that *eed* performs tumor suppressive function in hemopoietic cells. To this possibility, a large cohort of mice homozygous for a hypomorphic allele of *eed* ($eed^{1989/1989}$) or heterozygous for a null allele of *eed* ($eed^{3354/+}$) were given a single dose of ionizing radiation (600 RADs) at 5 weeks of age and monitored for the development of disease. Homozygous $eed^{1989/1989}$ mutant mice developed a high incidence of B- and T-cell lymphomas with shorter latency when compared with control littermates (n=7/13 vs n=1/16; 16±4 wks vs 34±0 wks post-irradiation, respectively, Fig. 4.2). Heterozygous $eed^{3354/+}$ null mice were also more susceptible to radiation-induced B- and T- cell lymphomas than control littermates (n=11/15 vs n=4/15), although after a much longer latency period than homozygous $eed^{1989/1989}$ mice (27±9 wks vs 16±4 wks, respectively), indicating that *eed* performs tumor-suppressive function in lymphoid cells. Histological analysis of hematoxylin and eosin stained sections confirmed the diagnosis of lymphoma in all the tumors recovered from these animals (data not shown). Flow cytometric analyses performed on hemopoietic cells derived from the bone marrow, thymus, spleen and lymph nodes of

hypomorphic *eed*$^{1989/1989}$ animals revealed that 29% of the irradiation-induced lymphomas were of CD4^{+}CD8^{+} double positive (DP) origin, whereas 71% of the tumors analyzed corresponded to a CD8^{+} phenotype (Fig. 4.3). Importantly, no B cell and myeloid tumors were induced by the irradiation treatment. Since each developing T- or B-cell clone contains unique rearrangements at the *T cell receptor β (TCRβ)* and *Immunoglobin (Ig)* gene locus, respectively, clonality in these lymphomas was assessed by the pattern of *TCRβ* and *kC* gene rearrangements by Southern blot analyses. Irradiation-induced tumors in hypomorphic *eed*$^{1989/1989}$ and heterozygous *eed*$^{3354/+}$ mutant mice were of monoclonal or oligoclonal origin, suggesting that neoplastic transformation of *eed* mutant cells arises as a result of multiple genetic lesions that collaborate to generate a malignant clone (Fig. 4.4 and 4.6). In most cases, the neoplastic T-cell clones had already infiltrated the spleen and or the bone marrow of the animals, including one *eed* $^{1989/1989}$ clone (identified as B5 in Fig. 4.4) which presented rearrangement of both *Jβ2B* alleles. As expected, all *eed*$^{1989/1989}$ tumors presented a germ-line configuration of the *Ig kC* chain. Although one of the *eed*$^{1989/1989}$ bone marrow-derived genomic DNA samples revealed the over-representation of a B-cell clone (identified as B2), this clone could not be detected in the spleen and thymus of this animal. Moreover, cytofluometric analyses demonstrated a normal distribution of the B220^{+}CD43^{+}, B220^{+}IgM^{+} and IgM^{+}IgD^{+} B-cell populations in the BM of this animal (data not shown), potentially suggesting aberrant rearrangement of the B-cell receptor (BCR) in these cells.

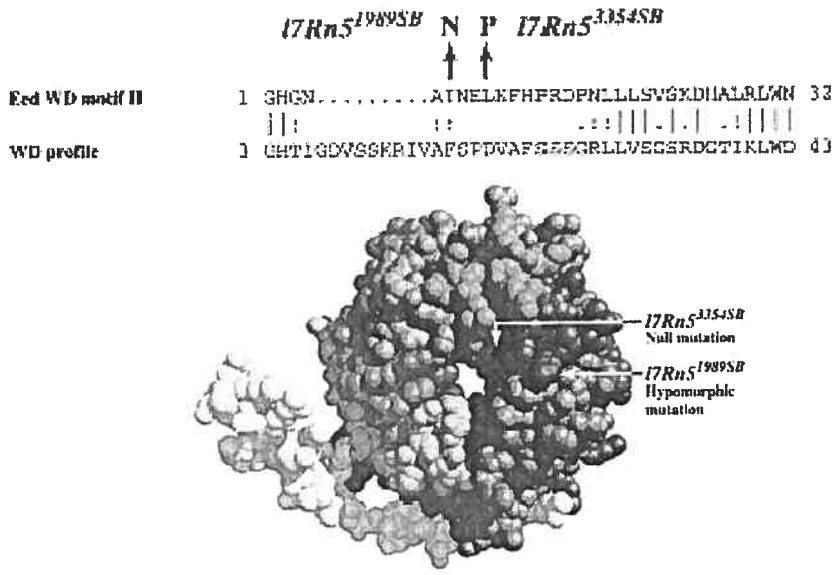

Fig. 4.1 Ethylnitrosourea (ENU)- induced point mutations at the *eed* locus in mouse. The proline substitution (L196P) in the second WD motif of the *l7Rn5*^{3354SB} null allele maps to the internal core of the inner end of the beta-propeller blade and is likely to disrupt protein folding. In contrast, the asparagine substitution (I193N) in the second WD motif of the hypomorphic *l7Rn5*^{1989SB} allele maps onto the surface of the beta propeller blade near the central cavity and may affect surface interactions without compromising propeller packing (Schumacher A. et al., (1998) *Genomics* 54(1):79-88).

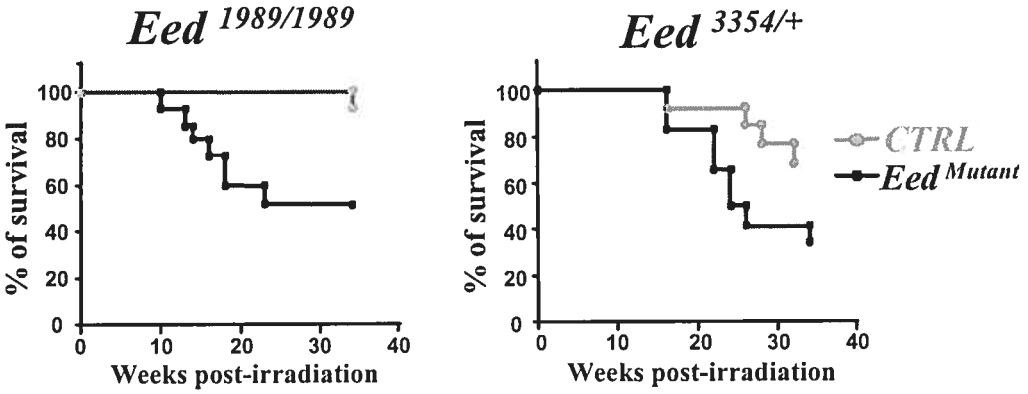

Fig. 4.2 Survival curves showing an increased insidence of irradiation-induced (600 RADs) lymphomas in *eed*$^{1989/1989}$ and *eed*$^{3354/+}$ mutant mice when compared to control littermates.

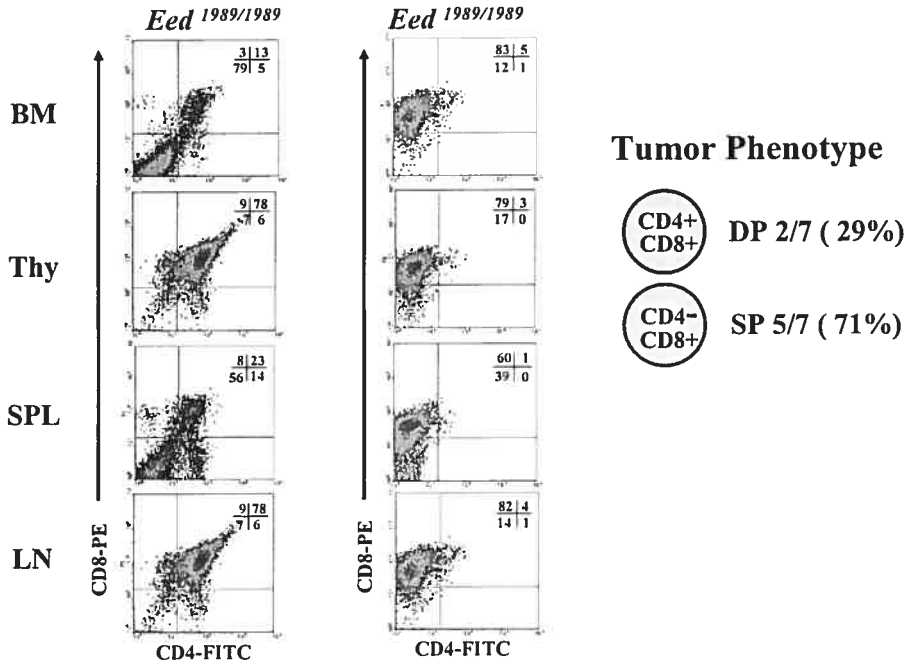

Fig. 4.3 Representative examples of T-cell lymphomas arising in irradiated *eed*$^{1989/1989}$ mutant mice. Left panel: a T-cell tumor of $CD4^+$ $CD8^+$ double positive (DP) origin. Right panel: T-cell lymphoma presenting a $CD8^+$ phenotype. Abbrev.: BM, bone marrow; Thy, thymus; SPL, spleen; LN, lymph node; PE, phycoerythrin; FITC, fluorescein isothiocyanate.

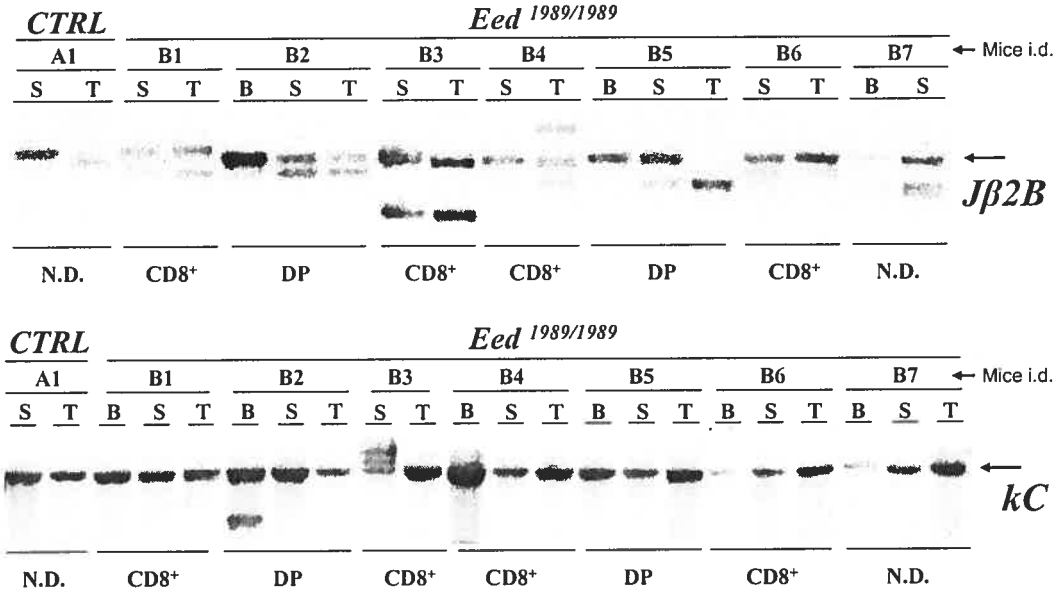

Fig. 4.4 Irradiation-induced lymphomas in *eed*$^{1989/1989}$ mice are of monoclonal or oligoclonal origin. Genomic DNA isolated from the hemopoietic organs of control and *eed*$^{1989/1989}$ irradiated animals was digested with *Hind*III (upper panel) or *Eco*RI/*Bam*H1 (lower panel) and hybridized to probes capable of detecting rearrangements at the *T cell receptor (TCR)* and *Immunoglobulin (Ig)* gene locus (*Jβ2B* probe, Malissen (1984) Cell 37: 1101 and *kC* probe, Leder (1986) Cell 45:485, respectively). Abbrev.: S, spleen; T, thymus; B, bone marrow; N.A., not analyzed; DP, double positive; i.d., identification.

As revealed by FACS analyses, 36% (n=4/11) of the *eed*$^{3354/+}$ irradiation-induced lymphomas analyzed expressed phenotypes corresponding to immature cortical thymocytes, whereas 27% (n=3/11) of the tumors were of B-cell origin (Fig. 4.5). *Eed*$^{3354/+}$-derived T-cell lymphomas included CD4$^+$CD8$^+$ double positive (DP) tumors (Fig. 4.5, right panel for representative example) and CD8$^+$ single positive (SP) tumors, expressing variable levels of CD4 (data not shown). One of the monoclonal B-cell lymphomas that developed in the *eed*$^{3354/+}$ background was strongly positive for the B220$^+$ and IgM$^+$ surface antigens (Fig. 4.5, left panel) and histological examination of hemopoietic cells derived from this animal confirmed the presence of malignant lymphoid cells presenting a fragmented nuclei, reminiscent of a mantle B-cell lymphoma (MCL) (data not shown). Clonal analysis performed on BM and thymus-

derived cells from this animal (identified as B3 in Fig. 4.6) revealed the presence of a unique rearrangement of the *kC* chain in those organs as well as 2 other animals, identified as B4 and B9 (Fig. 4.6, upper panel). Unique rearrangements at the *JB2β* gene locus were observed in at least one of the hemopoietic organs analyzed from three homozygous $eed^{3354/+}$ mice (identified as B2, B3 and B5, Fig. 4.6, lower panel) and two control littermates (A1 and A2). Finally, with the probes used, no clonal rearrangement of the *JB2β* and *kC* genes could be detected in the hemopoietic organs of three $eed^{3354/+}$ animals (identified as B1, B6 and B7).

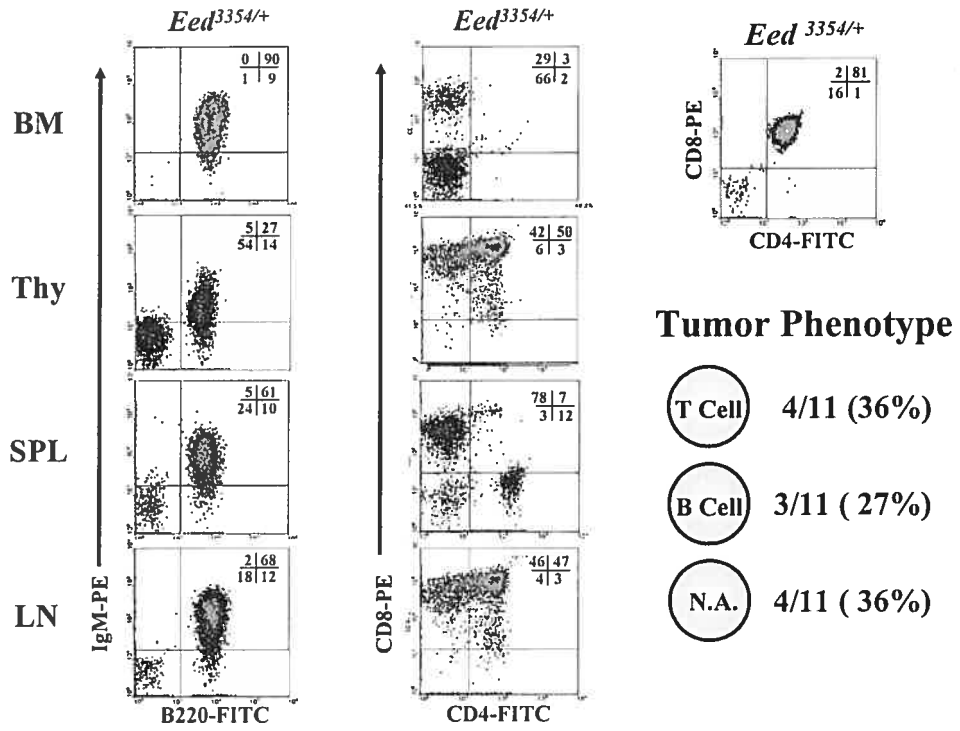

Fig. 4.5 Representative examples of B- and T-cell abnormalities arising in $eed^{3354/+}$ irradiated mice, as analyzed by fluorometric activated cell sorting (FACS). Left panel: B-cell tumor of $B220^{+}IgM^{+}$ origin, most likely of the mantle cell type (MCL). Right panel: $CD4^{+}CD8^{+}$ double positive (DP) T-cell lymphoma. Abbrev.: BM, bone marrow; Thy, thymus; SPL, spleen; LN, lymp node; PE, phycoerythrin; FITC, fluorescein isothiocyanate; N.A., not analyzed.

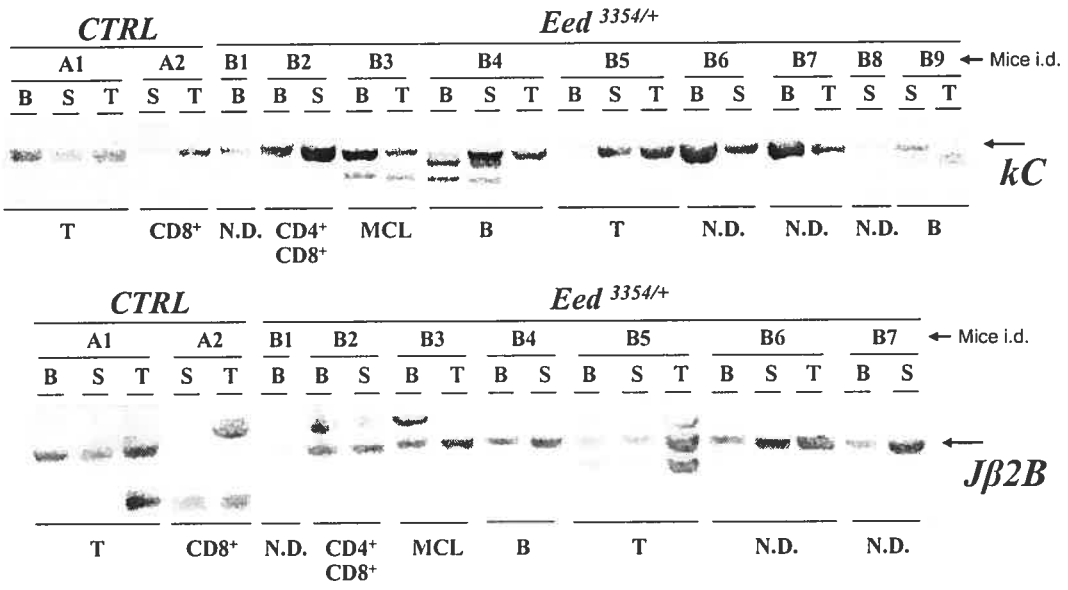

Fig. 4.6 Irradiation-induced lymphomas in *eed*$^{3354/+}$ mice are monoclonal or oligoclonal. Genomic DNA isolated from the hemopoietic organs of control and *eed*$^{3354/+}$ irradiated animals was digested with *Eco*RI/*Bam*H1 (upper panel) or *Hind*III (lower panel) and hybridized to probes capable of detecting rearrangement at the *Immunoglobulin (Ig)* and *T cell receptor (TCR)* gene locus (*kC* probe, Leder (1986) Cell 45:485 and, *Jβ2B* probe, Malissen (1984) Cell 37: 1101, respectively). Abbrev.: B, bone marrow; S, spleen; T, thymus; MCL, mantle cell lymphoma; N.D., not determined.

The observation that the onset and mean latencies to lymphomagenesis are decreased in the hypomorphic *eed*$^{1989/1989}$ background when compared to the heterozygous *eed*$^{3354/+}$ null background suggests that *eed* performs tumor-suppressive function in lymphoid cells. To answer this question, it would be imperative to verify whether the wild-type *eed* allele is lost and/or mutated in heterozygous *eed*$^{3354/+}$ null mice. As point mutations at the *eed* gene locus that account for the *eed*^{3354} (and *eed*^{1989}) alleles disrupt its interaction with both the Enx and HDAC partners [5,6], co-immunoprecipitation experiments could be performed in order to verify whether the Eed-Enx1 and/or the Eed-HDAC interaction is perturbed in heterozygous *eed*$^{3354/+}$ null tumors. Rescue experiments involving the overexpression of wild-type *eed* (versus *EGFP* as a control) in *eed*$^{1989/1989}$ and *eed*$^{3354/+}$ lymphomas will assess the cellular and molecular

mechanisms employed by the PcGe complex to suppress the proliferation of lymphoid cells. As the ability E(z) (the fly homologue of Enx) to methylate lysine 27 of histone 3 is dependent on the presence of Esc (Eed) in the complex [7], it is plausible that these include epigenetic modifications of chromatin structure, such as methylation of the core histones. Of note, we recently demonstrated a significant decrease in the levels of H3-K27 methylation in all (n=7/7) *eed*$^{1989/1989}$-derived T cell tumors analyzed when compared to that derived from control littermates (all MMLV-induced T-cell lymphomas, see Fig. 4.7). Furthermore, it is interesting to note that the chromosomal region containing the *eed* locus on human chromosome 11 (11q14.2-22.3) is encompassed by the *cyclin D1 (PRAD1)* (11q14.2) and *Ataxia Telangiectasia Mutated (ATM)* (11q22.3) genes, both of which are regarded as cytogenetic hallmarks of mantle cell lymphoma (MCL) in humans ([8-11] and Fig. 4.8). The presence of tumor suppressor genes in this region is strongly suggested by the recurrent deletion of distinct chromosomal segments at 11q in several reported cases of B-cell chronic lymphocytic leukemia (B-CLL), mantle cell lymphoma (MCL) and T-cell prolymphocytic leukemia (T-PLL) (reviewed in [12]). As compelling evidence indicates that *eed* controls the proliferative potential of lymphoid progenitor cells in mice by acting as a tumor suppressor, it is imperative to investigate whether these genes are allelic with *eed*. Finally, it will be important to determine whether *Enx1* and/or *Enx2* null animals also develop tumors, thereby ascribing the tumor-suppressor function to the Eed-Enx PcGe complex (Fig. 4.9).

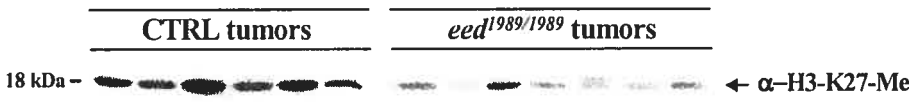

Fig. 4.7 Decreased methylation levels at the lysine 27 (K27) residue of histone H3 (H3-K27) in *eed*$^{1989/1989}$ MMLV-induced T-cell lymphomas when compared to that isolated from control littermates. Briefly, 55 ug of proteins from total cellular extracts were hybridized with an affinity purified chicken anti-H3-K27-me antibody recognizing specifically the dimethylated form of H3-K27 (kindly provided by Dr Yi Zhang, University of North Carolina).

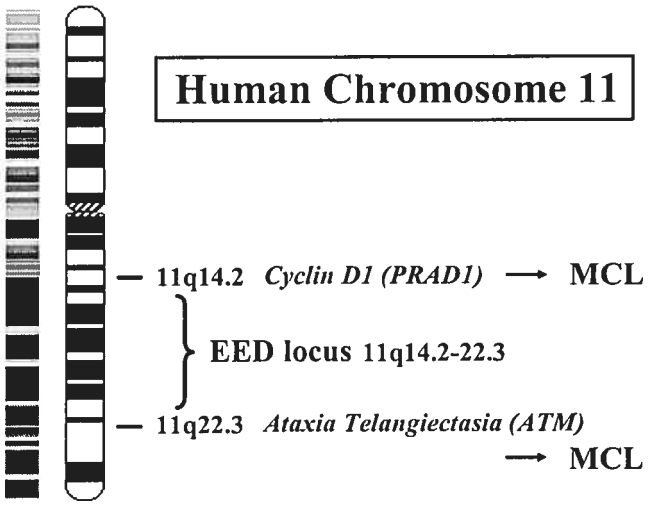

Fig. 4.8 Structural aberrations at 11q are among the most common aberrations involved in lymphoproliferative disorders in humans. The presence of tumor suppressor gene(s) in this region has been suspected and may involve *eed,* which is found at (11q14.2-22.3) on the human chromosome 11 (reviewed in Monni O., (2001) *Leuk Lymphoma* 40:259-66).

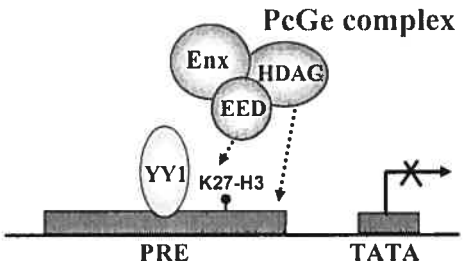

Fig. 4.9 Schematic representation of the Eed-containing PcGe complex in mammalian cells. Of note, *Drosophila* Eed (Esc) is essential for the ability of the complex to methylate the lysine 27 residue of histone H3 (through the SET domain-containing Enx protein), a key event in its ability to induce gene silencing through chromatin remodeling (Cao R et al., (2002) *Science* 298:1039-43). Whether this epigenetic

mechanism underlies the tumor suppressive function of Eed in lymphoid cells is currently being investigated.

The requirement for exposure to genetoxic agents and the long latency period before tumor development in *eed* mutants indicate that additional genetic events are required for "full transformation" of hemopoietic cells lacking a functional *eed* gene. Genetic screens for modifiers and oncogenic collaborators of *eed* function in hemopoiesis and tumorigenesis are eagerly awaited. A proviral insertional mutagenesis screen in hypomorphic $eed^{1989/1989}$ neonates, using the Moloney murine leukemia virus (MMLV), was recently undertaken by myself and is now pursued by a graduate student in the laboratory to identify genes that collaborate with *eed* in tumorigenesis. Proviral mutagenesis frequently disrupts transcription units by genomic rearrangement at the integration site and/or alters expression levels of nearby genes due to interference with regulatory elements. Cloning of collaborating oncogenes will take advantage of the retroviral tagging of the chromosomal integration site, which can be recovered by inverse PCR on genomic DNA isolated from these tumors. Initial validation of candidate oncogenic collaborators and potential modifiers of *eed* function will be elucidated in hemopoietic chimeras and/or transgenic mice.

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CHAPITRE 5

ARTICLE

Bmi-1 Determines the Proliferative Capacity of Normal and Leukaemic Stem Cells

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Chapter 5 describes a study which established the critical role of the *Bmi-1 PcG* gene in determining the proliferative capacity of both normal and leukaemic stem cells. Importantly, this work was the first to provide a molecular basis for the concept that stem cell function (whether normal or neoplastic) is regulated by common genetic determinants and, potentially, has broad implications in the field of stem cell biology. Julie Lessard generated all the results presented in this chapter and wrote the paper under the supervision of Dr Guy Sauvageau.

Abstract

An emerging concept in the field of cancer biology is that a rare population of 'tumour stem cells' exists among the heterogeneous group of cells that constitute a tumour. This concept, best described with human leukaemia, indicates that stem cell function (whether normal or neoplastic) might be defined by a common set of critical genes. Here we show that the *Polycomb group* gene *Bmi-1* has a key role in regulating the proliferative activity of normal stem and progenitor cells. Most importantly, we provide evidence that the proliferative potential of leukaemic stem and progenitor cells lacking *Bmi-1* is compromised because they eventually undergo proliferation arrest and show signs of differentiation and apoptosis, leading to transplant failure of the leukaemia. Complementation studies showed that *Bmi-1* completely rescues these proliferative defects. These studies therefore indicate that *Bmi-1* has an essential role in regulating the proliferative activity of both normal and leukaemic stem cells.

Introduction

In humans, the concept of "tumor stem cell" is best described in acute myeloid leukemias (AMLs) in which the majority of the leukaemic cells (blasts) display a limited proliferative capacity and must therefore be constantly regenerated by the rare "leukaemic stem cell" (L-HSC) population, which is itself capable of extensive self-renewal[1-3] (see also Fig. 5.1a). Interestingly, these L-HSCs share phenotypical similarities (but also some differences, such as CD123 expression[4,5]) with normal haematopoietic stem cells (HSCs)[1].

Several arguments suggest that the *Polycomb Group (PcG)* gene *Bmi-1* is a good candidate for regulating the proliferative activity of both normal and leukaemic HSCs. First, *Bmi-1* expression is restricted to primitive bone marrow cells in both humans and mice[6-8] and is expressed in all myeloid leukaemias analysed so far[6] including the L-HSC-enriched $CD34^{++}$ fraction (Fig. 5.1b). Second, homozygosity for a null allele of

Bmi-1 causes a profound and progressive failure of the entire haemopoietic system in mice that is probably responsible for their death in early adulthood or before[9]. This progressive and fatal depletion of all blood cells (including primitive progenitors[7]) indicates that *Bmi-1* is not required for the generation of HSCs derived from fetal liver but essential for adult HSCs. Third, in addition to this presumed function in HSCs, *Bmi-1* has a critical and dose-dependent role in regulating the proliferative output of primitive progenitors derived from bone marrow[7].

Here we provide evidence demonstrating that *Bmi-1* is dispensable for the generation of stem and progenitor cells derived from fetal liver, but is absolutely necessary for their full proliferative potential. Most interestingly, our studies indicate that this function of *Bmi-1* is preserved in leukaemic stem and progenitor cells and therefore provide a molecular basis for the concept that common genetic determinants regulate normal and leukaemic stem cells.

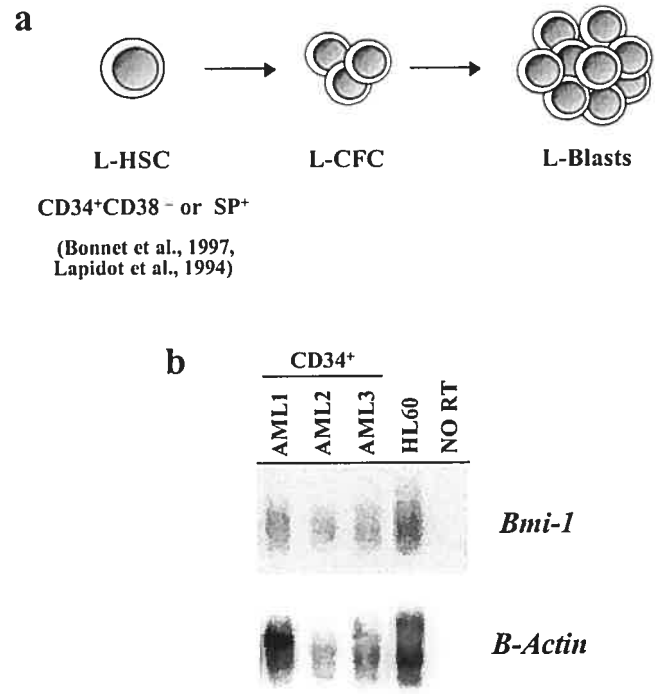


Fig. 5.1 *Bmi-1* is expressed in $CD34^+$ leukaemic cells. a, Cellular hierarchy in human (xenogenic) acute myeloid leukemia (AML) and phenotypic presentation of the leukaemic hemopoietic stem cells (L-HSCs). b, *Bmi-1* is expressed in purified human $CD34^{++}$ leukaemic cells isolated from AML patients. Proportion of $CD34^{++}$ cells represented 35% (AML1), 8% (AML2) and 83% (AML3) of total leukaemic bone marrow cells. *Bmi-1* signal intensity was not significantly different between $CD34^{++}$ cells vs unsorted cells. Twenty thousand cells were isolated from each leukemia sample (>98% purity for CD34 upon reanalysis), and their total RNA was reverse-transcribed and PCR-amplified (see methods). Human HL60 cells were used as a positive control for *Bmi-1* expression. Membranes were hybridized to probes specific for *Bmi-1* and *β*–Actin and exposed for 48 hrs on a PhosphoImager screen. L-CFC, leukaemic colony-forming cells; SP, side population; NO RT, no reverse transcriptase.

Results

Function of *Bmi-1* in stem and progenitor cells

Bmi-1$^{-/-}$ E14.5 fetal liver cells grown for 4 days *in vitro* (Fig. 5.2a) present a 27-fold reduction in their frequency of myeloid progenitors when compared to *Bmi-1*$^{+/+}$ controls (Fig. 5.2c, left panel). This progenitor cell defect was quantitatively and qualitatively rescued by the retroviral transduction (94-100% gene transfer) of *Bmi-1* in these cells (see Fig. 5.2c, right panel), indicating that *Bmi-1* is dispensable for the generation of myeloid progenitors derived from fetal liver but absolutely essential for their full proliferative activity.

On the basis of these results, it can be inferred that a similar situation might occur at the long-term repopulating cell (LTRC) level. An inability of bone marrow and E14.5 fetal-liver-derived *Bmi-1*$^{-/-}$ cells to contribute to long-term haematopoiesis was recently shown in reconstitution experiments, suggesting that *Bmi-1* plays a key role in self-renewal/proliferation of primitive haematopoietic cells[24]. Similarly, an experiment performed by our group (with the same group of cells as described in Fig. 5.2c, transplanted at about one LTRC per sublethally irradiated recipient) confirmed the presence of roughly normal numbers of cells with long-term repopulating potential in the fetal liver of *Bmi-1*$^{-/-}$ mice but the detection of these cells, at 16 weeks after transplantation, was strictly dependent on the retroviral expression of *Bmi-1* (Fig. 5.2d, three of three recipients were reconstituted with *Bmi-1*-transduced cells, whereas no reconstitution was observed in five of five recipients of a similar number of cells transduced with enhanced green fluorescent protein (EGFP)). As might be expected from previous experiments in which near-limiting numbers of stem cells are transplanted, the proportion of EGFP-expressing lymphoid and myeloid cells was at best 5.6% in the peripheral blood of these mice (nos 53-55 in Fig. 5.2d). The long-term (16 weeks) and pluripotent potential of these cells confirmed that the rescue was occurring at the HSC level.

Together, these complementation studies confirmed the critical function of *Bmi-1* once stem and progenitor cells have reached a given developmental stage. Considering the striking parallel between the organization of normal and leukaemic haematopoiesis and the knowledge that *Bmi-1* is expressed in all primary leukaemia and leukaemic cell lines analysed so far (see introduction and Fig. 5.1b), we sought to investigate the importance of *Bmi-1* in the biology of leukaemic stem and progenitor cells.

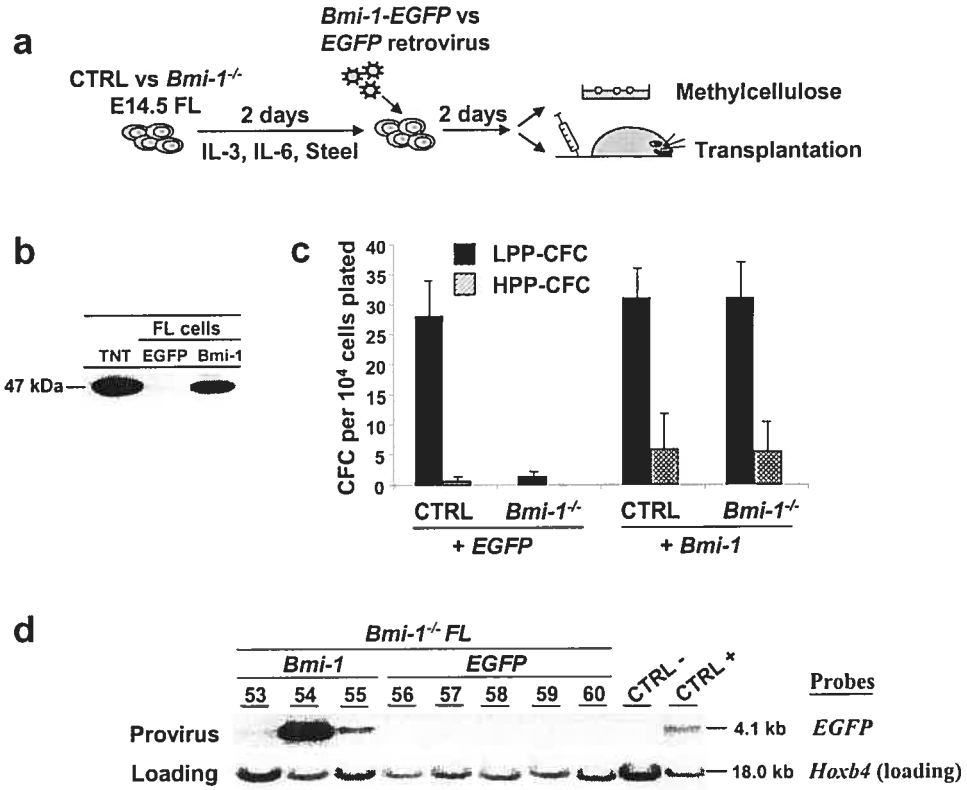


Fig. 5.2 *Bmi-1* regulates the proliferative potential of embryonic day (E) 14.5 fetal liver (FL)-derived haematopoietic cells. **a**, Overview of the experimental strategy. Ctrl, control; IL, interleukin. **b,** Western blot analysis of total cell extracts isolated from fetal liver cells engineered to express *Bmi-1* (lane 3) or not (lane 2). Lane 1, transcribed and translated (TNT) Bmi-1 protein. **c,** *Bmi-1*$^{-/-}$ and *Bmi-1*$^{+/+}$ fetal liver cells engineered to express *EGFP* or *Bmi-1-EGFP* were assayed for their content in colony-forming cells (CFC). HPP-CFC and LPP-CFC indicate CFC with high and low proliferative potential. **d,** Southern blot analysis to detect the integrated proviruses in DNA (digested with *Kpn*I) from the bone marrow of recipients of *Bmi-1*$^{-/-}$ fetal liver cells transduced with *Bmi-1* (lanes 1-3) or with *EGFP* only (lanes 4-8). kb, kilobases.

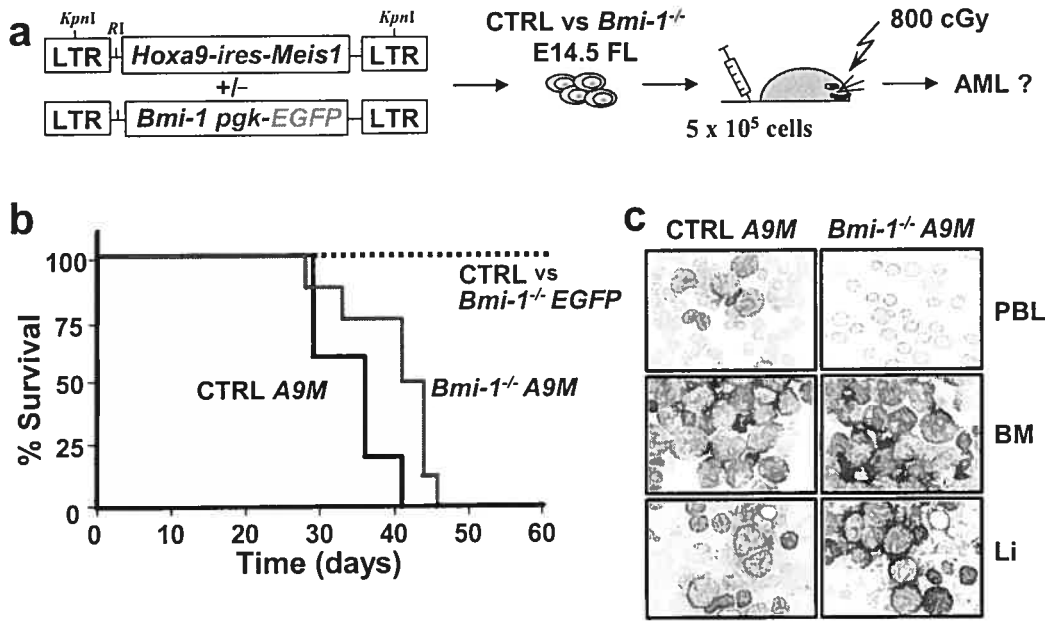
Generation of leukemia in *Bmi-1*$^{-/-}$ and *Bmi-1*$^{+/+}$ FL cells

To determine whether *Bmi-1* might also be essential in leukaemic haematopoiesis as it is in the normal situation, we first investigated whether stem or progenitor cells lacking *Bmi-1* could be transformed by a set of complementary oncogenes, and whether these leukaemic cells could self-renew and proliferate sufficiently to induce AML in mice. To achieve this, *Bmi-1*$^{-/-}$ fetal liver cells were infected with a retrovirus containing both the *Hoxa9* and *Meis1a* genes, and transduced cells were transplanted into sublethally irradiated syngeneic hosts (Fig. 5.3a). The selection of the *Hoxa9* and *Meis1* collaborating oncogenes was based on several criteria: first, these genes generate a well-characterized myeloid leukaemia in mice that displays a similar cellular hierarchy (namely L-HSCs and "mature" blasts) to that observed in human patients[10]; second, these two oncogenes are sufficient to induce full transformation of primary haematopoietic cells without the need for additional genetic events[10]; third, the HSCs, or a close progeny, are the target for the transformation induced by these genes (ref.10 and U. Thorsteinsdottir, P. Austin and G. Sauvageau, unpublished data); and last, the *Hoxa9* oncogene is involved in myeloid leukaemia in humans[11,12].

Regardless of the genotype (that is, *Bmi-1*$^{+/+}$ or *Bmi-1*$^{-/-}$), all recipients of *Hoxa9-Meis1*-transduced fetal liver cells developed AML within a similar latency period (Fig. 5.3b). The simultaneous overexpression of *Bmi-1* did not accelerate the occurrence of the leukaemia in all groups analysed, nor did it change their phenotype or clinical presentation (except for one parameter; see below and Fig. 5.3c-d). The leukaemic mice from all groups had a very similar and aggressive disease (see Fig. 5.3c-d for a detailed description). When analysed by fluorescence-activated cell sorting (FACS), the leukaemic blasts were strongly positive (more than 96%) for the Mac-1 myeloid cell surface antigen and 13-33% co-expressed Gr-1 (data not shown).

Although the *Bmi-1* status of the transformed fetal liver cells did not affect the phenotypical and clinical characteristics of the leukaemias, the presence of *Bmi-1* seemed to be important for the capacity of large numbers of leukaemic cells to accumulate in the peripheral blood (Fig. 5.3d, group A versus group B, eighth column). This phenotype was dependent on *Bmi-1* because it was complemented by the retroviral introduction of *Bmi-1* in *Bmi-1*$^{-/-}$ fetal liver cells (Fig. 5.3d, group A versus group D, eight column).

A northern blot analysis of total RNA isolated from the leukaemic cells confirmed high expression levels of retrovirally derived mRNAs (data not shown). DNA analysis of proviral integration sites in both wild-type and *Bmi-1*$^{-/-}$ leukaemic cells demonstrated that the *Hoxa9-Meis1*–induced AMLs were derived from two to four leukaemic clones (data not shown). Because a leukaemic mouse contains at least 10^9 leukaemic cells, this indicates that *Bmi-1*$^{-/-}$ leukaemic cells displayed a substantial proliferative capacity *in vivo*. However, the lower leukaemic cell numbers in the peripheral blood of recipients of *Bmi-1*$^{-/-}$ cells (Fig. 5.3d, group B) could indicate a progressive exhaustion of the *Bmi-1*$^{-/-}$ L-HSCs.



d

Characterization of Leukemias Developing in E14.5 Fetal Liver Transplantation Chimeras

	Retroviruses	Groups	Time to AML (days) *	Spleen weight (g)	Organ Infilt.†	%blast in BM‡	# WBC/ field‡
CTRL	*A9M* (5/5)	A	34±5	0.4±0.1	yes	80±12	22±25
	A9M + *Bmi-1* (3/3)	C	36±5	0.4±0.2	yes	84±6	46±40
Bmi-1$^{-/-}$	*A9M* (10/10)	B	37±8	0.3±0.1	yes	91±1	1±1
	A9M + *Bmi-1* (5/5)	D	31±11	0.3±0.1	yes	86±14	28±21

Fig. 5.3 *Bmi-1* is dispensable for the generation of AML in primary recipients. **a,** Summary of the experimental strategy and retroviruses used. Ctrl, control; FL, fetal liver; LTR, long terminal repeat. **b,** Survival graph of mice reconstituted with control or *Bmi-1*$^{-/-}$ E14.5 fetal liver cells engineered to express either *Hoxa9-Meis1* (*A9M*) or *EGFP*. **c,** Cytological preparations of peripheral blood leukocytes (PBL), bone marrow (BM) and liver (Li) of representative leukaemic mice from each group shown in **b**. **d,** Major characteristics of the leukaemia that occurred in each cohort. * Mean ± SD; † organs infiltrated were lung, liver, kidney; ‡ n = 200 cells counted from three representative mice. WBCs, white blood cells.

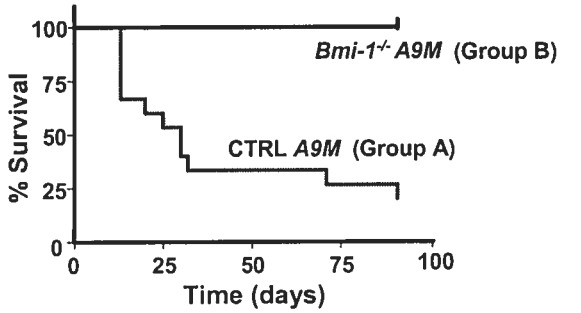
Bmi-1$^{-/-}$ AMLs do not repopulate secondary recipients

To evaluate a possible exhaustion of *Bmi-1*$^{-/-}$ L-HSCs, a dose of 10^5 leukaemic cells derived from the bone marrow of three different primary recipients from group A (control *Hoxa9-Meis1*) and group B (*Bmi-1*$^{-/-}$ *Hoxa9-Meis1*) were transplanted into syngeneic hosts (n = 5 secondary recipients per primary leukaemic mouse). Mice were then monitored over time for the development of AML. Strikingly, *Bmi-1* seemed to be essential for the maintenance of *Hoxa9-Meis1* L-HSCs *in vivo* because the leukaemias generated in *Bmi-1*$^{-/-}$ cells were not maintained on transplantation into secondary hosts (Fig. 5.4a, group B). However, animals bearing control *Hoxa9-Meis1* transplanted tumours developed AML within 30 ± 25 days after transplantation (Fig. 5.4a-b, group A). Clonal analyses of proviral integration sites indicated these leukaemias originated from several clones (Fig. 5.4c, and data not shown).

As mentioned above, none of the secondary recipients of *Bmi-1*$^{-/-}$ *Hoxa9-Meis1* leukaemic bone marrow cells developed AML (Fig. 5.4a) and no leukaemic cells were detected in long-term recipients analysed at 4 and 11 months after transplantation (Fig. 5.4b-c, and data not shown, respectively).

This difference in the "transplantability" between *Bmi-1*$^{-/-}$ and control AMLs is particularly significant considering that all (n = 3) of the secondary recipients receiving as few as 1 000 leukaemic cells from the control "group A" developed AML within 27 ± 9 days of transplantation (data not shown). In all cases, the invasive behaviour of the control *Hoxa9-Meis1* tumours was reproduced after transplantation, and the morphology and phenotype of the leukaemias were indistinguishable (data not shown). Interestingly, leukaemias developing in two of three primary recipients of *Bmi-1*$^{-/-}$ *Hoxa9-Meis1* fetal liver cells co-infected with *Bmi-1* (group D) were transferred to secondary recipients within a similar timeframe than control AMLs (group A) (data not shown). This suggests that the transplantation of the leukaemic phenotype is dependent on the continuous expression of *Bmi-1*.

a



b

Characterization of Secondary Recipients of 10^5 CTRL vs *Bmi-1*$^{-/-}$ *Hoxa9 + Meis1 (A9M) Transduced* Bone Marrow –Derived Leukemic Cells

FL cells day 14.5	Secondary Recipients	Time to AML (days) *	Spleen weight (g)	Organ infilt.†	% Blast in BM‡	Disease
CTRL	22.1 - 22.4	29±3	0.3±0.03	yes	87±8	AML
	24.1 - 24.5	13±0	0.4±0.05	yes	92±2	AML
	25.1 - 25.3	61±37	0.2±0.07	yes	85±6	AML
Bmi-1 $^{-/-}$	64.1 - 64.3	sacrificed	0.08±0.01	no	16±6	NED
	69.1 - 69.3	sacrificed	0.09±0.06	no	13±6	NED
	70.1 - 70 3	sacrificed	0.1±0.02	no	18±10	NED

c

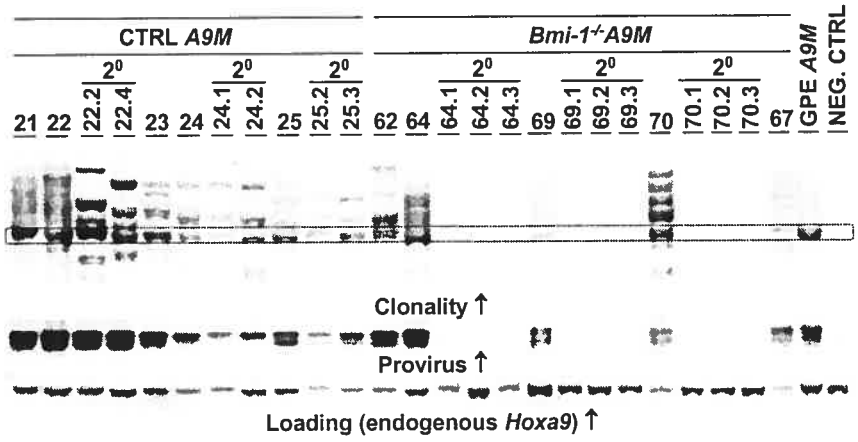


Fig. 5.4 *Bmi-1* is essential for the "transplantability" of AML into secondary hosts. **a,** Survival graph of secondary recipients of control (Ctrl) and *Bmi-1*$^{-/-}$ leukaemic cells (see Fig. 5.3). **b,** Characterization of secondary recipients shown in **a**. Abbreviations and details are as in Fig. 5.3d. FL, fetal liver; NED, no evidence of disease. **c,** Southern blot analyses of DNA isolated from the bone marrow of primary and secondary recipients detailed in Fig. 5.3d and 5.4b. 2^0: Secondary recipients. Top panel versus middle and lower panels show *Eco*RI versus *Kpn*I restriction, respectively. The probe was *Hoxa9*.

The bands enclosed in the dotted box in the top panel correspond to a specific fragment of proviral *Hoxa9*-derived DNA that does not represent clonality (inappropriate activity?). GPE *A9M*, viral producers of *Hoxa9-Meis1* retrovirus; Neg. Ctrl, GPE cells only.

In vitro properties of *Bmi-1*$^{-/-}$ leukaemic cells

To explain the apparent exhaustion of the *Bmi-1*$^{-/-}$ leukaemic clones *in vivo*, we monitored their survival and growth characteristics *in vitro*. After 10 days of growth, the cellularity of cultures initiated with *Bmi-1*$^{-/-}$ leukaemic cells was decreased 15 (±4)-fold relative to controls (namely *Bmi-1*$^{+/+}$; data not shown). Supporting these findings, a direct comparison between control and *Bmi-1*$^{-/-}$ leukaemic cells indicated that the latter had the following properties: first, they displayed accumulation in G1 (Fig. 5.5a-b) and reduction in S-phase (Fig. 5.5c); second, they underwent much greater morphological changes consistent with differentiation to butyrate-positive adherent layer-forming macrophages; third, they showed an increased proportion of apoptotic cells (Fig. 5.5d); and last, they were less efficient in generating leukaemic colony-forming cells (Fig. 5.5e).

Derivation of clones from *Bmi-1*$^{-/-}$ AMLs

To investigate further the impairment in proliferation of *Bmi-1*$^{-/-}$ leukaemic blasts, attempts were made to derive highly proliferative clones (HPCs) from cultures initiated with *Bmi-1*$^{-/-}$ and *Bmi-1*$^{+/+}$ leukaemic cells. Only 1% of colonies from the *Bmi-1*$^{-/-}$ cultures could generate more than 10^{10} cells (our minimal definition of HPC), whereas HPCs could be derived at a frequency of 24% in control cultures (Fig. 5.5f). In contrast to controls, all *Bmi-1*$^{-/-}$ HPCs initially presented the same characteristics as those described in the previous section for leukaemic bone marrow cells (namely reduced proliferation, monocytic differentiation and increased apoptotic index). However, within about 2 weeks of culture, the *Bmi-1*$^{-/-}$ HPCs were similar to controls (Fig. 5.5g-j).

Karyotype analysis (G-banding) performed on several independent control (group A, n = 3) and *Bmi-1*$^{-/-}$ (group B, n = 8) HPCs revealed a near-diploid content (containing 38-40 chromosomes) and the absence of recurrent structural chromosomal aberrations, indicating that the derivation of *Bmi-1*$^{-/-}$ HPCs *in vitro* involved neither overt genomic instability nor recurrent chromosomal lesions (data not shown). When analysed by FACS, about 100% of cells from HPCs from both groups A (n = 12) and B (n = 13) expressed the myeloid antigen Mac-1, whereas the expression of Gr-1 varied extensively (data not shown). Clonal analysis performed on several of the control (n = 37) and *Bmi-1*$^{-/-}$ (n = 25) HPCs revealed the presence of several integrations of an intact *Hoxa9*-IRES-*Meis1* provirus, where IRES stands for internal ribosome entry site (Fig. 5.6a, and data not shown).

The low frequency (1%) of clonal derivation from *Bmi-1*$^{-/-}$ AMLs suggested either that there was a marked decrease in their generation *in vivo* and/or that genetic or epigenetic events were required for their emergence (clonal evolution). On the basis that the *Ink4a-Arf* locus is a critical genuine target *in vivo* for *Bmi-1* in the regulation of haematopoietic cell proliferation[13], and increased p19^{ARF} levels induce a p53-mediated checkpoint response in G1 (refs 14,15), we verified whether inactivation of Cdkn2a (p16^{INK4a}), Cdkn2b (p19^{ARF}) and other regulator(s) of the G1 cell cycle checkpoint was present in *Bmi-1*$^{-/-}$ *Hoxa9-Meis1* HPCs. Most of the control *Hoxa9-Meis1* (group A) HPCs analysed presented normal levels of most of the G1 cyclin-dependent kinase inhibitors (CKIs) tested, including p19^{ARF}, p57^{Kip2}, p27^{Kip1} and p21^{Cip1} (Fig. 5.6b, left panel). In sharp contrast, most of the *Bmi-1*$^{-/-}$ HPCs (group B) analysed showed low levels or complete loss of expression of several of these CKIs (n = 11 of 14 HPCs; Fig. 5.6b, right panel, and data not shown). Together with the initial delay (2 weeks) in proliferation of each of these *Bmi-1*$^{-/-}$ HPCs (see above), these results indicate that clonal evolution, through epigenetic or genetic loss of CKI expression, might represent the "driving force" that allowed the derivation of HPCs from *Bmi-1*$^{-/-}$ AMLs *in vitro*.

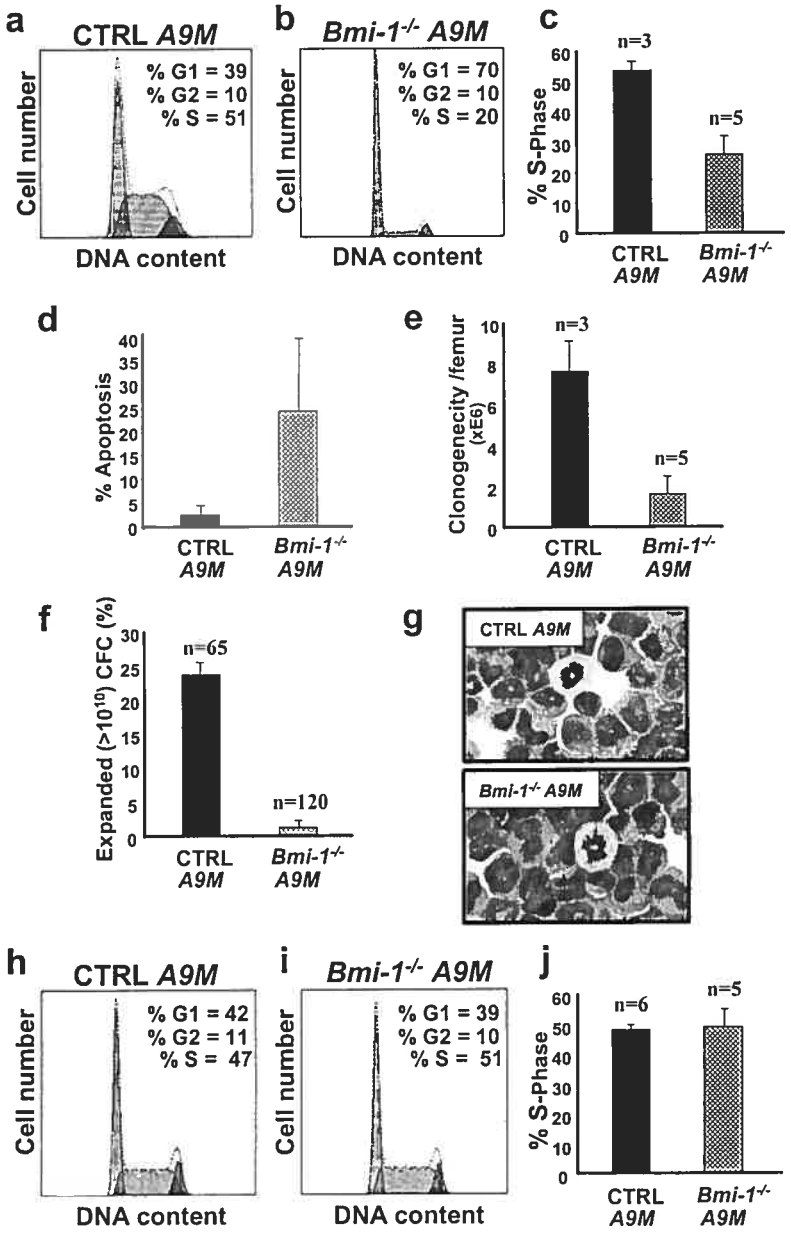


Fig. 5.5 Characterization of control and *Bmi-1*$^{-/-}$ leukaemic cells *in vitro*. **a-c,** Cell cycle analysis performed as described[22]. Ctrl, control. **d,** Apoptotic index determined by the percentage of PI-positive/annexinV-positive cells. **e,** Absolute number of leukaemic colony-forming cells (CFCs) per femur in primary recipients. **f,** Percentage of CFCs able to generate HPCs. **g,** Wright staining of representative HPCs from **f. h, i,** Cell cycle analysis (as in **a** and **b**) of exponentially growing HPCs. **j,** Proportion of cells in S-phase in exponentially growing HPCs. For **c-f** and **j**, results are presented as means ± s.d.

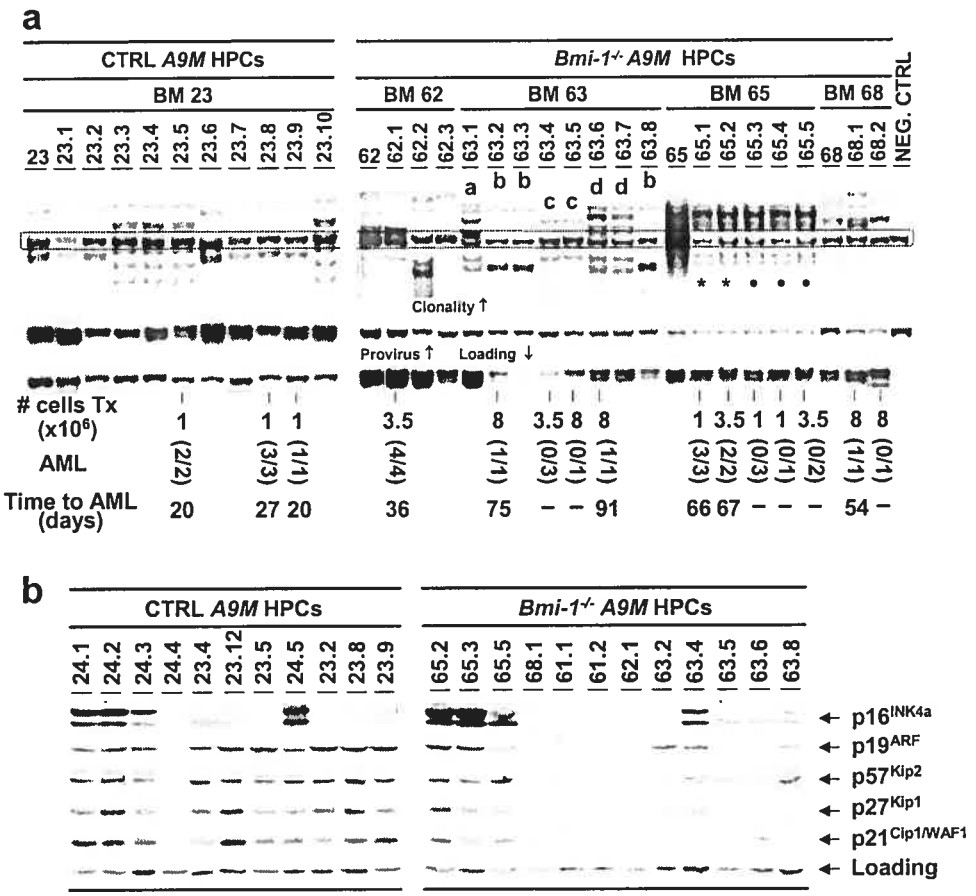


Fig. 5.6 Loss of expression of several CKIs in *Bmi-1*$^{-/-}$ *Hoxa9-Meis* HPCs. **a,** Southern blot analyses of *Hoxa9-Meis1* proviral integration sites in genomic DNA isolated from control (Ctrl) and *Bmi-1*$^{-/-}$ HPCs. Restriction digest, neg. control and probe are as described in Fig. 5.4c. Letters a-d in the top panel indicate four different clones from the same primary recipient. The leukaemogenic potential of several clones was evaluated. Below each clone: number of cells transplanted (Tx) per recipient, the occurrence of AML reported and the number of mice transplanted (parentheses) and the time to clinical leukaemia. **b,** Western blot analyses showing the protein expression levels of several CKIs in control and *Bmi-1*$^{-/-}$ HPCs.

Bmi-1$^{-/-}$ HPCs are weakly leukaemogenic

The ability of these HPCs to induce leukaemia *in vivo* was investigated by transplanting between 10^6 and 8 x 10^6 cells per clone into sublethally irradiated syngeneic recipients (Fig. 5.7a). Strikingly, all recipients of control *Hoxa9-Meis1* HPCs (n = 6, one to four mice per group) succumbed to AML within 16 ± 6 days of transplantation. However, all of the HPCs that could be derived from *Bmi-1*$^{-/-}$ *Hoxa9-Meis1* AMLs either did not induce AML (6 of 12; Fig. 5.6a and 5.7b) or induced the disease after a significant delay (see below). DNA analysis of proviral integration sites from selected HPCs indicated that clones such as 68.2 or 65.3-65.5, which represented dominant leukaemic clones in primary recipients, failed to induce AML *in vivo,* whereas clones 65.1 and 65.2 (see asterisk in Fig. 5.6a), which could transmit AML to secondary recipients after long latencies (66-67 days), had the same retroviral integration profile as clones 65.3-65.5 (see filled circles in Fig. 5.6a), which could not.

Rescue of *Bmi-1*$^{-/-}$ HPCs AML-inducing capacity

To distinguish between clonal evolution and selection as the mechanism responsible for leukaemic progression in certain *Bmi-1*$^{-/-}$ HPCs, we engineered retroviral expression of *Bmi-1* or *EGFP* (control) in three HPCs (two non-leukaemic identified as HPC-63.5 and HPC-65.4, and one poorly leukaemic identified as HPC-63.2 in Fig. 5.7c; see also Fig. 5.6a-b) and transplanted these cells immediately after gene transfer, at a dose of 3.5 x 10^6 cells, into irradiated syngeneic recipients (n = 2 or 3 mice per group, except HPC-63.2 plus *EGFP* (n = 1)). The three clones engineered to express *Bmi-1* readily induced AML within short latencies, whereas no leukaemias were detected until day 85 in recipients of similar numbers of cells from the same clones infected with the control *EGFP* virus (Fig. 5.7c). The leukaemias generated by the three HPCs expressing *Bmi-1* were highly polyclonal (see clonality in lanes 5-17 in Fig. 5.7d) and contained an intact *Bmi-1* provirus. The contribution of these *Bmi-1*$^{-/-}$ clones expressing *Bmi-1* to AML (chimaerism) was evaluated by FACS analysis for EGFP expression (the *Bmi-1* virus contains an *EGFP* reporter gene) (Fig. 5.7d) and by Southern blot analysis, by assessing the ratio of the inactivated *Bmi-1* allele (donor-derived) relative to the wild-type *Bmi-1*

allele (host-derived) in various haematopoietic organs of the leukaemic animals (Fig. 5.7d, bottom panel). Donor-derived cells corresponded to 25%-95% of the total haematopoietic cell population, confirming infiltration of the neoplastic cells into all of these organs (Fig. 5.7d). The demonstration that *Bmi-1* was sufficient to fully rescue the leukaemogenic potential of *Bmi-1*$^{-/-}$ HPCs further suggests that clonal evolution, rather than selection, is the underlying mechanism responsible for leukaemic progression in *Bmi-1*$^{-/-}$ HPCs.

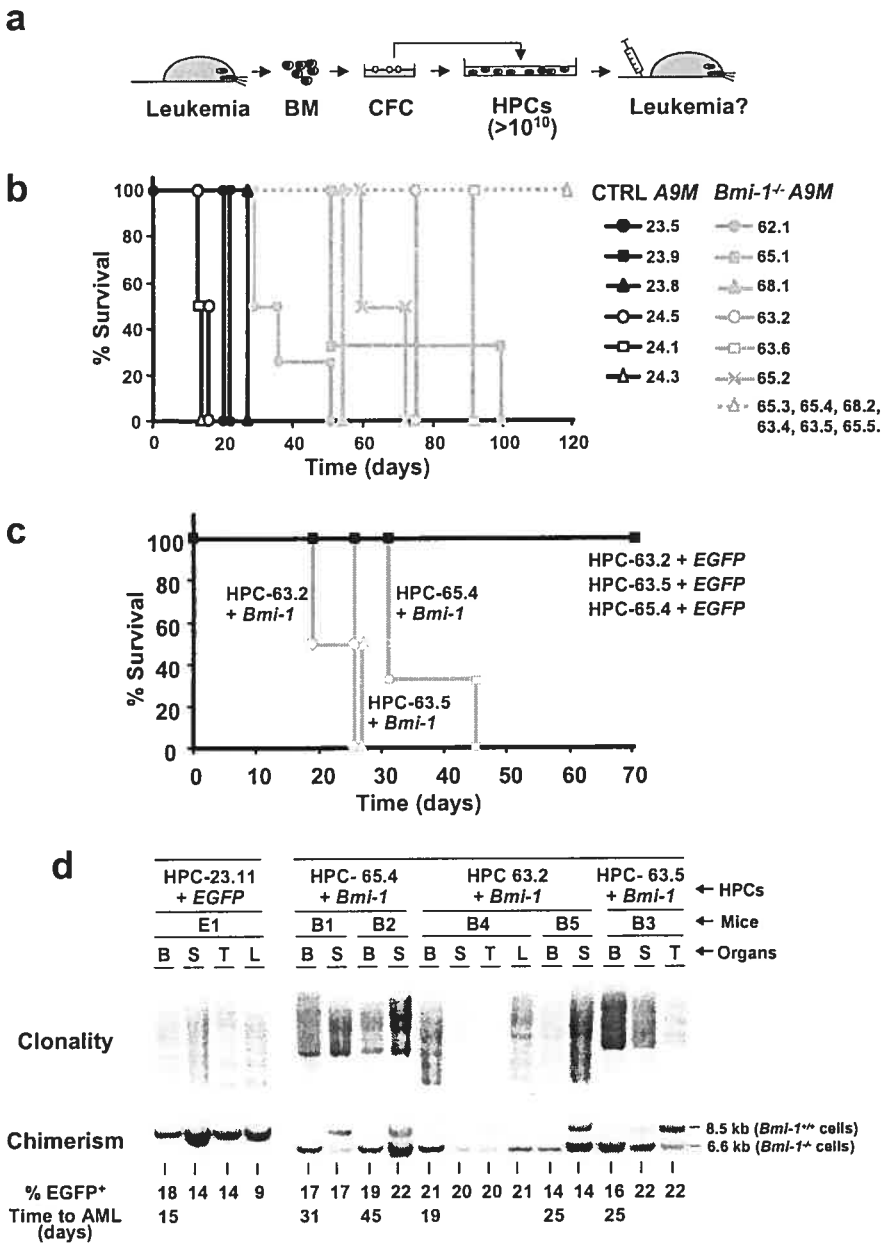


Fig. 5.7 *Bmi-1* rescues the weak leukaemogenic potential of *Bmi-1*$^{-/-}$ HPCs. **a,** Experimental procedures. BM, bone marrow; CFC, colony-forming cells. **b, c,** Survival graph of recipients of selected HPCs (see Fig. 5.6) transduced (**c**) or not (**b**) with *Bmi-1-EGFP* or *EGFP*. **d,** Southern blot analyses showing the polyclonality of the leukaemias. Upper panel: genomic DNA isolated from the BM (B), spleen (S), thymus (T) and/or lymph nodes (L) was digested with *Eco*RI; the probe used was *EGFP* (see Fig. 5.3a). Lower panel: genomic DNA was hybridized to a DNA probe specific for *Bmi-1* to assess

chimaerism. Time to leukaemia and proportion of EGFP-expressing cells present in the haematopoietic organs of the leukaemic animals are shown below.

Discussion

In summary, the findings reported here reinforce the notion of a structure in leukaemic hierarchy where "stemness" would be conferred by the continual expression of *Bmi-1* (see Fig. 5.8). Importantly, it was possible under conditions of stimulation with high concentrations of growth factors *in vitro* to generate non-leukaemogenic *Bmi-1*$^{-/-}$ clones with an impaired expression of $p19^{ARF}$ and $p16^{INK4a}$, both of which are known functional targets of Bmi-1 (ref.13). Retroviral introduction of *Bmi-1* into these clones deficient in $p19^{ARF}$ and $p16^{INK4a}$ ($n = 3$) readily rescued their tumorigenic properties, suggesting that Bmi-1 has one or more additional functions in L-HSCs besides repression of these CKIs. The expression of currently known regulators of early haematopoiesis (such as *tal-1/SCL* (ref.16) and *Hoxb4* (ref. 17)) was not altered by the status of *Bmi-1* in leukaemic and non-leukaemic clones (See Fig. 5.9).

The apparent difficulties of bypassing the requirement for Bmi-1 in leukaemic stem/progenitor cells *in vivo* suggests that adroit molecular targeting of Bmi-1 in leukaemic stem/progenitor cells might have potent and specific therapeutic effects. Interestingly, *BMI-1* expression was recently reported in several cases of human non-small-cell lung cancer[18], breast cancer cell lines[19] and immortalized mammary epithelial cells (MECs)[19]. It will therefore be interesting to determine whether the findings reported here might also extend to other types of "cancer stem cells".

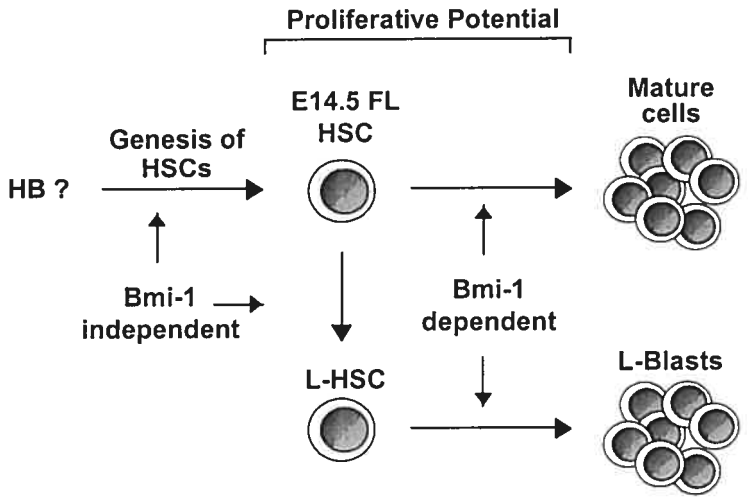


Fig. 5.8 *Bmi-1* regulates the proliferative capacity of stem and progenitor cells, whether normal or leukaemic. This also indicates that *Bmi-1* is dispensable for the genesis of FL (E14.5) HSCs and for their leukaemic transformation. HSC, haematopoietic stem cell; L-HSC, leukaemic haematopoietic stem cell; L-blasts, leukaemic blasts; FL, fetal liver.

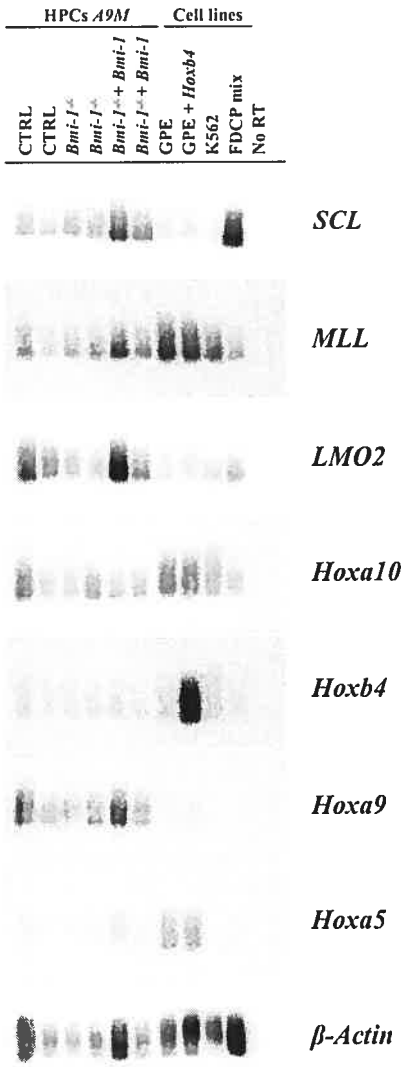


Fig. 5.9 Expression of selected genes in *Bmi-1*$^{+/+}$ and *Bmi-1*$^{-/-}$ *Hoxa9-Meis1* highly proliferative clones overexpressing or not *Bmi-1*. Cell lines were also included as specificity control for hybridization. Total RNA isolated from each sample was reverse-transcribed and PCR-amplified (see methods). Membranes were hybridized to probes specific for *SCL, MLL, LMO2, Hoxa10, Hoxb4, Hoxa9, Hoxa5,* β–Actin and *GATA-2* (not shown) and exposed for 36-48 hrs on a PhosphoImager screen. Note that *GATA-2* expression was undetectable in HPCs.

Methods

Mice and genotyping

The *Bmi-1*$^{-/-}$ mice (129Ola/FVB/N hybrid background) used in these studies[9] had been backcrossed 10-12 times in the C57Bl/6J background.

Retroviral generation, infection and transplantation of E14.5 fetal liver and bone marrow cells

Details of the construction of the *MSCV-Hoxa9-IRES-Meis1a* bicistronic retroviral vector and the MSCV-*Bmi-1-pgk-EGFP* vector are available from the authors on request. Production of vesicular stomatitis virus-pseudotyped retroviruses, infection of haematopoietic cells and transplantation into mice were done as described[20].

Methylcellulose cultures, flow cytometry and cell cycle analyses

These assays were performed essentially as described[10,21,22].

Generation, amplification and analysis of complementary DNA

Semi-quantitative PCR with reverse transcription was performed with a previously described method that amplifies all the cDNAs derived from as few as 1-1 000 cells[23]. The amplified (total) cDNAs were then transferred to a nylon membrane and hybridized to specific probes as indicated. See Table IV for a detailed description of each probe used.

Southern, northern and western analyses

All of these were essentially done as described[10,22]. Information on the antibodies used in these studies is available from the authors on request.

In vitro generation of HPCs

Myeloid progenitors derived from methylcellulose cultures of leukaemic bone marrow cells isolated from primary recipients (n = 3 for wild-type and 5 for *Bmi-1*$^{-/-}$ leukaemic mice) were plucked on day 6 and expended *in vitro* in liquid culture media containing DMEM (Invitrogen), 15% fetal calf serum (pre-selected, Invitrogen), 6 ng/ml Interleukin (IL)-3, 10 ng/ml IL-6, 100 ng/ml Steel factor, 100 ng/ml IL-11 (all generated and titrated at IRCM from COS cell supernatants) and $1x10^{-5}$ M β-Mercaptoethanol. Clones that could generate more than 10^{10} progeny cells are referred to as highly proliferative clones (HPCs).

Table IV

Generation of gene-specific probes.

Fig.	Probes	Enzymes	Fragments	Bank Number	Accession; Nucleic acids
2, 9	*hHOXB4*	*SalI/PmlI*	408 bp	178	AF307160; 1512-1920
4, 6, 9	*mHoxa9*	*PstI/Hin*dIII	243 bp	321	AB005457; 1267-1510
9	*hHOXA10*	*Apa*I	247 bp	84	AF040714; 603-850
9	*hHOXA5*	*Sac*II/*Hin*dIII	230 bp	185	as described[17]
1, 9	*hBMI-1*	*Eco*RV/*Eco*RI	310 bp	508	L13689; 1938-2248
9	*hLMO2*	*Bam*H1	476 bp	1587	X61118; 1037-1513
9	*mSCL*	*PstI/Hin*dIII	437 bp	1590	U01530; 6272-6709
9	*hGATA-2*	*Eco*RI	252 bp	1589	M68891; 1463-1715
9	*hMLL*	*XbaI/Sma*I	936 bp	1586	L04284; 10974-11910
1, 9	*β-Actin*	*Pst*I	1.8 kb	212	as described[35]
2, 7	*EGFP*	*Eco*RI/*Hin*dIII	719 bp	719	U76561; 289-1008
7	*mBmi-1*	*Sac*I	1.3 kb	675	as described[11]

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Supplementary Information accompanies the paper on *Nature*'s website (http://www.nature.com).

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CHAPITRE 6

Identification of Novel Co-factors of Bmi-1 in Hemopoietic Stem Cells

Julie Lessard, Sherry Niessen, Simon Girard and Guy Sauvageau

(Ongoing studies)

Chapter 6 summarizes the results of a yeast-two-hybrid screen which was aimed at identifying HSC-specific partners for the Bmi-1 protein. Julie Lessard conducted the screen which led to the identification of 13 Bmi1-interactive clones, representing 8 different gene products. She is responsible for the results presented in Figures 6.1 to 6.3, 6.7 and 6.11 to 6.16. Sherry Niessen, a graduate student in the laboratory, is working at elucidating the functional interaction between Bmi-1 and one of these molecules, namely E4F-1. She is responsible for the results presented in Figures 6.5, 6.6., and the generation of the E4F-1 deletion mutants presented in Figure 6.8. Simon Girard, a technical assistant on the laboratory, is working at investigating the role of BHIP-1, a novel gene product identified in the screen and its biochemical and functional interaction with Bmi-1. He generated the results presented in Figures 6.4, 6.9 and 6.10 under the supervision of Julie Lessard and Sherry Niessen.

Bmi-1, which is essential for the proliferation (self-renewal) of embryonic and adult hemopoietic stem cells (HSCs, see Chapters 3 and 4) participates in the formation of a multimeric complex with other PcG proteins including Mph1/HPH1, Mph2/HPH2, M33, RING1A, RING1B MPc2, MPc3 and Mel-18 [1-5]. The RING domain of Bmi-1 (together with its nuclear localization signal (NLS)) is essential for its proliferative/oncogenic function, whereas homeotic transformations of skeletal structures require the HTHTHT region of the protein [6,7]. Direct interactions between Bmi-1 (or *Drosophila* Psc) and other PcG proteins have been examinated mainly through the use of yeast-two-hybrid (Y-2-H) screens. *Drosophila* Pc interacts with Psc through an extended HTHTHT region which is also required for its interaction with Ph [8]. Mammalian Bmi-1 interacts through its RING domain with the RING finger-containing Mel-18, Ring1A and Ring1B proteins [2,4,5] and through its HTHTHT region with the MPh1 and MPh2 proteins [2,4]. Importantly, these interactions are evolutionary conserved, as the human HPH1 and HPH2 gene products (homologues of *Drosophila* Ph and mouse Mph1/Mph2) also interact with Bmi-1[3]. The association of Bmi-1 with M33 is mediated through a region encompassing both its RING and HTHTHT domains [2].

We have previously reported a curious dissociation between the expression profile of *Bmi-1* (mostly restricted to primitive human $CD34^+CD71^-CD45^-$ and mouse $Sca\text{-}1^+$ Lin^- bone marrow cells) and that of other gene products with which Bmi-1 normally interacts (mostly expressed in mature bone marrow cells, see Chapter 2), Therefore, we postulated that the function of Bmi-1 in primitive bone marrow cells is mediated through its interaction with yet unidentified proteins. To identify such gene products (in particular those involved in its recruitment to relevant DNA target loci), a yeast-two-hybrid screen was performed (MatchMaker III, Clontech) using the full-length Bmi-1 coding sequence (CDS) as a bait in a yeast expression library (1.2×10^6 clones screened) made from week 14-18 human fetal livers (Clontech) enriched for primitive stem/progenitor cells (see Fig. 6.1 for details on the experimental procedure). The Bmi-1 bait vector did not result in non-specific activation of any of the 3 yeast reporter genes (histidine, adenine and LacZ, data not shown). A total of 13 clones, which represented 8 different Bmi-1-interacting factors, were identified (Fig. 6.2). Two of those encoded

novel gene products and were named BHIP-1 and BHIP-2 for Bmi-1 Hemopoietic Interacting Protein-1 and 2. Four of the clones corresponded to three different fragments of the human vacuolar protein sorting 11 (hVPS11) gene product, a RING/Zinc finger domain-containing protein. The remaining 4 clones identified included: the PcG Bmi-1-interacting protein HPH1, two pRb-interacting proteins (Trip230 and E4F-1), the RED protein (also known as IK factor) and the H^+-ATPase (n=3 clones out of 13).

A list of criteria was established to prioritize the study of proteins that specifically interacted Bmi-1 in primitive bone marrow (BM) cells. These included: (i) confirmed interaction with Bmi-1 in yeast; (ii) coexpression with *Bmi-1* in primitive BM cells; (iii) known DNA-binding activity; (iv) proof of interaction and/or colocalization with Bmi-1 in mammalian cells and, (v) potential to participate in chromatin remodeling and/or to possess trans-repressing activity. Based on these criteria, the E4F-1 and BHIP-1 gene products were selected.

The studies presented in this Chapter will extend our initial efforts to characterize the Bmi-1-containing complex present in primitive bone marrow cells. This is most important considering the unique and essential role played by Bmi-1 in early hemopoiesis, together with the inability of this protein to intrinsically bind DNA (Bmi-1 DNA-tethering proteins must be present in primitive cells). Below, I will provide a short description of the proteins identified in the screen, together with some of the work that has been accomplished thus far.

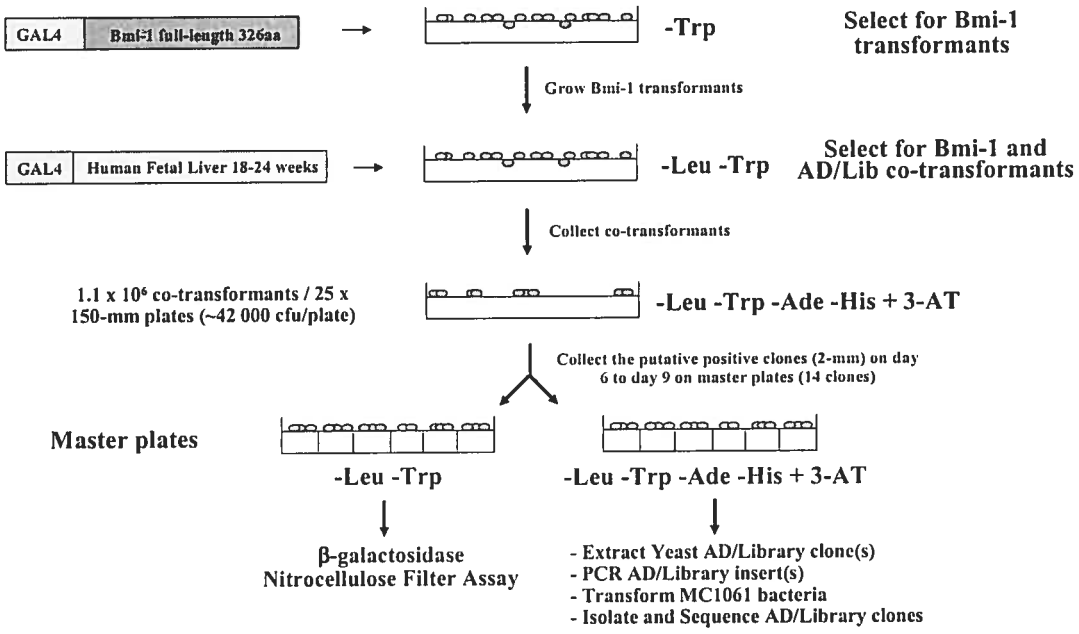

Fig. 6.1 Yeast transformation and screening protocol. Abbrev.: Trp, tryptophane; Leu, leucine; Ade, adenine; His, histidine; 3-AT, 3-amino-1,2,4-trazole; a competitive inhibitor of the His3 protein; AD, activation domain; Lib, library.

Y2H Clone	Domain(s)	DNA binding?	Repression?
HPH1	Zn finger	no	yes
E4F-1	Zn finger + Rb	yes	yes
RED protein	RED motif	RNA	?
TRIP 230	Rb + TR	no	yes (Rb)
BHIP-1	PEST	?	?
BHIP-2	KRAB + ZF	?	?
hVPS11 (4)	RING finger	?	?
H^+-ATPase (3)	ATPase	no	?

Fig.6.2 Bmi-1-interacting clones identified in our yeast-two-hybrid (Y2H) screen. The protein interaction domain(s), DNA binding and/or repressive functions for each of the clones is indicated if known. Abbrev.: Zn, zinc finger domain; KRAB, kruppel-

associated box; RED, domain rich in arginine (R) , glutamic acid (E) and aspartic acid (D) residues; Rb, retinoblastoma protein; TR, thyroid hormone receptor; PEST, a sequence rich in proline, aspartate or glutamate, serine, and threonine residues found in many unstable proteins.

E4F-1, a potential mediator of Bmi-1 function in HSCs?

E4F-1 ($p120^{E4F}$) is an ubiquitously expressed GLI-Kruppel-related zinc finger phosphoprotein with 86% sequence identity to the murine nuclear factor øAP3 [9]. E4F-1 was initially cloned as a transcriptional repressor of the E4 adenoviral gene through the direct recognition of a specific promoter element (5'-RTGACGTC/AAY-3' sites) [9-11]. In response to adenovirus E1A, E4F-1 becomes hyperphosphorylated and undergoes a reduction in both DNA binding and transcriptional repressor activities [9,12], possibly to permit activation of (adeno) viral genes during early infection. Although cellular target genes for E4F-1 remain to be identified, overexpression of E4F-1 in primary fibroblasts inhibits cell cycle progression and cell growth [11-14]. This inhibitory activity of E4F-1 correlates with the post-transcriptional elevation of several cell cycle regulatory proteins, including the CDK inhibitors $p21^{Waf1}$ and $p27^{Kip1}$, cyclin E, and cyclin B1, with reduced Cdk2, Cdk4/6, and Cdc2 kinase activities and with the down-regulation of *cyclin A2* gene expression [11,12,15]. E4F-1-induced cell cycle arrest is enhanced by its interaction with the p53 transcription factor and hypophosphorylated pRb [13,14]. Although *cyclin A2* expression is down-regulated by E4F-1, it is likely that other cell cycle regulatory molecules are regulated by E4F-1, as ectopic expression of $p120^{E4F}$ can induce cell cycle arrest at the G1-S and G2-M transitions, even when *cyclin A* mRNA and protein levels are not reduced [12,15].

A recent study demonstrated that E4F-1 forms a ternary complex together with $p14^{ARF}$ and p53 and enhances $p14^{ARF}$-induced G2 cell cycle arrest in a p53-dependent manner [16]. These findings suggest that the interaction of Bmi-1 with this trimeric complex is part of a network regulating cellular proliferation that is disrupted in cancer and following viral infection.

Using semi-quantitative RT-PCR studies, we showed that, together with *Bmi-1*, *p120^{E4F}* is preferentially expressed in primitive subsets of mouse bone marrow cells (Fig. 6.3). E4F-1 specifically associates with Bmi-1 in yeast (confirmation tests, see Fig. 6.4). Co-immunoprecipitation experiments demonstrated a specific association between Bmi-1 and E4F-1 in human 293T cells. Using a monoclonal antibody against Bmi-1, we showed that both an overexpressed HA-tagged version of the E4F-1 clone identified in our Y-2-H screen (Fig. 6.5) and the endogeneous E4F-1 protein (Fig. 6.6) can be coimmunoprecipitated together with endogenous Bmi-1 in these cells. Of note, recent experiments revealed that the interaction is cytoplasmic-specific (data not shown). Pull-down experiments using *in vitro* transcribed and translated (TNT) ^{35}S-labeled proteins indicate that Bmi-1 and E4F-1 directly associate (Fig. 6.7). A panel of Bmi-1 and E4F-1 deletion mutants (subcloned in yeast and retroviral (MSCV)) expression vectors) was used to map the minimal interaction domains between Bmi-1 and E4F-1 in yeast (Fig. 6.8). For Bmi-1, these included: (1) deletion of the RING finger domain (n.t. 52-167); (2) deletion of the HTHTHT domain (n.t.493-659); (3) deletion of the C-terminal PEST domain (n.t. 706-975), and (4) an inactive RING finger mutant of Bmi-1 corresponding to two cycteine to phenylalanine substitutions (C→F, Fig. 6.9). For E4F-1, these included: (1) a C-terminal deletion including the zinc-fingers 3 to 6 (n.t. 473-783); (2) a N-terminal deletion including the zinc fingers 1 and 2 (n.t. 1-471); (3) a specific deletion of the zinc fingers 1 and 2 (n.t. 194-240), and (4) a specific deletion of the zinc fingers 3 to 6 (n.t. 437-567, Fig. 6.10). These studies identified the HTHTHT domain of Bmi-1 and the zinc finger domains 3 to 6 of p120^{E4F} as being required for their interaction (Fig. 6.9-6.10). The functionality (i.e. proper folding) of the Bmi-1 deletion mutants was assessed by confirming their ability to interact with other known Bmi-1 interacting partners (HPH1 and RING1B; Fig. 6.9) and that of E4F-1 by performing gel-shifts experiments using an E4F-1 specific DNA probe (data not shown). The presence of a E4F-1 DNA response element at position -1687 to -1658 bp on the human *p14^{ARF}* promoter leads to the hypothesis that down-regulation of p14^{ARF} levels, through the interaction with E4F-1, may be essential for Bmi-1 function in HSCs. Trans-repression assays using a panel of p14^{ARF} promoter deletion mutants failed to demonstrate an

ability of E4F-1 to modulate $p14^{ARF}$ levels in 293T cells (data not shown). Most importantly, we recently demonstrated that E4F-1 cytoplasmic levels progressively increase in primary murine embryonic fibroblasts (MEFs) undergoing senescence. This is most interesting considering that the E4F-1-Bmi-1 interaction is cytoplasmic-specific (see above) and the aging of primary human fibroblasts is accompanied by decreased *Bmi-1* expression (both at the RNA and protein level) and accumulation of $p16^{INK4a}$ and $p14^{ARF}$ ([17,18] and see Chapter 1 for details).

The detailed analysis of $p120^{E4F}$ function in regulating hemopoietic cell proliferation and senecence is now being pursued by Sherry Niessen, a PhD student in the laboratory.

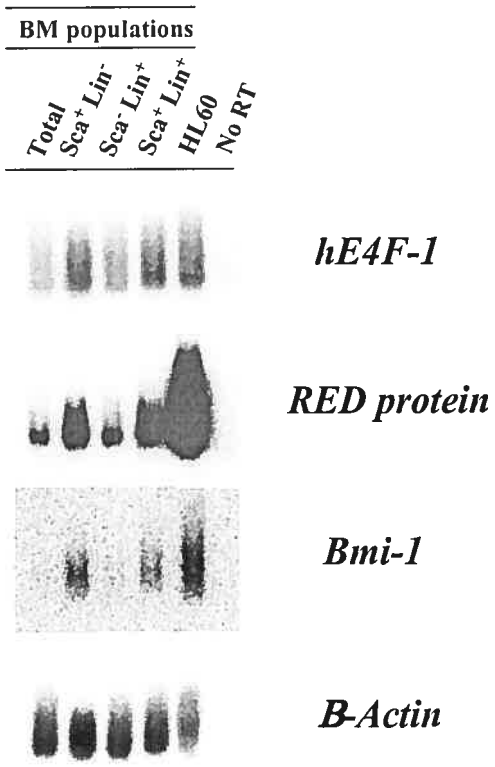

Fig. 6.3 *$p120^{E4F}$* and *RED protein* are co-expressed together with *Bmi-1* in primitive bone marrow cells and hemopoietic cell lines. Ten thousand cells were isolated from each subpopulation (>98% purity upon reanalysis), and their total RNA was reverse-transcribed and PCR-amplified as described (see Material and Methods, Chapter3).

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-1^+ Lin^- subpopulation (lane 2); Sca-1^- Lin^+ subpopulation (lane 3). Exposure times are as follow: *p120*^{E4F} (22 hrs), *RED protein* (12 hrs), *Bmi-1* (20 hrs) on Phosphor-Imager screen. β-Actin (25 min at -70^0).

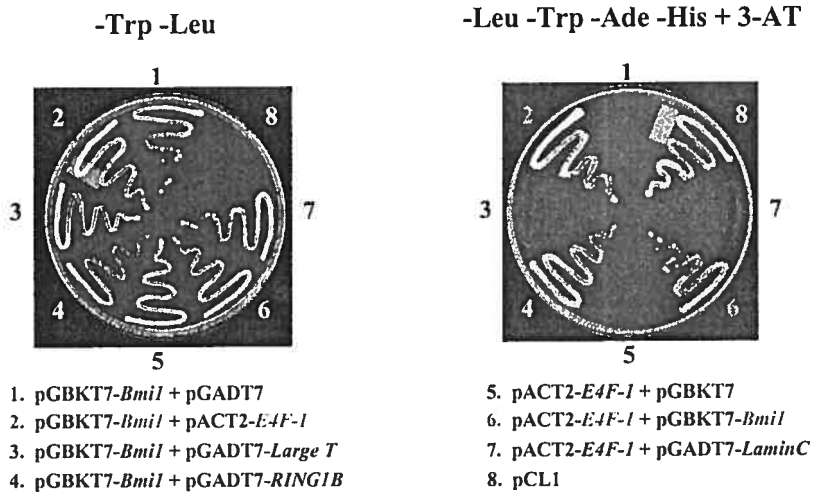

Fig. 6.4 Bmi-1 specifically associates with E4F-1 in yeast. AH109 yeast were co-transfected with GAL4-DNA binding (pGBKT7) and GAL4-transactivation domain (pGADT7 or pACT2) expression vectors encoding Bmi-1, E4F-1, RING1B (positive control) and Large T antigen or Lamin C (both negative controls) and grown on selection media (left panel), depleted in the amino acids (a.a.) tryptophane (Trp) and leucine (Leu), allowing the growth of any yeast co-transformant and (right panel), depleted in the a.a. adenine (Ade), histidine (His), Trp and Leu, and allowing the growth of yeast interactors only. pCL1 is used as a positive control as it encodes a functional GAL4 protein. Abbrev.: 3-AT, 3-amino-1,2,4-triazole.

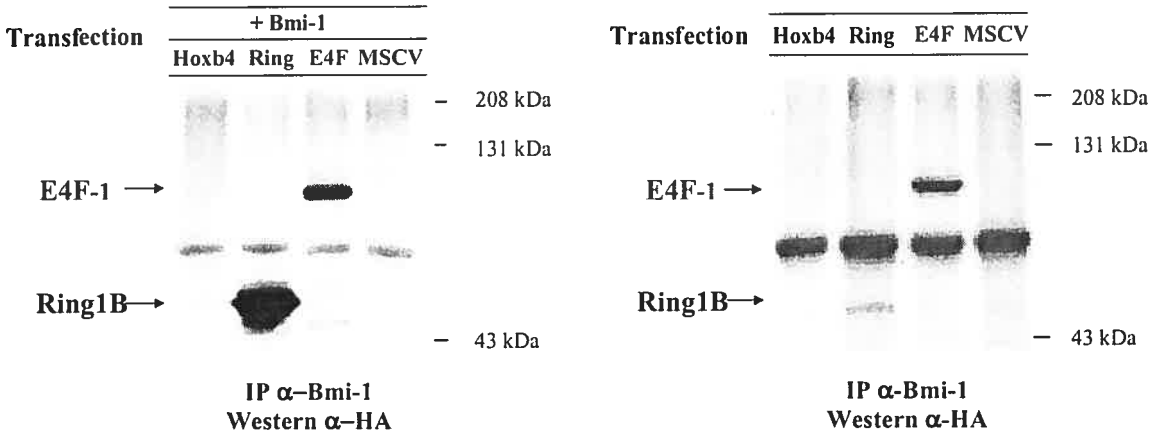

Fig. 6.5 Bmi-1 and E4F-1 specifically coimmunoprecipitate in human 293T cells. 293T cells were transfected with 20 μg of either HA-Hoxb4 (negative control), HA-RING1B (positive control), a HA-tagged version of the C-terminal E4F-1 fragment clone isolated in the screen with (left panel) or without (right panel) Bmi-1. Immunoprecipitations were performed overnight on total cellular extracts using a monoclonal anti-Bmi-1 (F6) antibody. Western blots were revealed using a monoclonal anti-HA antibody (clone 12CA5, Boehringher). Abbrev.: IP, immunoprecipitation; MSCV, empty MSCV retroviral vector; HA, peptide epitope derived from the hemagglutin protein.

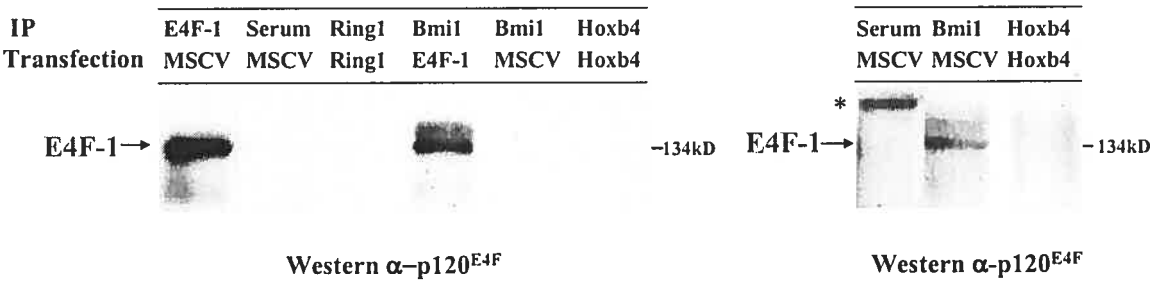

Fig. 6.6 Immunoprecipitation of an endogenous Bmi-1 and E4F-1 complex in human cells. 293T cells were transfected with 20 ug of empty MSCV retroviral vector or MSCV-HA-RING1B (positive control), HA-E4F-1 or HA-Hoxb4 (negative control). Immunoprecipitations were performed overnight using rabbit anti-E4F-1 serum, rabbit pre-immune serum, a mouse monoclonal anti-Bmi-1 or anti-HA antibodies (same isotype). Samples were separated by SDS-PAGE and blots were revealed using the anti-E4F-1 serum. Two different exposure times are shown: left panel, 15 secondes; and right panel, 2 minutes. (*), Background; MSCV, empty retroviral vector.

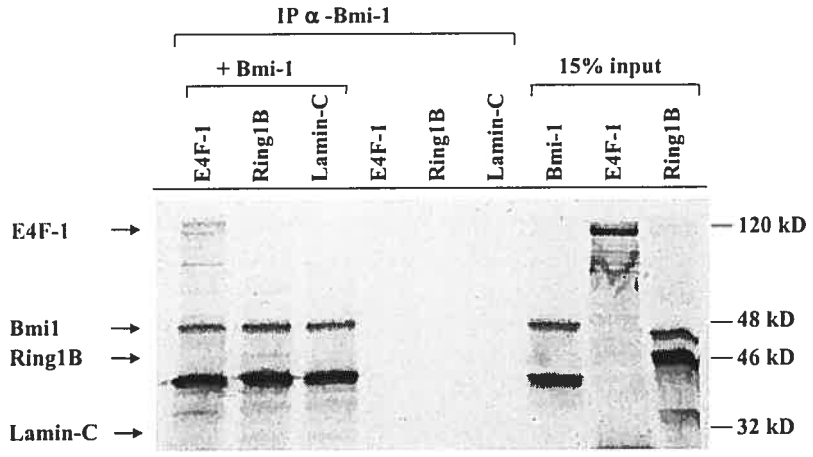

Fig. 6.7 Direct physical association between Bmi-1 and E4F-1. *In vitro* transcribed-translated ^{35}S-labeled proteins were immunoprecipitated for 2 hrs in ELB binding buffer with a monoclonal anti-Bmi1 (F6) antibody, washed and separated by SDS-PAGE. Abbrev.: IP, immunoprecipitation.

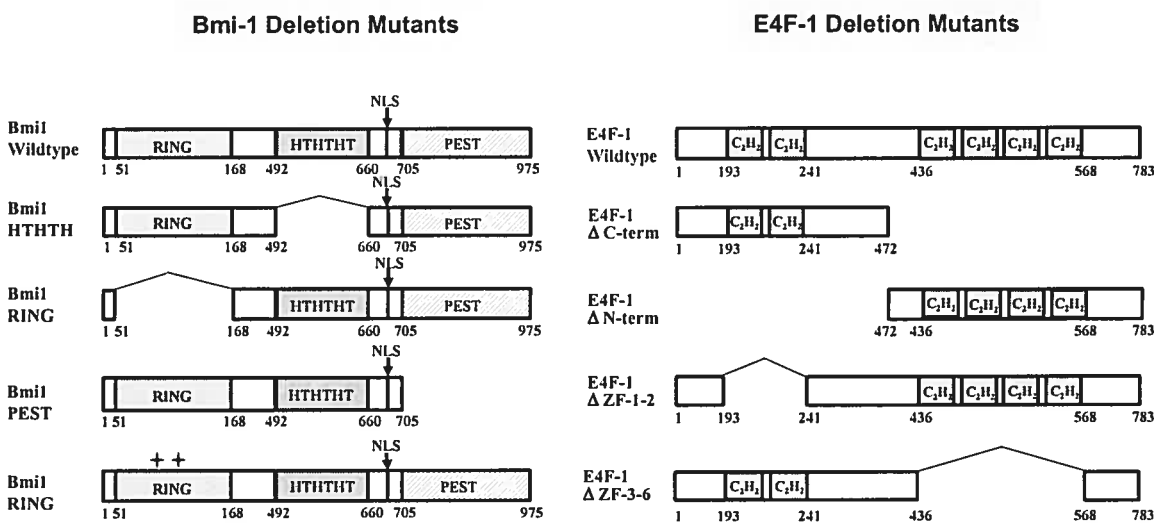

Fig. 6.8 Mapping the minimal interaction domains of E4F-1 and Bmi-1 using a panel of deletion mutants. See text for details. (*), inactivating point mutations (cysteine to phenylalanine substitutions) in the RING finger domain of Bmi-1. Abbrev.: HTHTHT, helix-tur-helix-turn-helix domain; C_2H_2, zinc finger domain of the C_2H_2 subtype.

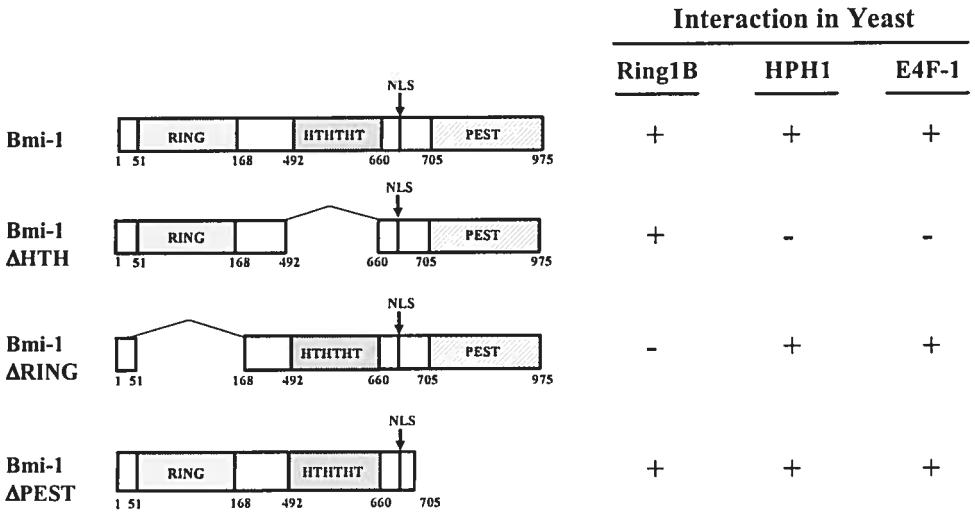

Fig. 6.9 The HTHTHT domain of Bmi-1 is essential for its interaction with E4F-1 in yeast. AH109 yeast were co-transformed with expression vectors encoding the different Bmi-1 deletion mutants (as described in text) together with either full-length E4F-1, Ring1B (a known interactor of Bmi-1 through its RING finger domain) or HPH1 (a known interactor of Bmi-1 through its HTHTHT domain) and their growth assessed in selection media depleted in the amino acids TRP, Leu, Ade and His in presence of 3-AT (see below).

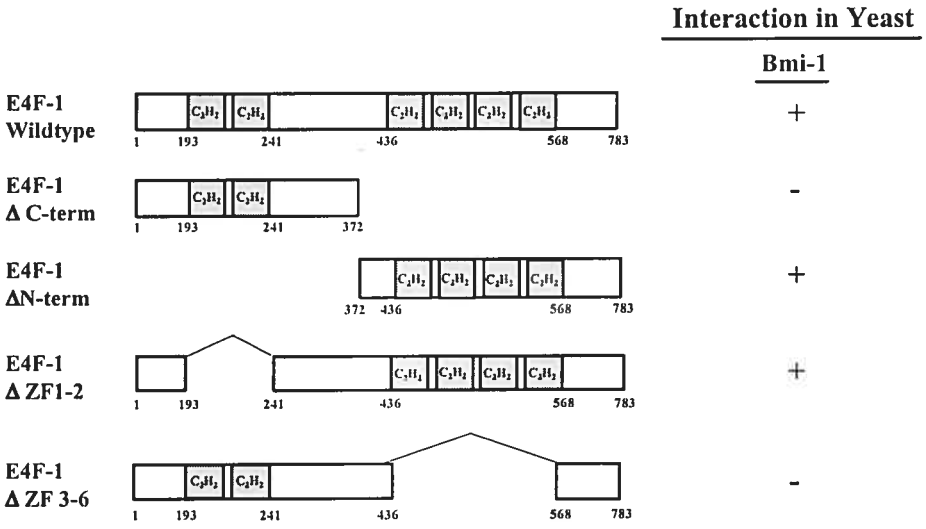

Fig. 6.10 A domain comprising the zinc finger 3 to 6 of E4F-1 is essential for its ability to interact with Bmi-1. AH109 yeast were co-transformed with expression vectors encoding the different E4F-1 deletion mutants (as described in text) together with full-length Bmi-1 and their growth assessed in selection media depleted in the amino acids TRP, Leu, Ade and His in presence of 3-AT (see below).

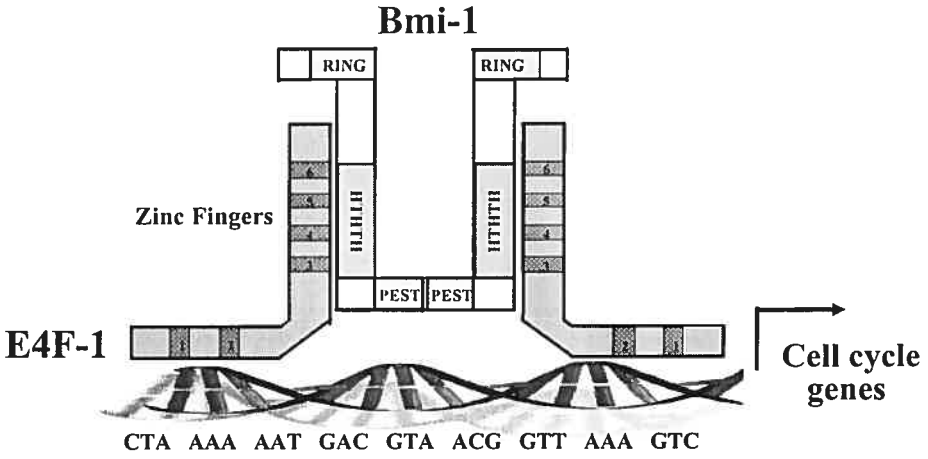

Fig. 6.11 Schematic representation of our current working model. Bmi-1 associates with E4F-1 and modulates its ability to repress transcription of cell cycle regulatory genes.

BHIP-1: a novel Bmi-1 interacting partner in HSCs

Bmi-1 Hemopoietic Interacting Protein-1 (BHIP-1) is a novel gene product identified in our screen which, together with *Bmi-1,* is preferentially expressed in the stem/progenitor cell compartment of mouse bone marrow cells (data not shown). BHIP-1 expression (2 transcripts) was also observed in all the mouse adult tissues analyzed thus far, including the bone marrow, spleen and thymus (data not shown). BHIP-1 is evolutionarily conserved, as homologues have been identified in several species including mouse and human. Of note, BHIP-1 (1059 a.a) does not encode any conserved protein-interaction motif, but contains two PEST domains (a motif found in many short-lived eukaryotic proteins that plays a role in their degradation, see Fig. 6.12). Yeast-two-hybrid assays using a panel of positive and negative controls (as presented in Fig. 6.4 for E4F-1) confirmed the specificity of the interaction between Bmi-1 and BHIP-1 (data not shown). Simon Girard, a research assistant in the laboratory, is pursuing the project. He recently cloned the human full-length BHIP-1 cDNA (3179 bp) from K562 cells and developed a polyclonal rabbit antisera to BHIP-1, which recognizes a single protein of approximately about 155 kDa in both mouse and human cells. He also demonstrated that a monoclonal antibody against endogenous Bmi-1 specifically co-immunoprecipitates a full-lenght FLAG-tagged form of BHIP-1 as well as endogenous BHIP-1 in human 293T cells (data not shown). The function of BHIP-1 in the hemopoietic system is being investigated using a conditional allele of BHIP-1 in the mouse.

```
   1  MAAVAGSGAA AAPSSLLLVV GSEFGSPGLL TYVLEELERG IRSWDVDPGV
  51  CNLDEQLKVF VSRHSATFSS IVKGQRSLHH RGDNLETLVL LNPSDKSLYD
 101  ELRNLLLDPA SHKLLVLAGL CLEETGELLL QTGGFSPHHF LQVLKDREIR
 151  DILATTPPPV QPPILTITCP TFGDWAQPAP AVPGLQGALR LQLRLNPPAQ
 201  LPNSEGLCEF LEYVAESLEP PSPFELLEPP TSGGFLRLGR PCCYIFPGGL
 251  GDAAFFAVNG FTVLVNGGSN PKSSFWKLVR HLDRVDAVLV THPGADSLPG
 301  LNSLLRRKLA ERSEVAAGGG SWDDRLRRLI SPNLGVVFFN ACEAASRLAR
 351  GEDEAELALS LLAQLGITPL PLSRGPVPAK PTVLFEKMGV GRLDMYVLHP
 401  PSAGAERTLA SVCALLVWHP AGPGEKVVRV LFPGCTPPAC LLDGLVRLQH
 451  LRFLREPVVT PQDLEGPGRA ESKESVGSRD SSKREGLLAT HPRPGQERPG
 501  VARKEPARAE APRKTEKEAK TPRELRKDPK PSVSRTQPRE VRRAASSVPN
 551  LKKTNAQAAP KPRKAPSTSH SGFPPVANGP RSPPSLRCGE ASPPSAACGS
 601  PASQLVATPS LELGPIPAGE EKALELPLAA SSIPRPRTPS PESHRSPAEG
 651  SERLSLSPLR GGEAGPDASP TVTTPTVTTP SLPAEVGSPH STEVDESLSV
 701  SFEQVLPPSA PTSEAGLSLP LRGPRARRSA SPHDVDLCLV SPCEFEHRKA
 751  VPMAPAPASP GSSNDSSARS QERAGGLGAE ETPPTSVSES LPTLSDSDPV
 801  PLAPGAADSD EDTEGFGVPR HDPLPDPLKV PPPLPDPSSI CMVDPEMLPP
 851  KTARQTENVS RTRKPLARPN SRAAAPKATP VAAAKTKGLA GGDRASRPLS
 901  ARSEPSEKGG RAPLSRKSST PKTATRGPSG SASSRPGVSA TPPKSPVYLD
 951  LAYLPSGSSA HLVDEEFFQR VRALCYVISG QDQRKEEGMR AVLDALLASK
1001  QHWDRDLQVT LIPTFDSVAM HTWYAETHAR HQALGITVLG SNSMVSMQDD
1051  AFPACKVEF*
```

Fig.6.12 Amino acids sequence of the human BHIP-1 protein. The protein sequence was obtained from Genbank (1059 a.a, accession XP_038599). Putative nuclear localization signals (NLS) are underlined. The colored boxes represent the putative PEST domains identified by the PESTfind program (**http://vienna.at.embnet.org**). The first amino acid of the yeast-two-hybrid clone (arginine (R) residue), clone 60.9) is in purple.

Bmi-1 interacts with the thyroid hormone receptor (TR) coactivator protein Trip230

Trip230 is a specific coactivator of the thyroid hormone receptor (TR) that is negatively regulated by interacting with the retinoblastoma tumor-suppressor (pRB) protein ([19], Fig. 6.13 for schematic representation). Thyroid hormone receptor (TR) bound to thyroid hormone (T3)-responsive elements in the absence of ligand decreases transcription, due to its interaction with the nuclear co-repressors NcoR and SMRT [20,21]. Ligand-dependent activation of TR results in the replacement of the corepressor by a coactivator complex including pCIP/SRC1/NcoA-1/pCAF and CBP/p300 (reviewed in [22,23]). Trip230, which is predominantly a cytoplasmic protein, is such an activator that becomes phosphorylated and translocates to the nucleus in presence of T3 to potentiate

transcription of T3-responsive genes [24]. The association of Bmi-1 with a TR-specific coactivator molecule is most interesting considering that TR has a role in regulating such diverse cellular processes as proliferation, differentiation, and programmed cell death. We first confirmed the specificity of the interaction between Bmi-1 and Trip230 in a yeast-two-hybrid assay (data not shown). Expression studies performed on purified subpopulation of murine bone marrow cells revealed that Trip230 is expressed at very low levels in mouse bone marrow cells (either in total or purified primitive ($Sca\text{-}1^+$ Lin^-) and mature ($Sca\text{-}1^-$ Lin^+) populations, Fig. 6.14). A polyclonal antiserum to Trip230 (raised against a.a. 1099-1372 of Trip230, obtained from Dr Yumay Chen, Texas) was used in coimmunoprecipitation experiments to characterize the interaction between Bmi-1 and Trip230 in human 293T cells (data not shown). A monoclonal antibody directed against endogenous Bmi-1 specifically immunoprecipitated an overexpressed FLAG-tagged version of Trip230 (the partial cDNA isolated in the screen) in these cells, as well as FLAG-Ring1b (a PcG protein; positive control) but not FLAG-Hoxb4 (negative control, data not shown). Using the anti-Trip230 antiserum, the presence of an endogenous E4F-1-Bmi-1 complex was also demonstrated in the human K562 hemopoietic cell line (data not shown). We decided to hand these results to our collaborator, Dr Maarten van Lohuizen, at The Netherlands Cancer Institute, The Netherlands.

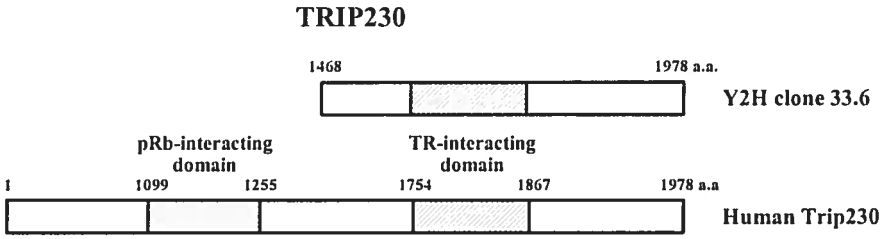

Fig. 6.13 Schematic representations of the human Trip230 protein and the Trip230 clone identified in our yeast-two-hybrid screen. The clone 33.6 corresponds to the amino acids 1468 to 1978 of human Trip230 (accession AF007217) and encodes the thyroid hormone receptor (TR)-interacting domain. Abbrev.: Y-2-H, yeast-two-hybrid; pRb, retinoblastoma protein; a.a., amino acids.

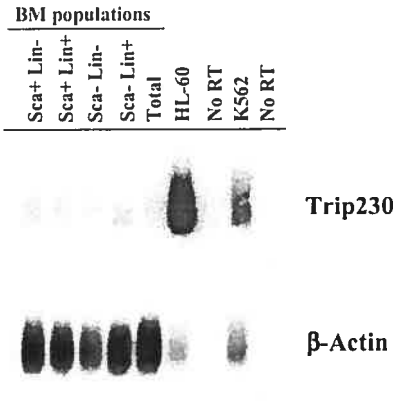

Fig.6.14 *Trip230* is expressed at low levels in purified subpopulations of murine bone marrow cells. Note that *Trip230* expression is very high in the HL-60 and K562 hemopoietic cell lines. Exposure time, *Trip230*, 23.5 hrs; *β-Actin*, 1 hr. See Fig. 6.3 (this Chapter) for a detailed description of the purified subpopulations and experimental procedure.

Human Vacuolar protein sorting 11 (hVPS11) specifically associates with Bmi-1 in yeast.

```
  1 MAAYLQWRRF VFFDKELVKE PLSNDGAAPG ATPASGSAAS KFLCLPPGIT
 51 VCDSGRGSLV FGDMEGQIWF LPRSLQLTGF QAYKLRVTHL YQLKQHNILA
101 SVGEDEEGIN PLVKIWNLEK RDGGNPLCTR IFPAIPGTEP TVVSCLTVHE
151 NLNFMAIGFT DGSVTLNKGD ITRDRHSKTQ ILHKGNYPVT GLAFRQAGKT
201 THLFVVTTEN VQSYIVSGKD YPRVELDTHG CGLRCSALSD PSQDLQFIVA
251 GDECVYLYQP DERGPCFAFE GHKLIAHWFR GYLIIVSRDR KVSPKSEFTS
301 RDSQSSDKQI LNIYDLCNKF IAYSTVFEDV VDVLAEWGSL YVLTRDGRVH
351 ALQEKDTQTK LEMLFKKNLF EMAINLAKSQ HLDSDGLAQI FMQYGDHLYS
401 KGNHDGAVQQ YIRTIGKLEP SYVIRKFLDA QRIHNLTAYL QTLHRQSLAN
451 ADHTTLLLNC YTKLKDSSKL EEFIKKKSES EVHFDVETAI KVLRQAGYYS
501 HALYLAENHA HHEWYLKIQL EDIKNYQEAL RYIGKLPFEQ AESNMKRYGK
551 ILMHHIPEQT TQLLKGLCTD YRPSLEGRSD REAPGCRANS EEFIPIFANN
601 PRELKAFLEH MSEVQPDSPQ GIYDTLLELR LQNWAHEKDP QVKEKLHAEA
651 ISLLKSGRFC DVFDKALVLC QMHDFQDGVL YLYEQGKLEQ QIMHYHMQHE
701 QYRQVISVCE RHGEQDPSLW EQALSYFARK EEDCKEYVAA VLKHIENKNL
751 MPPLLVVQTL AHNSTATLSV IRDYLVQKLQ KQSQQIAQDE LRVRRYREET
801 TRIRQEIQEL KASPKIFQKT KCSICNSALE LPSVHFLCGH SFHQHCFESY
851 SESDADGPTC LPENRKVMDM IRAQEQKRDL HDQFQHQLRC SNDSFSVIAD
901 YFGRGVFNKL TLLTDPPTAR LTSSLEAGLQ RDLLMHSRRG T*
```

Fig. 6.15 Amino acid sequence of the human Vacuolar protein sorting 11 (hVPS11) protein. The human protein contains 941 a.a (accession number Q9H270). The first

amino acid encoded by the yeast-two-hybrid clones 41.7 and 53.7 (identical clones) is in pink, that of clone 42.7 is in grey and that of clone 43.7 is in yellow. The green colored boxes correspond to the cysteine (C) and histidine (H) residues involved in coordinating the C-terminal RING/zinc finger domain.

BHIP-2: a KRAB and Zinc finger domain-containing protein that associates with Bmi-1 in yeast.

The KRAB domain (or Kruppel-associated box), which is present in about a third of zinc finger proteins containing C2H2 fingers, is found to be involved in protein-protein interactions. This domain is generally encoded by two exons, corresponding to the KRAB-A and KRAB-B subdomains.

The C2H2 zinc finger is the classical zinc finger domain. The two conserved cysteines and histidines co-ordinate a zinc ion. The following pattern describes the zinc finger. #-X-C-X(1-5)-C-X3-#-X5-#-X2-H-X(3-6)-[H/C], where X can be any amino acid, and numbers in brackets indicate the number of residues. The positions marked # are those that are important for the stable fold of the zinc finger. The final position can be either an histidine (H) or cysteine (C). The C2H2 zinc finger is composed of two short beta strands followed by an alpha helix. The amino terminal part of the helix binds the major groove in DNA binding zinc fingers.

```
1   METQADLVSQ EPQALLDSAL LSKVPAFSDK DSLGDEMLAA ALLKAKSQEL
51  VTFEDVAVYF IRKEWKRLEP AQRDLYRDVM LENYGNVFSL DRETRTENDQ
101 EISEDTRSHG VLLGRFQKDI SQGLKFKEAY EREVSLKRPL GNSPGERLNR
151 KMPDFGQVTV EEKLTPRGER SEKYNDFGNS FTVNSNLISH QRLPVGDRPH
201 KCDECSKSFN RTSDLIQHQR IHTGEKPYEC NECGKAFSQS SHLIQHQRIH
251 TGEKPYECSD CGKTFSCSSA LILHRRIHTG EKPYECNECG KTFSWSSTLT
301 HHQRIHTGEK PYACNECGKA FSRSSTLIHH QRIHTEALPT FVTLIRLLPS
351 VDPIVTNEAA FPAESLATIF ALIWRLFCVH SLMFKKV*
```

Fig. 6.16 Amino acid sequence of the human Zinc finger protein 3 (ZNF3) protein. The human protein contains 387 amino acids (accession number NP060185). The KRAB box is underlined in yellow. The four zinc finger domains (C2H2 type) are underlined.

The conserved cysteines (C) and histidines (H) residues that co-ordinate the zinc ions are in green. The glycine residue in pink is the first amino acid of the yeast-two-hybrid clone identified in our screen (clone 46.7). Of note, no mouse homologue for ZNF3 has been identified thus far.

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CHAPITRE 7

Conclusions, Perspectives and Future Directions

Chapter 7 is a discussion of the relevance of the findings presented in this thesis and adresses potential future studies that could be performed to further understand the roles of *Bmi-1* and *eed* in the regulation of normal and leukemic hemopoiesis.

"Epigenetics" and the regulation of hemopoiesis

The unique hierarchical and unidirectional nature of the hemopoietic system makes it a powerful model system to study the molecular mechanisms involved in the regulation of cell fate. Everyday, billions of mature blood cell elements must be replenished from a rare population of highly primitive multipotent hemopoietic stem cells (HSCs) capable of extensive self-renewal. The processes of lineage commitment and ensuing differentiation/proliferation outcomes of the gradually maturing blood-forming units along a particular lineage pathway are tightly regulated both at the extracellular and intracellular levels. Amongst the intrinsic influences, chromatin structure appears to contain the molecular imprint underlying the epigenetic inheritance of lineage/cell-type specific transcription programs. Modification of the protruding histone tails –by the addition of methyl, acetyl or phosphate groups- is known to alter chromatin structure, which in turn influences the activity of neighboring genes. While the consequences of individual histone modifications have begun to be addressed, the great challenge now resides in deciphering how a sequential and possibly combinatorial network of such modifications –known as the histone code- can direct gene expression, either in a synergistic or antagonistic fashion.

Epigenetic regulation of cell fate by the *Polycomb group (PcG)* genes

The *Polycomb (PcG)* and the *Trithorax (TrxG) group* genes are part of a widely conserved epigenetic cellular memory system responsible for regulating cell fate in several developmental systems [1,2] (see also Chapter 1). Based on biochemical characteristics, at least two distinct types of PcG complexes with different properties/functions can be distinguished both in *Drosophila* and mammals (see Chapter 1) [3-11]. The Extra Sex Combs (Esc) and Enhancer of Zeste (E(z)) PcG proteins form the core of an evolutionary conserved PcG complex that appears to be involved in making the "epigenetic mark" necessary for the establishment and memory trace of the silent state and is therefore referred to as "PcGe" [6,8]. The mechanisms by which the PcGe complexes find their way onto chromatin and convey epigenetic inheritance are being

unraveled. Biochemical evidence links PcGe complexes to histone deacetylases (HDACs), although the data gathered to date suggests that the interaction may be transient or that biochemically distincts PcGe complexes may exist [7,11,12]. Further insights into the mechanisms underlying PcGe silencing came from the recent finding that E(z)/EZH2 displays methyltransferase activity towards lysine (K) 9 and K27 of histone H3 (with a strong preference for the latter), a modification that might contribute to the stability of the PcGe complex and its spreading to neighboring target loci, particularly in the early stages of assembly at *Polycomb* response elements (PREs) [11,13,14]. Experiments performed in the fly demonstrated that the trimethylation mark at K27 is lost well before the binding of the PcGe proteins and revealed the presence of PcGe complexes at some transcriptionally active *Polycomb* response elements (PREs) [15]. Thus, the trimethyl mark could represent the distinction between the mere recruitment of the PcGe complex (which may be constitutive) and its ability to trigger the silent state. Importantly, the trimethyl K9 and dimethyl K27 on histone H3 appear to be a specific mark for PcG chromatin, as this pattern is highly enriched at PcG target sites in polytene chromosomes and poorly represented in regions of constitutive heterochromatin [13]. Notably, H3-K27 methylation is found in a variety of multi-cellular organisms including human, chicken, and *Drosophila,* but has not been observed in the budding yeast *Saccharomyces cerevisiae* [12], were orthologs of Eed and E(z) are absent. Deciphering the "histone code" accountable for PcGe complex recruitment will be instrumental in understanding how defined chromatin states are self-propagated through DNA replication and mitosis and will assess whether PcGe-mediated histone H3 methylation and/or deacetylation constitute an "ancient" molecular mechanism of gene silencing in the metazoan era.

The second type of PcG complexes appears to be required for stable maintenance (PcGm) of gene silencing and is exemplified by Polycomb repressive complex 1 (PRC1), a ~3 MDa complex purified from *Drosophila* embryos [3,16]. While its *in vivo* mode of action remains elusive, the PRC1 complex, which is widely conserved, harbors the capacity to counteract SWI/SNF chromatin remodeling complexes and stabilize a repressive chromatin structure *in vitro*. While a recent study suggested that the

association of PRC1-like (PcGm) complexes with the SuV39H1 histone methyltransferase enzyme (specific for H3-K9) may be involved in its ability to silence gene expression [17], PcGm complexes appear to recognize a pre-existent epigenetic repressional state generated by EZH2-containing PcGe complexes [11-13]. Accordingly, elegant experiments in the fly revealed that the establishment of heritable PcG silencing early in development requires a transient interaction between the PcGe and PcGm complexes [14]. It will be important to verify whether this transitory interaction between the complexes is evolutionarily conserved and takes place in other cellular and developmental contexts.

PcG function in regulating hemopoiesis: a role for the _hox_ cluster genes?

Although *Hox* genes are best known for their role in the specification of the antero-posterior (A/P) skeletal axis during embryonic development, it is now becoming clear that *Hox* genes also play a part in determining cell fate in several other tissues, including blood (reviewed in [18,19]). By analogy to their role in skeletal development, the studies presented in Chapter 2 raise the possibility that the *PcG* complexes might be involved in regulating hemopoiesis through the transcriptional silencing of the *Hox* cluster genes. In sharp contrast to the expression of *HoxA* and *B* cluster genes which is highest in the most primitive bone marrow cells, differentiation of primary human bone marrow cells is accompanied by a marked up-regulation in *PcG* gene expression levels including *M33, MEL-18, HPH1* and *ENX-1/EZH2*. Only *BMI-1* exhibits a different pattern of expression with high levels in primitive $CD34^+$ cells and very low levels in mature $CD34^-$ cells (see below). These data are consistent with a role for the PcG complexes in regulating the differentiation and/or proliferation of human bone marrow cells by silencing *Hox* gene expression [20]. Similarly, the *Mel-18, Bmi-1, M33 and Mph1/Rae-28 PcG* genes were shown to be rapidly activated upon antigenic stimulation of lymphoid B cells and are considered as immediate early genes. This contrast with the ubiquitous expression of the *PcG* genes in *Drosophila* syncytial blastoderms (reviewed in [21]) and might represent a critical mechanism of PcG function in adult hemopoiesis. Interestingly however, no evidence for *Hox* gene misexpression in the hemopoietic tissue of *PcG*

mutant mice has been reported yet, even though such a role has been suspected [10,22]. Extensive analyses of *Hox* gene expression levels in highly purified subpopulations of hemopoietic cells isolated from *PcG* mutant mice is awaited in order to resolve this issue.

Importantly, the above findings raise the intriguing possibility that the heterogeneity in PcG complex composition contributes to the establishment of lineage/cell-type specific transcription programs in differentiating bone marrow cells. In this view, *PcG* gene function in adult hemopoiesis would result, at least in part, from temporal and cell-type specific expression of the *PcG* genes [20,23-26], their modifications at the post-translational level [9,27,28], their subcellular distribution [27,29,30], the competition between PcG sub-family members with highly homologous protein interacting motifs (see Chapter 2 and 6) and their physical association with co-regulatory molecules displaying intrinsic enzymatic properties [17,31,32]. Furthermore, owing to the apparent absence of DNA-binding activity amongst the *PcG* machinery, the selective recruitment of PcG complexes to relevant target loci in hemopoietic cells relies on sequence-specific tethering molecules (see Chapter 6), their ability to recognize definite repressive "histone codes" [17,31], their interaction with components of the general transcriptional machinery [15,33] and chromatin condensation proteins [34,35]. Boundary elements that establish "nuclear domains" of transcriptional regulation through modulation of the chromatin environment most likely also impact on *PcG* gene function in regulating hemopoiesis [36,37].

The proliferation of hemopoietic progenitor cells is regulated by the combined action of Eed and Bmi-1-containing PcG protein complexes

The murine *Polycomb Group (PcG)* proteins Eed and Bmi-1 govern axial patterning during embryonic development by segment-specific repression of *Hox* gene expression [38-41]. The two proteins engage in biochemically distinct multimeric complexes that are known to functionally interact to render the regulatory regions of *Hox* and other downstream target genes inaccessible to transcriptional activators ([42], Chapter 1 and S. Paylor and A. Schumacher, unpublished). The finding that *eed* and *Bmi-1* are co-

expressed in both murine Sca-1^{+}Lin^{-} and human CD34^{+}CD45^{-}CD71^{-} primitive bone marrow cells [10,20], lead us to propose that Eed and Bmi-1 might similarly interact to regulate some critical aspects of hemopoietic cell development. In the studies reported in Chapter 3, evidence is presented that is consistent with an antagonistic function of *eed* and *Bmi-1* in hemopoietic cell proliferation. Heterozygosity for an *eed* null allele in mice causes marked myelo- and lympho-proliferative defects (3-fold increase in primitive (LTC-IC and WW-IC) and 19-fold increase in late (myeloid and pre-B-CFC) bone marrow progenitor cell numbers on average), indicating that *eed* is involved in the negative regulation of the pool size of both lymphoid and myeloid progenitor cells. This antiproliferative function of eed does not appear to be mediated by the *Hox* genes or the *Ink4a-Arf* tumor suppressor locus, as the expression of these genes was not altered in *eed* mutants. Conversely, a loss-of-function allele of *Bmi-1* causes a profound decrease in the numbers and proliferative potential of both highly primitive myeloid (LTC-IC) and lymphoid (WW-IC) bone marrow cells (4% and 1% of wild-type levels, respectively). Moreover, the absolute number of mature B220^{+} B cells generated per WW-IC at limiting dilution was reduced 8-fold in *Bmi-1*$^{+/-}$ mice relative to control animals, indicating that *Bmi-1* is haplo-insufficient for the proliferative potential of this subset of B cell precursors. In order to verify whether Eed and Bmi-1 functionally interact to regulate hemopoietic cell proliferation, inter-cross experiments between *eed* and *Bmi-1* mutant mice were performed. *Eed/Bmi-1* double mutant mice were severely pancytopenic and their number of early (WW-IC and LTC-IC) and late (myeloid and pre-B CFC) bone marrow progenitor cells were identical to those of *Bmi-1*$^{-/-}$ mice, indicating that *Bmi-1* 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 loss of one or two functional alleles of *eed* trans-complements the *Bmi-1* deficiency in pre-B bone marrow cells. Thus, the function of the two types of PcG complexes seems to have evolved from a common repressive effect on *Hox* gene expression in axial patterning to a more divergent activity in hemopoietic cells. Whereas the PcGm complex is involved in enhancing the proliferation of BM progenitor cells, the PcGe complex is a negative regulator of BM progenitor cell proliferation. Ongoing experiments suggest that the latter displays tumor suppressive function in hemopoietic

cells, possibly through an epigenetic mechanism involving H3-K27 methylation (see Chapter 4). Therefore, we believe that proper level of proliferation of several subsets of hemopoietic cells is regulated by the relative contribution of Eed (repressive) and Bmi-1 (enhancing)-containing PcG gene complexes.

Bmi-1 as a regulator of embryonic and adult HSC function

During mouse ontogeny, the first bloods cells, embryonic (or primitive) erythrocytes, arise within the blood islands of the extraembryonic yolk sac at embryonic day 7.5 (E7.5). By 11.5, hemopoiesis shifts to the fetal liver (FL), where adult (or definitive) red cells, as well as cells of other hemopoietic lineages, first appear. The site of origin of the hemopoietic stem cells (HSCs) has been less certain. Whereas it was previously accepted that HSCs and progenitors migrate from the yolk sac to the fetal liver during development, more recent studies relying on cell transplantation to reconstitute hemopoiesis in adult recipients assign an intraembryonic source for definitive (adult) hemopoiesis within the intraembryonic para-aortic splanchnopleura (PS) and aortic-gonadal-mesonephros (AGM) regions [43,44] [45]. HSCs arising in these areas are believed to migrate to and colonize the fetal liver and spleen, where they continue to differentiate into recognizable hemopoietic precursors. After birth, definitive hemopoiesis is primarily confined to bone marrow, and in some pathological conditions, also to extramedullary sites such as the spleen, the liver and occasionally the lung and brain. The presence of multipotential progenitors in the blood of E10 embryos suggests that migration and colonization are mediated via the circulation [46]. A unique origin of HSCs is challenged by recent evidence demonstrating long-term repopulation by yolk sac (extra-embryonic) progenitors as assayed by reconstitution of fetal recipient animals [47]. Hence, the origin of adult hemopoietic cells (and in particular definitive HSCs) within the specific vascular regions of the mammalian embryo body remains highly speculative. Nevertheless, the development of a stable functioning hemopoietic system reflexes complex processes involving cellular differentiation, as well as temporal and spatial control of migration, homing, self-renewal/proliferation and survival of HSCs.

During embryonic (FL) [93] and adult ([10,20] and see Chapter 2) hemopoiesis, expression of the *Bmi-1* gene is highly enriched in stem and multipotent progenitor cells, consistent with a role for this gene in regulating HSC function. In the studies presented in Chapter 3, we showed, using a knock-out allele of the *Bmi-1* gene and techniques of limiting-dilution analysis, that *Bmi-1* is absolutely essential for the identity and function of adult hemopoietic stem and progenitor cells. In Chapter 5, we next examined the consequences of *Bmi-1* inactivation on embryonic (FL) stem and multipotent progenitor cell function, using hemopoietic chimeras generated from *Bmi-1*$^{-/-}$ E14.5 fetal liver (FL)-derived cells engineered to overexpress either *Bmi-1* or the *EGFP* gene. First, these studies demonstrated that the absolute number and proliferative potential of myeloid progenitors derived from *Bmi-1*$^{-/-}$ E14.5 fetal liver (FL) cells is significantly impaired relative to controls. This progenitor cell defect was quantitatively and qualitatively rescued by the retroviral transduction *Bmi-1* in these cells, indicating that *Bmi-1* is dispensable for the generation of fetal liver-derived myeloid progenitors, but absolutely essential for their full proliferative activity. Similarly, transplantation studies performed at limiting dilution in sub-lethally irradiated recipients confirmed the presence of similar numbers of cells with long-term repopulating potential (LRC) in E14.5 *Bmi-1*$^{-/-}$ fetal livers relative to controls. Importantly, the detection of the *Bmi-1*$^{-/-}$ FL-derived HSCs, at 16 weeks post-transplantation, was strictly dependent on the retroviral expression of *Bmi-1*, indicating that *Bmi-1* is dispensable for the genesis of fetal liver-derived HSCs, but absolutely required for their proliferative capacity. The long-term (16 wks) and pluripotent potential of these cells confirmed that the rescue was occurring at the HSC level [94]. Clarke and coll. also demonstrated recently an inability of bone marrow (BM) and E14.5 FL-derived *Bmi-1*$^{-/-}$ cells to contribute to long-term hemopoiesis in reconstitution (FL and BM) as well as competitive (FL) experiments, suggesting a cell autonomous impairment of their self-renewal/proliferation potential [93]. Of note, the proliferative defect in progenitors derived from *Bmi-1*$^{-/-}$ E14.5 fetal livers (FLs) was much less pronounced than that observed in the bone marrow, suggesting a progressive impairment of the proliferative potential of hemopoietic cells lacking this gene ([10] and Chapter 5). The apparent progressive instability of the HSC phenotype in the absence of *Bmi-1* may reflect complex epigenetic regulatory circuits established in a context-

dependent manner during hemopoiesis. Because our results are derived from the analysis of transplantation chimeras, we infer that the fetal liver and bone marrow HSC defects are cell-autonomous and hemopoietic cell specific. Our findings, however, do not exclude additional functions of *Bmi-1* specific to cells of the microenvironment, as these would not be detected in our assays.

The transcriptional machinery governing hemopoietic stem cell biology is undoubtedly very complex. Genes involved in regulating HSC function in early embryogenesis include: *SCL/tal-1* [48] (stem cell leukemia hemopoietic transcription factor), *GATA-2* [49] and *Rbtn2* [50] (also known as *Lmo-2* or *ttg-2*), which are essential for primitive and definitive hemopoiesis and *AML-1* [51,52] (*also known as RUNX1/CBFA2 and PEBP2B*), that is specifically required for definitive hemopoiesis. In the adult hemopoietic system, HSC homeostasis is maintained, at least in part, by the *TEL/ETV6* [53], *Hoxa9* [54] and *Hoxb4* [55-57] genes. Examination of the contribution of these genetic axes to Bmi-1's function in regulating the proliferation of embryonic and adult HSCs is eargely awaited.

Using a conditional gene targeting approach, Orkin and colleagues recently established that *SCL1/tal-1* is critical for the genesis of HSCs, but its continued expression is dispensable for HSC function [58]. Our studies now suggest that *Bmi-1* is dispensable for the identity of fetal liver-derived embryonic HSCs, but absolutely required for their *in vivo* proliferative capacity. Thus, we propose that two distinctive classes of hemopoietic "stem cell" regulatory factors exist: those required for HSC genesis, and those required for later HSC properties, such as long-term repopulating activity and multipotency.

The studies presented in Chapters 2 and 3 reported an inquisitive dissociation between the expression profile of *Bmi-1* (mostly restricted to primitive human $CD34^{+}CD71^{-}$ $CD45^{-}$ and mouse $Sca\text{-}1^{+}$ Lin^{-} bone marrow cells), and that of other known gene products with which Bmi-1 interacts (mostly expressed in the mature compartment of bone marrow cells). Therefore, we postulated that Bmi-1 function in early hemopoietic cells is mediated through its interaction with yet unidentified proteins. Chapter 6 summarizes the results of a yeast-two-hybrid screen which led to the identification of 8 Bmi-1

interacting factors in human stem and progenitor cells. These included: (1) $p120^{E4F-1}$, a known inhibitor of cellular proliferation and growth which seems to functionally interact with Bmi-1 in the regulation of cell proliferation and senescence; (2) Trip230, a coactivator of the thyroid hormone receptor (TR) that is inhibited through its interaction with pRb; (3) HPH1, a PcG protein already known to interact with Bmi-1 and (4) 2 novel gene products that were named BHIP-1 and BHIP-2 for Bmi-1 Hemopoietic Interacting Proteins 1 and 2. Characterizing the Bmi-1-containing complex(es) in early hemopoietic cells is most important considering the inability of this protein to intrinsically bind DNA and its essential role in regulating the proliferation of stem and progenitor cells.

Bmi-1 determines the proliferative activity of normal and leukemic hemopoietic stem cells

Compelling evidence indicates striking similarities in the organization of the cellular hierarchy of normal and neoplastic tissues [59], i.e. a rare stem cell population with extensive self-renewal capacity results in mature cells representing the bulk of the resulting tissue. Based on this, a current hypothesis suggests that genes essential for the integrity of normal stem cells are also required for "cancer stem cells".

The similarity in the hierarchical organization of cancerous and normal tissues is best characterized in the hemopoietic system. In humans, evidence suggests that acute myeloid leukemias (AMLs) originate from a population of primitive cells including hemopoietic stem cells (HSCs) [59-61]. Most immature-looking leukemic cells (blasts) display a limited proliferative capacity and therefore must be constantly replenished by the rare "leukemic stem cells" (L-HSCs) capable of extensive self-renewal [59]. Interestingly, these L-HSCs are morphologically indistinguishable from mature leukemic blasts, but they display surface antigens similar to normal HSCs. In the study presented in Chapter 5, we focused on the functional characterization of *Bmi-1*, a gene which appears to qualify as a new member of genetic determinants required for the maintenance of both normal and neoplastic "stem cells" in the hemopoietic tissue.

Several arguments suggest that the *Polycomb Group (PcG)* gene *Bmi-1* represents a good candidate for regulating the proliferative activity of both normal and leukemic HSCs. First, *Bmi-1* regulates the expression of *Hox* genes which influence the expansion of HSCs and leads to their transformation when overexpressed [55,62-64]. Second, *Bmi-1* expression is restricted to primitive bone marrow cells in both humans and mice (see Chapters 2 and 3) and is expressed in all myeloid leukemias analyzed to date ([10,20], Chapters 2, 3 and 5). Third, homozygosity for a null allele of *Bmi-1* causes a profound decrease in the numbers and the proliferative potential of both primitive myeloid (LTC-IC) and lymphoid (WW-IC) progenitor cells (4% and 1% of wild-type levels, respectively, ([10] and Chapter 3) as well as adult HSCs (Chapter 5). Fourth, although not investigated in stem cells, genetic ablation of the *INK4a/ARF* locus dramatically reduced the proliferative defects observed in *Bmi-1* deficient cells, indicating that *INK4a/ARF* is a critical *in vivo* downstream target for Bmi-1 in the regulation of hemopoietic cell proliferation [65]. This is potentially important considering that recent evidence indicated that HSCs' homeostasis/quiescence is maintained, at least in part, by a dominant antiproliferative tone mediated by critical molecular checkpoints in the cell cycle machinery [66,67].

In the studies presented in Chapter 5, we show that *Bmi-1* is a significant determinant of the proliferative activity of normal and leukemic stem and progenitor cells. Although *Bmi-1* is dispensable for the initial establishment of *Hoxa9-Meis1*-induced AML, replicative exhaustion of *Bmi-1*$^{-/-}$ L-HSCs is reached upon transplantation into secondary recipients, leading to rapid proliferative arrest, differentiation, apoptosis of the leukemic blasts and resumption to normal host hemopoiesis. However, through high *in vitro* selective pressure which induced epigenetic alterations of critical regulators of proliferation (including both $p19^{ARF}$ and $p16^{INK4a}$), rare immortalized *Bmi-1*$^{-/-}$ highly proliferative clones (HPCs) could be derived from *Bmi-1*$^{-/-}$ AMLs (at a frequency of 1% vs 24% without selection in control AMLs). When assessed *in vivo*, some of these *Bmi-1*$^{-/-}$ HPCs, initially non-leukemogenic, eventually acquired the capacity to induce AML, pointing to clonal evolution rather than selection, as the underlying mechanism

responsible for leukemic progression in certain *Bmi-1*$^{-/-}$ HPCs. Retroviral introduction of *Bmi-1* in these "immortal" but non-leukemic *Bmi-1*$^{-/-}$ HPCs could induce AML within similar latency periods than HPCs derived from control AMLs. The highly polyclonal nature and short latencies of these leukemias indicated that *Bmi-1* is sufficient to fully restore the leukemogenic potential of these clones. This demonstrates that *Bmi-1*'s function in L-HSCs involves more than cellular proliferation, which was apparently similar between the several selected HPCs derived from control and *Bmi-1*$^{-/-}$ AMLs [94].

Importantly, epigenetic and genetic abrogation of *CDKN2A* (*p16*^{INK4a}) and *CDKN2B* (*p19*^{ARF}) are common lesions associated with poor prognosis in several human leukemias and mouse leukemia models [68-72]. The observation that the leukemogenic potential of *Bmi-1*$^{-/-}$ clones correlated with the loss of expression of *p16*^{INK4a} and *p19*^{ARF} underscores the importance of investigating whether these CKIs represent downstream regulators of *Bmi-1* function in normal and neoplastic HSCs. However, the inability of a several *Bmi-1*$^{-/-}$ clones, which lacked expression of both p16^{INK4a} and p19^{ARF} (and other CKIs), to induce AML when transplanted indicates that *Bmi-1*'s function in leukemic stem cells involves additional targets [94]. Together, our studies indicate that *Bmi-1* is dispensable for the generation of normal and leukemic fetal liver-derived HSCs, but is absolutely necessary for their full proliferative potential.

Although it has been assumed that cancer may arise from a seemingly endless combination of genetic and epigenetic alterations, our observations suggest that leukemic transformation occurs when a limited number of critical molecular events converge. One such event causes deregulated cell proliferation (or immortalization), which, together with the necessary compensatory suppression of apoptosis needed to support it, provides a minimal platform necessary to support further neoplastic progression. The challenge is to identify and to understand the molecular anatomy of such pivotal steps in tumor progression and to develop therapies that directly attack these points of convergence. The crucial role played by a single molecule, Bmi-1, in maintaining leukemic stem cell pool kinetics *in vivo* suggests that Bmi-1 is involved in a pathway that is critical in integrating such processes. Thus, adroit targeting of its

components should have potent and specific therapeutic consequences. Among other consequences, transgenic systems that allow for induction and reversion of gene expression will be invaluable in testing the requirement for sustained Bmi-1 activity in the *in vivo* maintenance of L-HSCs. Such a system would also enable investigation of the leukemogenic potential of other types of complementary oncogenes in the absence of *Bmi-1*. The recent demonstration that continuous expression of the offending oncogene is required to maintain the tumor phenotype, despite the accumulation of spontaneous secondary mutations in other oncogenes and tumor suppressor genes, emphasizes the importance of such future studies in the elaboration of novel therapeutic strategies [73-76].

Bmi-1: a common regulator of stem cell function?

Recent studies implicated the Notch [77,78 79], Wnt [80,81] and Shh [82-84] signaling pathways in promoting stem cell self-renewal in a variety of different epithelia in addition to HSCs. In particular, mutations of these pathways have been associated with a number of human neoplasia, including colon carcinoma [85] and epidermal tumors (Wnt) [86], medulloblastoma [87] and basal call carcinoma (Shh) [88], and T-cell leukemias (Notch) [89]. Supporting a potential role for *Bmi-1* in regulating the self-renewal/proliferation of stem cells others than the HSCs, severe skeletal and neuronal developmental defects were reported in the *Bmi-1* knockout mouse [40]. Moreover, as mentioned in Chapter 5, the overexpression of *Bmi-1* was observed in several cases of human non-small cell lung cancer [90], breast cancer cell lines [91] as well as immortalized mammary epithelial cells (MECs) [91]. High-level DNA amplifications and gains of the region encompassing the human *Bmi-1* gene locus (10p13) have also been reported in several cases of head and neck carcinomas [92].

In summary, with the research described in this thesis, we established that the *Polycomb Group (PcG)* genes are expressed in the hemopoietic system and play a central role in regulating hemopoietic cell proliferation. Secondly, we were able to identify the *Bmi-1* gene as a master regulator of HSC proliferation in both embryonic and adult hemopoiesis. Most importantly, we found that *Bmi-1* function is preserved in leukemic

hemopoietic stem cells (L-HSCs), providing the first molecular basis for the concept that stem cell function (whether normal or neoplastic) is regulated by a common set of genetic determinants. These above findings have broad implications in the fields of stem cell biology and cancer.

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