Regulation of hematopoietic stem cells by their mature progeny

Carolyn A. de Graaf[a,b], Maria Kauppi[c], Tracey Baldwin[a], Craig D. Hyland[c], Donald Metcalf[c,1], Tracy A. Willson[a], Marina R. Carpinelli[a], Gordon K. Smyth[d,e], Warren S. Alexander[c,b,2], and Douglas J. Hilton[a,b,1,2]

[a]Molecular Medicine Division, [c]Cancer and Haematology Division, and [d]Bioinformatics Division, The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3052, Australia; and Departments of [b]Medical Biology and [e]Mathematics and Statistics, University of Melbourne, Parkville, Victoria 3010, Australia

Contributed by Donald Metcalf, October 27, 2010 (sent for review August 23, 2010)

Thrombopoietin (TPO), acting through its receptor Mpl, has two major physiological roles: ensuring production of sufficient platelets via stimulation of megakaryocyte production and maintaining hematopoietic stem cell (HSC) quiescence. Mpl also controls circulating TPO concentration via receptor-mediated internalization and degradation. Here, we demonstrate that the megakaryocytosis and increased platelet mass in mice with mutations in the *Myb* or *p300* genes causes reduced circulating TPO concentration and TPO starvation of the stem-cell compartment, which is exacerbated because these cells additionally exhibit impaired responsiveness to TPO. HSCs from $Myb^{Plt4/Plt4}$ mice show altered expression of TPO-responsive genes and, like HSCs from *Tpo* and *Mpl* mutant mice, exhibit increased cycling and a decline in the number of HSCs with age. These studies suggest that disorders of platelet number can have profound effects on the HSC compartment via effects on the feedback regulation of circulating TPO concentration.

The control of circulating platelet number is critical to health. Deregulated platelet production accompanies some leukemias and high platelet counts characterize many myeloproliferative disorders. Conversely, low platelet count, or thrombocytopenia, can occur as the result of autoimmune disorders or hematological diseases such as idiopathic thrombocytopenia purpura and is often a side effect of cytotoxic cancer treatments, where episodes of bleeding can put patients at risk as well as interrupt and compromise therapy (1). Thrombopoietin (TPO) is the primary regulator of platelet production. Loss-of-function mutations in the genes for *Tpo* or its receptor, *Mpl*, result in a severe reduction in platelets, megakaryocytes, and megakaryocyte progenitor cells (2, 3). In mice, reduced TPO signaling also leads to increased hematopoietic stem cell (HSC) cycling, reduced HSC function in transplantation assays and, over time, a deficit in HSC numbers (4–7). Likewise, in humans, mutations in *Mpl* result in congenital amegakaryocytic thrombocytopenia and, eventually, aplastic anemia (8). Taken together, these observations highlight the key role for TPO signaling in control of platelet number and regulation of HSCs.

TPO is produced primarily in the liver (9) and Mpl is expressed on platelets, megakaryocytes and their progenitors, and within the HSC compartment, including on cells with long-term hematopoietic reconstituting capacity (6, 10, 11). Several lines of evidence support a model in which TPO production is constant and that the concentration of available circulating TPO is controlled by the mass of Mpl receptors available to internalize the cytokine. For example, TPO transcription remains relatively constant during periods of thrombocytopenia or thrombocytosis (12–14), and serum TPO levels correlate with platelet and megakaryocyte mass rather than absolute platelet count (15, 16). In healthy individuals, this system provides an effective feedback mechanism in which the availability of TPO reflects platelet need.

The transcription factor Myb and its coregulator p300 have diverse roles in hematopoietic regulation. Knockout mouse studies have demonstrated an absolute requirement for Myb/p300 in hematopoiesis and a series of elegant conditional mutant studies in mice have defined key roles for Myb in progression of erythropoiesis and lymphopoiesis (17–19). In contrast, Myb/p300 appears to play a repressive role in megakaryocytopoiesis. We recently identified the hypomorphic alleles Myb^{Plt3}, Myb^{Plt4}, and $p300^{Plt6}$, which each cause a marked thrombocytosis (20, 21). This phenotype has also been observed in independently derived mutant alleles of *Myb* and *p300* (22–24) and appears to arise from excessive production of megakaryocytes and their progenitor cells from multipotent precursors (25).

In this article, we show that mice with mutations in *Myb*/*p300* not only have a low circulating TPO level, consistent with the thrombocytosis characteristic of these mutants, but that $Myb^{Plt4/Plt4}$ stem cells also express subnormal amounts of *Mpl* and respond poorly to TPO. Consistent with reduced TPO signaling, *Myb*/*p300* mutant HSCs exhibit altered expression of TPO-responsive genes, are more actively cycling than normal stem cells in vivo, and become depleted with age, phenotypes that are ameliorated by transgenic overexpression of TPO. Our data highlight a unique link between platelet number and the regulation of HSCs and suggests that unrestrained elevation in platelet production may lead to significant perturbations of the stem-cell compartment via TPO depletion.

Results

***Myb/p300* Deficiency-Induced Thrombocytosis Leads to a Reduction of Serum TPO Levels.** To explore the relationship between platelet number and circulating TPO level, serum TPO concentrations and platelet counts were measured in $Myb^{Plt4/Plt4}$ mice on either wild-type or $Mpl^{-/-}$ genetic backgrounds and compared with control cohorts of wild-type, $Mpl^{-/-}$, and *Tpo* transgenic (Tpo^{Tg}) mice (Fig. 1 *A* and *B*). As expected, TPO levels were high in $Mpl^{-/-}$ mice and unaffected by the thrombocytosis in $Mpl^{-/-}$ $Myb^{Plt4/Plt4}$ mutants. This result is because of the inability to actively clear circulating TPO in the absence of *Mpl* expression. In Tpo^{Tg} mice, which constitutively express TPO from a liver-specific transgene (*SI Materials and Methods*), TPO production was elevated beyond the consumption ability of even the large numbers of platelets found in these mice, resulting in a high circulating concentration. In $Mpl^{+/+}$ $Myb^{Plt4/Plt4}$ mice, TPO levels were approximately half that observed in wild-type mice, consistent with the marked

Author contributions: C.A.d.G., M.K., G.K.S., W.S.A., and D.J.H. designed research; C.A.d.G., M.K., T.B., C.D.H., D.M., T.A.W., and M.R.C., performed research; C.A.d.G., M.K., C.D.H., D.M., G.K.S., W.S.A., and D.J.H. analyzed data; and C.A.d.G., W.S.A., and D.J.H. wrote the paper.

Conflict of interest: D.J.H. and W.S.A. hold shares in Murigen Therapeutics and Murigen Pty. Ltd., both of which funded part of this work.

Data deposition: Microarray data generated in this study have been deposited in ArrayExpress http://www.ebi.ac.uk/arrayexpress/ (accession no. E-TABM-1050).

[1]To whom correspondence may be addressed. E-mail: metcalf@wehi.edu.au or hilton@wehi.edu.au.

[2]W.S.A. and D.J.H. contributed equally to this work.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1016166108/-/DCSupplemental.

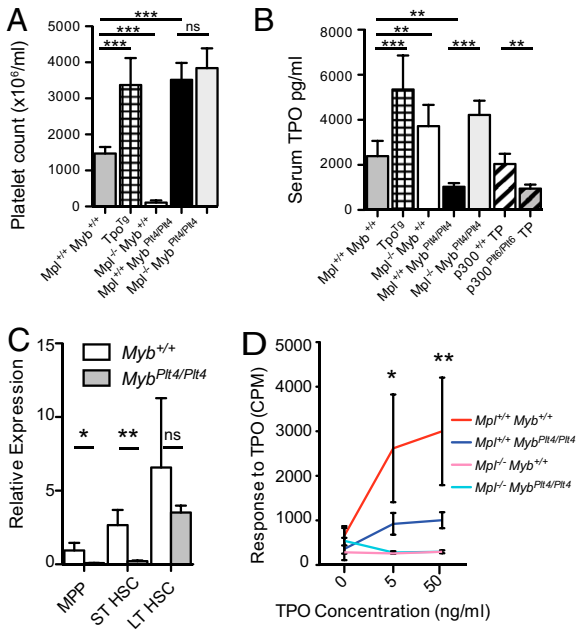

Fig. 1. Deficiencies in the TPO/Mpl pathway in $Myb^{Plt4/Plt4}$ mice. (*A*) Platelet counts and (*B*) circulating serum TPO levels in 8- to 12-wk-old mice of each genotype; TP indicates data from mice 16 wk after bone marrow transplant with bone-marrow donors as indicated. n = 6–21 mice per genotype. (*C*) *Mpl* expression measured by quantitative real time RT-PCR in cell populations purified by flow cytometry. Data shown are expression of *Mpl* relative to *Hprt* expression, measured by the ΔΔCt method. n = 5–8 mice per genotype. (*D*) Thymidine incorporation in lineage-negative (Lin^-) cells from mice of the indicated genotypes after 4 d of culture in TPO. n = 5 for $Mpl^{+/+}$, n = 2 for $Mpl^{-/-}$ mice. Data are shown as means ± SDs. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; and ns, not significant, for comparisons between $Mpl^{+/+}$ $Myb^{+/+}$ and $Mpl^{+/+}$ $Myb^{Plt4/Plt4}$ data.

thrombocytosis typical of *Myb* mutant mice driving increased TPO clearance. A similar result was observed for irradiated mice reconstituted with stem cells from $p300^{Plt6/Plt6}$ mice, which are also thrombocytotic (21). This finding reinforces the TPO-independent nature of the expanded thrombopoiesis in *Myb* and *p300* mutant mice and demonstrates the capacity of excessive numbers of platelets to consume circulating TPO.

$Myb^{Plt4/Plt4}$ Cells Respond Poorly to TPO. In addition to controlling megakaryocyte and platelet production, TPO also has a role in HSC regulation (4–7). Because thrombocytosis in $Myb^{Plt4/Plt4}$ mice resulted in a significant reduction in circulating TPO, we examined TPO-dependent activities in $Myb^{Plt4/Plt4}$ HSCs. The lineage $(Lin)^-$ $Sca\text{-}1^+$ $c\text{-}Kit^+$ (LSK) compartment of the bone marrow has been shown to contain all of the HSCs capable of hematopoietic reconstitution (26), but this population is heterogeneous and can be divided by cell-surface markers into subsets with differing repopulation potential. Quantitative RT-PCR analysis of phenotypically defined LSK $CD34^-$ $Flt3^-$ long term (LT)-HSCs, LSK $CD34^+$ $Flt3^-$ short term (ST)-HSCs, and LSK $CD34^+$ $Flt3^+$ multipotent progenitors (MPPs) (27, 28) revealed that *Mpl* expression was reduced in all three subsets of LSK cells in $Myb^{Plt4/Plt4}$ mice compared with that in wild-type cells (Fig. 1*C*).

We next compared responses to TPO of Lin^- cells collected from $Myb^{Plt4/Plt4}$ and wild-type mice. We found significantly less proliferation of $Myb^{Plt4/Plt4}$ Lin^- cells compared with their wild-type counterparts at each of two concentrations of TPO analyzed (Fig. 1*D*). As a control, we confirmed that both $Mpl^{-/-}$ $Myb^{+/+}$ and $Mpl^{-/-}$ $Myb^{Plt4/Plt4}$ Lin^- cells were unable to respond to TPO. This finding shows that the TPO/Mpl pathway is highly perturbed in $Myb^{Plt4/Plt4}$ mice: $Myb^{Plt4/Plt4}$ LSKs have reduced *Mpl* expression, are exposed to lower TPO concentrations, and respond poorly to TPO in vitro.

Composition of the LSK Populations in $Myb^{Plt4/Plt4}$ Mice. Flow cytometric analysis revealed that $Mpl^{+/+}$ $Myb^{Plt4/Plt4}$ femurs had five times the number of LSK cells than observed in wild-type controls (Fig. 2), with no significant change in overall cellularity. The overall increase in LSK number was primarily because of increased ST-HSCs, which were 7.5 times more numerous in $Mpl^{+/+}$ $Myb^{Plt4/Plt4}$ bone marrow than $Mpl^{+/+}$ $Myb^{+/+}$ bone marrow (Fig. 2); however, there were also four times as many MPPs in $Myb^{Plt4/Plt4}$ bone marrow. No significant alteration in the number of LT-HSCs in $Mpl^{+/+}$ $Myb^{Plt4/Plt4}$ mice was observed compared with wild type, but as in $Mpl^{-/-}$ mice, they constituted a reduced proportion of the LSK compartment. As expected from previous studies (5), the numbers of LSKs and LSK subsets were significantly decreased in $Mpl^{-/-}$ bone marrow compared with wild type. The effect of *Myb* mutation on these populations was not influenced by the presence or absence of Mpl (Fig. 2).

***$Mpl^{-/-}$* and *Myb/p300*-Deficient LSKs Share a Common Set of Gene-Expression Changes.** To explore gene-expression changes in the stem-cell compartment, we collected LSK cells from wild-type, $Mpl^{-/-}$, $Myb^{Plt4/Plt4}$, and TPO^{Tg} mice, as well as mice reconstituted with $p300^{Plt6/Plt6}$ bone marrow (and as a control, from mice reconstituted with $p300^{+/+}$ bone marrow), and isolated RNA that was then hybridized to Illumina Mouse-WG microarrays. Although there were many genes that were differentially expressed when comparing LSKs from each mutant to LSKs from wild-type mice (380 for $Myb^{Plt4/Plt4}$ vs. $Myb^{+/+}$; 333 for $p300^{Plt6/Plt6}$ vs. $p300^{+/+}$; 446 for $Mpl^{-/-}$ vs. $Mpl^{+/+}$), we focused on genes for which expression

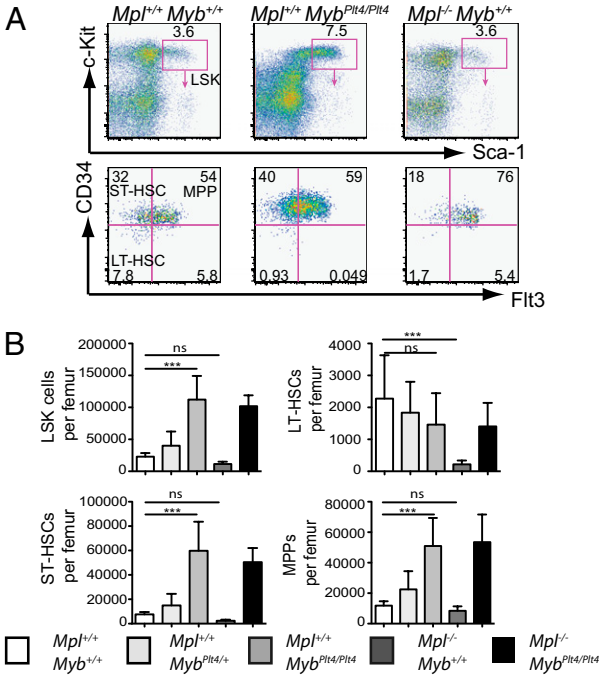

Fig. 2. $Myb^{Plt4/Plt4}$ mice have more prospective ST-HSCs and MPPs. (*A*) Representative flow cytometric analyses of viable Lin^- cells (*Upper*) and LSK cells (*Lower*) from bone marrow of mice of the indicated genotypes. The gates used to distinguish prospective LT-HSCs ($CD34^-$ $Flt3^-$ LSK), ST-HSCs ($CD34^+$ $Flt3^-$ LSK), and MPPs ($CD34^+$ $Flt3^+$ LSK) are shown. Percentages of gated cell populations within the parent populations are indicated. (*B*) Graphs show mean ± SD of the number per femur of total LSKs and LSK subsets. n = 5–6 mice per genotype. ***$P < 0.001$; ns, non significant.

was consistently deregulated in all of the mutants. We found 137 probes that were significantly up- or down-regulated in each of $Myb^{Plt4/Plt4}$, $p300^{Plt6/Plt6}$, and $Mpl^{-/-}$ LSKs compared with wild type (Fig. 3*A* and Dataset S1). These genes, the vast majority of which were down-regulated in mutant LSKs, tended to be up-regulated in cells from TPO^{Tg} mice. Thus, the changes in expression of this gene set in $Myb^{Plt4/Plt4}$ and $p300^{Plt6/Plt6}$ LSK cells is likely because of reduced TPO exposure. The data imply that normal expression of these genes not only requires TPO, but that they are inducible by excess TPO. We have termed this gene set the "TPO-response signature."

Subsets of the LSK compartment, LT-HSCs, ST-HSCs, and MPPs, are known to exhibit differential gene expression (29). Of five TPO-response signature genes tested, *Col4a1*, *Esam1*, *Ryk*, *Socs2*, and *Zfp532*, each showed reduction in expression when tested by RT-PCR in $Myb^{Plt4/Plt4}$ ST-HSCs and MPPs compared with wild-type cells. In LT-HSCs, two of these genes, although detected in $Myb^{+/+}$ cells, were no longer expressed in $Myb^{Plt4/Plt4}$ cells, and two genes were modestly, but not significantly, reduced in mutant cells (Fig. 3*B*). The majority of the genes in the TPO-response signature displayed intermediate expression in $Myb^{Plt4/+}$ LSK cells (Fig. 3*A*), in which the relative frequency of LT-HSCs, ST-HSCs, and MPPs is normal (Fig. 2). These data suggest that genes in the TPO-response signature were detected as differentially expressed as a result of expression changes across the mutant LSK subpopulations, rather than selective expression in a disproportionately represented cell subset. Although a number of targets of Myb have been identified, with diverse roles including differentiation, cell survival, and proliferation, none of the genes in the TPO-response signature are known to be direct targets of Myb (30–32). One member of the TPO-response signature, *Cdkn1c*, was reported to be up-regulated in human $CD34^+$ cells following Myb expression knockdown (32), but has been independently reported to be inducible by TPO (7). The down-regulation of *Cdkn1c* in *Myb*-mutant LSK cells implies that the influence of TPO on expression may outweigh any effects of Myb activity. Other genes within the TPO-response signature have not previously been associated with TPO signaling.

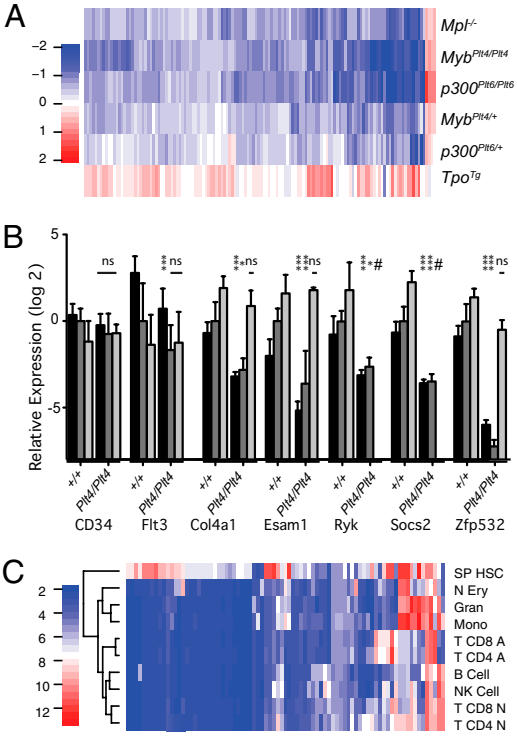

Fig. 3. Common gene-expression signature in Myb/p300 deficient and $Mpl^{-/-}$ LSK cells. (*A*) Heat map visualization of genes that are differentially expressed in $Myb^{Plt4/Plt4}$, $p300^{Plt6/Plt6}$, and $Mpl^{-/-}$ LSK cells in the same direction (either up or down) compared with wild type ($P < 0.05$). Genes are distributed horizontally and grouped vertically according to genotype. The scale represents $\log_2$-fold change differences for each mutant compared with wild type. (*B*) Quantitative real-time RT-PCR of gene expression in MPPs (black bars), ST-HSCs (dark gray bars), and LT-HSCs (light gray bars) purified by flow cytometry from $Myb^{+/+}$ mice ($^{+/+}$) and $Myb^{Plt4/Plt4}$ mice (*Plt4/Plt4*). Expression is presented as relative to that in $Myb^{+/+}$ ST-HCS, set as 1. Data, expressed as mean and SD, represent one experiment with two to five independent cDNA samples. *CD34* and *Flt3* were used to define the populations, but not shown to be differentially expressed on the microarrays. All other genes were identified as down-regulated by microarray analysis. Genes that were unable to be detected as expressed in a particular subset are marked with #. $***P < 0.001$; $*P < 0.05$; ns, not significant in comparison with $Myb^{+/+}$ to $Myb^{Plt4/Plt4}$. (*C*) Hematopoietic expression pattern of genes selected as down-regulated in $Myb^{Plt4/Plt4}$, $p300^{Plt6/Plt6}$ and $Mpl^{-/-}$ LSK cells. The scale represents normalized expression across the cell types for each gene. Cell types are murine, as defined in Chambers et al. (33), and sourced from http://franklin.imgen.bcm.tmc.edu/loligag/, where SP HSC is side population LSK, N Ery, nucleated erythrocyte; Gran, granulocytes; Mono, monocytes; T CD8 A and T CD4 A, activated T cell; T CD8 N and T CD4 N, naive T cell; NK Cell, natural killer cell.

Next, we investigated the expression of genes in the TPO-response signature in a publicly available collection of microarray data (33) from diverse hematopoietic cell types (Fig. 3*C*). This collection included the "side population" (SP) LSKs, which are highly enriched for HSCs. Our analysis revealed that the TPO-response signature is most highly expressed in the SP-LSKs and down-regulated as cells mature. Gene sets derived from gene ontology terms (34) sourced from MSigDB (35) were tested with Fisher's exact test to see if any were over represented in the down-regulated set of genes. We found that genes from the "Hematopoietic Stem Cell" [up-regulated in mouse HSCs compared with differentiated brain and bone marrow cells (36)] and "Cell Proliferation" (34) gene sets were overrepresented among the genes down-regulated in the TPO response signature (Table 1). This finding implies that the TPO-response signature genes are associated with proliferation, are enriched for those that are down-regulated as stem cells mature, and that these genes have specificity to the hematopoietic system.

Increased Cycling of $Myb^{Plt4/Plt4}$ LSK Cells. To explore cell cycle status in $Myb^{Plt4/Plt4}$ LSKs, we used a combination of DAPI to measure cell DNA content and an antibody specific for K_i-67, a nuclear antigen associated with proliferation (Fig. 4*A*). We found that a decreased proportion of $Mpl^{-/-}$ and $Myb^{Plt4/Plt4}$ LSKs were in the quiescent G0 phase, compared with wild-type mice and, reciprocally, that there was a modest increase in the proportion of LSKs in G0 in TPO^{Tg} mice (Fig. 4*B*). In contrast, the Lin^- $Sca\text{-}1^-$ $c\text{-}Kit^+$ fraction of the bone marrow, which only expresses low levels of *Mpl* (Fig. 4*C*), showed no difference in the number of cells that were in cycle for any of the genotypes examined (Fig. 4*D*).

Decline in HSC Number in $Myb^{Plt4/Plt4}$ Mice with Age. TPO regulates HSC quiescence and maintenance of HSC number throughout life (5, 7). In wild-type mice, LSK cell number did not vary greatly between 7 wk and 13 mo of age; however, in $Myb^{Plt4/Plt4}$ mice there was a marked expansion in LSK number that peaked at ≈9 mo of age and subsequently declined between 9 and 13 mo (Fig. 5*A*). Although the wild-type LT-HSC compartment showed a fivefold increase in numbers from 7 wk to 13 mo of age, no significant increase was observed in $Myb^{Plt4/Plt4}$ mice (Fig. 5*B*). Unlike wild-type bone marrow, in which progenitor cell numbers increased from 7 wk to 13 mo of age, in $Myb^{Plt4/Plt4}$ marrow, progenitor cell numbers declined modestly with age (Fig. 5*C*). These observations suggest that increased cycling of LT-HSCs in

Table 1. Gene sets over-represented in the TPO-response signature

Category	Genes
Cell proliferation	*Mpl, Ephb4, Fscn1, Ndn, Cdkn1c, Serpinf1, Dlg5, Ebi3, Maged1*
Hematopoietic stem cell	*Irf6, Traf1, Tcf15, Trib3, Tgm2, Emcn, Pkia, Efna1, Il11ra1, Galt, Tie1, Ppap2b, Mpl, Gjb3, Foxa3, Zfp30, Tead2, Cdkn1c, Phlda2, Vegfc, Col4a1, Col4a2, Cx3cl1, Ryk, Socs2, Serpinf1, Chrnb1, Aldoc, Ptrf, Ndrg2, Mapk12, Ltb, Ebi3, Ctsf, Fhl1, Bgn, Maged1*

Gene sets from MSigDB (35).

Myb mutant mice leads to a transitory expansion of the ST-HSC and MPP compartments at the expense of long-term maintenance of the LT-HSC compartment. Over time, this process leads to reduced ability to maintain the hematopoietic system.

TPO Rescues Loss of Quiescence in $Myb^{Plt4/Plt4}$ Mice. To confirm that the loss of HSC quiescence observed in $Myb^{Plt4/Plt4}$ mice was a result of TPO starvation, we introduced the *TPO* transgene onto the $Myb^{Plt4/Plt4}$ background, which exacerbated thrombocytosis above that observed in either the $Myb^{Plt4/Plt4}$ or the TPO^{Tg} mice (Fig. S1*A*). Despite the marked thrombocytosis in these mice, and hence increased TPO clearance, TPO production from the transgene was sufficient to ensure that the cytokine remained at supraphysiological levels in the serum (Fig. S1*B*). As described above, $Myb^{Plt4/Plt4}$ LSKs showed a decrease in quiescence, with fewer cells in G0 than evident in wild-type populations. TPO^{Tg} LSKs had a similar cell cycle profile to the wild-type mice, suggesting that additional TPO over that in wild-type mice does not influence HSC cycling. On a $Myb^{Plt4/Plt4}$ background, the additional TPO produced from the transgene resulted in partial rescue of quiescence, with more LSK cells in G0 and fewer in G1, compared with $Myb^{Plt4/Plt4}$ mice (Fig. 4*B*). Additionally, the number of LT-HSCs in compound $Myb^{Plt4/Plt4}$ TPO transgenic mice was increased compared with that observed in $Myb^{Plt4/Plt4}$ mice (Fig. S1*C*). These data support a model in which reduced circulating TPO contributes significantly to the HSC defects observed in $Myb^{Plt4/Plt4}$ mice.

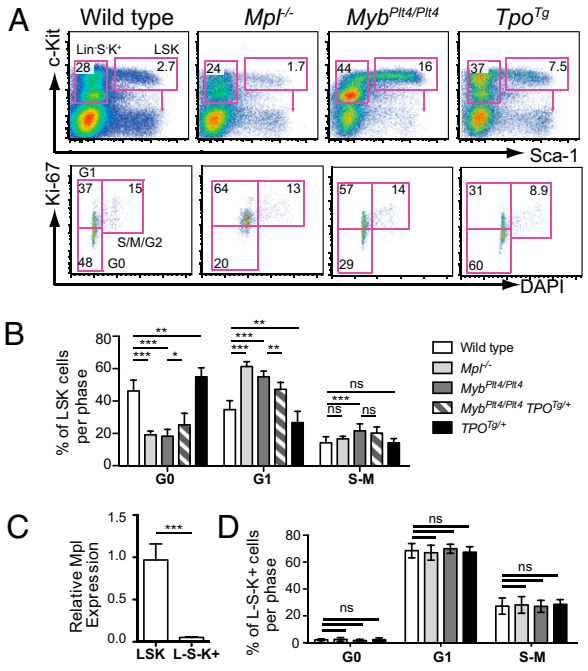

Fig. 4. Cycling is enhanced in $Myb^{Plt4/Plt4}$ cells. (*A*) Representative flow cytometric analyses of bone marrow from wild-type, $Mpl^{-/-}$, $Myb^{Plt4/Plt4}$, and TPO^{Tg} mice showing LSK and Lin-S-K$^+$ gates (*Upper*) and K_i-67/DAPI staining profiles for LSK cells (*Lower*). (*B*) Mean percentages of LSK cells that are in G0, G1, or S-M phase of the cell cycle. n = 4–8 mice of each genotype. (*C*) Relative expression of *Mpl* in hematopoietic progenitor populations as determined by qRT-PCR. Expression is relative to *Hprt* expression as determined by the ΔΔCt method. n = 3 independent samples per genotype. (*D*) Mean percentages of Lin$^-$ Sca-1$^-$ c-Kit$^+$ cells that are in G0, G1, or S-M phase. n = 6–10 mice of each genotype. Data shown are mean ± SD. ***$P < 0.001$; ns, not significant.

Discussion

Production of platelets is regulated by a negative feedback loop in which the concentration of free TPO in the circulation is controlled by the rate of its internalization via Mpl receptors expressed on the surface of megakaryocytes and platelets. Under thrombocytopenic conditions, this process leads to an accumulation of TPO and greater production of megakaryocytes via stimulation of megakaryocyte progenitors in the bone marrow. Reciprocally, in situations of thrombocytosis there are reduced TPO levels and hence a reduction in megakaryocyte production. However, TPO not only has effects on cells committed to the megakaryocyte lineage, but is also required to maintain the appropriate balance of quiescence and activity in HSCs (5, 7). Thus, fluctuations in circulating TPO concentration are likely to effect stem cell behavior.

As expected, thrombocytosis driven by *Myb* or *p300* mutations resulted in low circulating TPO concentrations. The likelihood that HSCs in these mice experience TPO deprivation was further supported by the observation that Mpl expression on $Myb^{Plt4/Plt4}$ LSK cells was reduced. Previous studies have noted reduced Mpl expression on platelets in mice administered with TPO, concluding that TPO has little or no effect on *Mpl* transcription or translation but that excess cytokine can stimulate greater receptor catabolism (37). In contrast, our data suggest that in the chronic low-TPO environment of $Myb^{Plt4/Plt4}$ mice, HSCs express reduced *Mpl* RNA. This finding may reflect differences in regulation of receptors in stem cells and platelets, or might reflect an intrinsic contribution of Myb deficiency to altered regulation of Mpl ex-

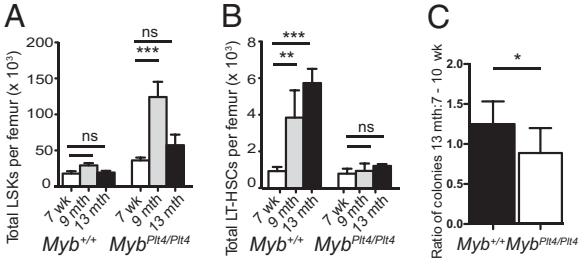

Fig. 5. Age-related deficits in $Myb^{Plt4/Plt4}$ bone marrow. Counts of (*A*) LSK cells per femur and (*B*) LT-HSCs per femur from $Myb^{Plt4/Plt4}$ and $Myb^{+/+}$ mice at 7 wk, 9 mo, and 13 mo of age. (*C*) Ratio of the number of bone marrow-derived colony-forming cells in $Myb^{Plt4/Plt4}$ and $Myb^{+/+}$ mice at 13 mo vs. 7 to 10 wk of age. Bone-marrow cells were cultured in SCF/IL3/EPO. Meg, megakaryocyte; G/M/Eo, Granulocyte/Macrophage/Eosinophil. Data shown are mean ± SD. n = 3–9 mice per genotype and age. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; ns, not significant.

pression. In any event, reduced receptor expression on stem cells from $Myb^{Plt4/Plt4}$ mice appeared functionally significant because $Myb^{Plt4/Plt4}$ LSK cells proliferated poorly in response to TPO in vitro. $Myb^{Plt4/Plt4}$ stem cells also showed multiple signs of TPO deprivation in vivo. Transcriptional profiling revealed changes in gene expression in $Myb^{Plt4/Plt4}$ LSK cells that were shared with cells from $Mpl^{-/-}$ mice and reciprocally regulated in mice exposed to excess TPO. These studies allowed definition of a unique TPO-response signature, a pattern of gene expression reflecting the degree of stimulation of hematopoietic stem cells by TPO. The majority of the genes within this TPO-response signature have not previously been implicated in TPO signaling; however, there was an overrepresentation of genes previously associated with HSCs, and a preponderance of proliferation-associated genes.

Studies in mice lacking TPO or Mpl have established that in the absence of TPO signaling, a greater than normal proportion of HSCs are in cycle at steady state (5, 7). Increased cycling characterizes HSCs in $Myb^{Plt4/Plt4}$ mice and was also observed in $Myb^{M303V/M303V}$ mice (24). Increased HSC cycling is consistent with the fact that many of the cell proliferation-associated genes that were down-regulated in the TPO-response signature are cell cycle related; our data suggest that genes such as *Ndn* (38), *Cdkn1c* (39), and *Maged1* (40) are important mediators of the balance between HSC quiescence and activity regulated by TPO. In support of this regulatory pathway, changes in HSC *Cdkn1c* expression have independently been associated with administration of TPO in mice (7). Consistent with TPO deprivation playing a key role in the HSC phenotype in $Myb^{Plt4/Plt4}$ mice, transgenic supplementation of TPO expression resulted in amelioration of the cell cycle deregulation characteristic of $Myb^{Plt4/Plt4}$ LSK cells. Also consistent with observations in TPO-deficient mice (41), the age-related increase in the number of phenotypically defined LT-HSCs typical of wild-type animals was absent in $Myb^{Plt4/Plt4}$ mice. The decline in the numbers of progenitor cells in aging $Myb^{Plt4/Plt4}$ mice is consistent with reduced stem cell activity, but the precise changes in functional capacity of $Myb^{Plt4/Plt4}$ stem cells with age requires further studies.

We conclude that the loss of stem-cell quiescence phenotype associated with *Myb* mutation arises primarily because of a feedback loop in which platelet numbers, through regulation of available TPO levels, regulates the entry of HSCs into cycle. Such a feedback mechanism may provide protection in times of hematopoietic stress associate with bleeding. Platelets will be lost directly following an episode of bleeding and indirectly as a result of concomitant vessel repair. The resulting increase in the levels of TPO will drive committed progenitor cells to produce megakaryocytes and platelets to replace those lost. Because emergency demand on the hematopoietic system runs the risk of exhausting the stem-cell compartment and compromising future hematopoietic production, the action of TPO to promote the quiescence of the most primitive stem cells may represent the mechanism by which acute demand for new blood cells can be balanced against future requirement.

It is clear from these experiments, and that of others, that deficits in the Myb/p300 complex mediate other changes to stem cell function that are TPO independent, such as the expansion of the ST-HSC population and bias in lineage commitment. The complexity of hematopoietic regulation makes it difficult to tease out which of these phenotypes are driven by direct functions of the Myb/p300 complex, and which result from feedback loops. The data presented here demonstrates that the enhanced cycling of stem cells is likely to be largely indirect and to arise from a unique feedback loop in which platelet number regulates the behavior of stem cells via control of circulating TPO concentration. To isolate the direct effects of Myb/p300 loss on the function of HSCs and progenitors from those caused by the bone-marrow environment, it may be useful to perform reciprocal transplant experiments using mice and cells deficient for Myb, Mpl, and TPO.

Materials and Methods

Mice and Hematological Analysis. Mice carrying the *Myb*, *p300*, and *Mpl* alleles were derived as previously described (3, 20, 21). *TPO* transgenic mice were created as described in *SI Materials and Methods*. All animal experiments were approved by the Walter and Eliza Hall Institute of Medical Research Animal Ethics Committee.

Manual or automated blood cell counts were performed on blood collected from the retro-orbital plexus into sample tubes coated with EDTA (Sarstedt). Mice with hematopoiesis derived from engrafted $p300^{Plt6/Plt6}$ stem cells were generated as previously described (21). Clonal analysis of bone marrow cells in semisolid agar cultures were performed as previously described (42) using a combined stimulus of 100 ng/mL SCF, 10 ng/mL IL-3 (both prepared in our laboratories), and 4 IU/mL EPO (Janssen-Cilag).

For serum TPO measurement, mice were bled by cardiac puncture, the blood was allowed to clot at room temperature, and serum was separated by centrifugation. Commercial monoclonal antibody-based ELISA test kits were used to detect TPO (Quantikine M mouse TPO, MTP00; R&D Systems).

Proliferation Assays. Twenty-thousand flow-sorted Lin^- cells were collected into DMEM with 10% FCS with recombinant TPO and incubated at 37 °C in 5% CO_2 in air. Cells were cultured for 4 d before they were pulsed with 1 μCi (0.037 MBq) [3H]-thymidine for 16 h, harvested onto Inotech glass filters, and counted in a TopCount NXT Microplate Scintillation Counter (Packard).

Cell Cycle Analysis. Bone-marrow suspensions were enriched for mononuclear cells by centrifugation over a Ficoll-Paque gradient. Cells were then incubated with biotinylated CD4, CD8, B220, Gr-1 and Ter119 antibodies (Pharmingen), before staining with fluorochrome-conjugated Sca-1, c-Kit, and streptavidin (Pharmingen). Cells were made permeable by incubation with Cytofix/Cytoperm solution (BD Biosciences) on ice before washing with Perm/Wash solution (Pharmingen) and incubating with K_i-67 antibodies for 18 h at 4 °C. DAPI (4′,6-diamidino-2-phenylindole, 5 mg/mL) was then added to each sample for room temperature incubation for 30 min. Cells were then resuspended and filtered before flow cytometric analysis.

Gene Expression Analysis. RNA for microarrays was extracted from LSK cells using MicroRNeasy (Qiagen) purification columns according to the manufacturer's protocol, then labeled, amplified, and hybridized to Illumina MouseWG-6 V1.1 or V2.0 Expression BeadChips according to Illumina standard protocols at the Australian Genome Research Facility, Melbourne. Each sample was derived from bone-marrow LSK cells from at least six donor mice. Data were analyzed in R and subjected to a variance stabilizing transformation and quantile normalization (43). Linear modeling using an empirical Bayes approach was applied to the data (44). Data were corrected for multiple testing using Benjamini and Hochberg correction. Microarray data are available at Array Express (www.ebi.ac.uk/arrayexpress/) under accession number E-TABM-1050. The $Myb^{Plt4/Plt4}$ and $Myb^{+/+}$ microarrays data were those previously described (19). Methods for real-time quantitative RT-PCR were described in *SI Materials and Methods*.

Statistical Tests. The two-sided Student *t* test was used for all statistical analyses of two means. One-way ANOVA was used when three or more means were compared. *P* values were adjusted for multiple testing by the Holm-Bonferroni correction, unless otherwise specified (45).

ACKNOWLEDGMENTS. The authors thank Jason Corbin for technical assistance and Kelly Trueman and Shauna Ross for animal husbandry. This work was supported by Program Grant 461219, fellowships (to D.J.H. and W.S.A.), Independent Research Institutes Support Scheme Grant 361646 from the Australian National Health and Medical Research Council of Australia, Grant R01 HL080019 from the National Heart, Lung, and Blood Institute, a fellowship from the Cancer Council, Victoria (to D.M.), an Australian Cancer Research Fund grant, and a Victorian State Government Operational Infrastructure Support grant and MuriGen Pty Ltd. C.A.d.G. was the recipient of an Australian Government Postgraduate Award, an Australian Stem Cell Centre Postgraduate Supplementary Scholarship, and the Sydney Parker Smith Postdoctoral Research Fellowship from the Cancer Council, Victoria.

1. Kuter DJ, Begley CG (2002) Recombinant human thrombopoietin: Basic biology and evaluation of clinical studies. *Blood* 100:3457–3469.
2. de Sauvage FJ, et al. (1996) Physiological regulation of early and late stages of megakaryocytopoiesis by thrombopoietin. *J Exp Med* 183:651–656.
3. Alexander WS, Roberts AW, Nicola NA, Li R, Metcalf D (1996) Deficiencies in progenitor cells of multiple hematopoietic lineages and defective megakaryocytopoiesis in mice lacking the thrombopoietic receptor c-Mpl. *Blood* 87: 2162–2170.
4. Kimura S, Roberts AW, Metcalf D, Alexander WS (1998) Hematopoietic stem cell deficiencies in mice lacking c-Mpl, the receptor for thrombopoietin. *Proc Natl Acad Sci USA* 95:1195–1200.
5. Qian H, et al. (2007) Critical role of thrombopoietin in maintaining adult quiescent hematopoietic stem cells. *Cell Stem Cell* 1:671–684.
6. Solar GP, et al. (1998) Role of c-mpl in early hematopoiesis. *Blood* 92:4–10.
7. Yoshihara H, et al. (2007) Thrombopoietin/MPL signaling regulates hematopoietic stem cell quiescence and interaction with the osteoblastic niche. *Cell Stem Cell* 1: 685–697.
8. Ballmaier M, Germeshausen M, Krukemeier S, Welte K (2003) Thrombopoietin is essential for the maintenance of normal hematopoiesis in humans: Development of aplastic anemia in patients with congenital amegakaryocytic thrombocytopenia. *Ann N Y Acad Sci* 996:17–25.
9. Lok S, et al. (1994) Cloning and expression of murine thrombopoietin cDNA and stimulation of platelet production in vivo. *Nature* 369:565–568.
10. Buza-Vidas N, et al. (2006) Cytokines regulate postnatal hematopoietic stem cell expansion: Opposing roles of thrombopoietin and LNK. *Genes Dev* 20:2018–2023.
11. Debili N, et al. (1995) The Mpl receptor is expressed in the megakaryocytic lineage from late progenitors to platelets. *Blood* 85:391–401.
12. Cohen-Solal K, et al. (1996) Constitutive expression of Mpl ligand transcripts during thrombocytopenia or thrombocytosis. *Blood* 88:2578–2584.
13. Fielder PJ, et al. (1996) Regulation of thrombopoietin levels by c-mpl-mediated binding to platelets. *Blood* 87:2154–2161.
14. Stoffel R, Wiestner A, Skoda RC (1996) Thrombopoietin in thrombocytopenic mice: Evidence against regulation at the mRNA level and for a direct regulatory role of platelets. *Blood* 87:567–573.
15. Emmons RV, et al. (1996) Human thrombopoietin levels are high when thrombocytopenia is due to megakaryocyte deficiency and low when due to increased platelet destruction. *Blood* 87:4068–4071.
16. Shivdasani RA, Fielder P, Keller GA, Orkin SH, de Sauvage FJ (1997) Regulation of the serum concentration of thrombopoietin in thrombocytopenic NF-E2 knockout mice. *Blood* 90:1821–1827.
17. Fahl SP, Crittenden RB, Allman D, Bender TP (2009) c-Myb is required for pro-B cell differentiation. *J Immunol* 183:5582–5592.
18. Vegiopoulos A, García P, Emambokus N, Frampton J (2006) Coordination of erythropoiesis by the transcription factor c-Myb. *Blood* 107:4703–4710.
19. Greig KT, et al. (2010) Critical roles for c-Myb in lymphoid priming and early B-cell development. *Blood* 115:2796–2805.
20. Carpinelli MR, et al. (2004) Suppressor screen in Mpl−/− mice: c-Myb mutation causes supraphysiological production of platelets in the absence of thrombopoietin signaling. *Proc Natl Acad Sci USA* 101:6553–6558.
21. Kauppi M, et al. (2008) Point mutation in the gene encoding p300 suppresses thrombocytopenia in Mpl-/- mice. *Blood* 112:3148–3153.
22. Mukai HY, et al. (2006) Transgene insertion in proximity to the c-myb gene disrupts erythroid-megakaryocytic lineage bifurcation. *Mol Cell Biol* 26:7953–7965.
23. Kasper LH, et al. (2002) A transcription-factor-binding surface of coactivator p300 is required for haematopoiesis. *Nature* 419:738–743.
24. Sandberg ML, et al. (2005) c-Myb and p300 regulate hematopoietic stem cell proliferation and differentiation. *Dev Cell* 8:153–166.
25. Metcalf D, et al. (2005) Anomalous megakaryocytopoiesis in mice with mutations in the c-Myb gene. *Blood* 105:3480–3487.
26. Okada S, et al. (1992) In vivo and in vitro stem cell function of c-kit- and Sca-1-positive murine hematopoietic cells. *Blood* 80:3044–3050.
27. Adolfsson J, et al. (2005) Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. *Cell* 121:295–306.
28. Yang L, et al. (2005) Identification of Lin(−)Sca1(+)kit(+)CD34(+)Flt3− short-term hematopoietic stem cells capable of rapidly reconstituting and rescuing myeloablated transplant recipients. *Blood* 105:2717–2723.
29. Forsberg EC, et al. (2005) Differential expression of novel potential regulators in hematopoietic stem cells. *PLoS Genet* 1:e28.
30. Lang G, White JR, Argent-Katwala MJ, Allinson CG, Weston K (2005) Myb proteins regulate the expression of diverse target genes. *Oncogene* 24:1375–1384.
31. Greig KT, Carotta S, Nutt SL (2008) Critical roles for c-Myb in hematopoietic progenitor cells. *Semin Immunol* 20:247–256.
32. Bianchi E, et al. (2010) c-Myb supports erythropoiesis through the transactivation of KLF1 and LMO2 expression. *Blood*, in press.
33. Chambers SM, et al. (2007) Hematopoietic fingerprints: An expression database of stem cells and their progeny. *Cell Stem Cell* 1:578–591.
34. Ashburner M, et al.; The Gene Ontology Consortium (2000) Gene ontology: Tool for the unification of biology. *Nat Genet* 25:25–29.
35. Subramanian A, et al. (2005) Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA* 102:15545–15550.
36. Ramalho-Santos M, Yoon S, Matsuzaki Y, Mulligan RC, Melton DA (2002) "Stemness": Transcriptional profiling of embryonic and adult stem cells. *Science* 298:597–600.
37. Cohen-Solal K, Vitrat N, Titeux M, Vainchenker W, Wendling F (1999) High-level expression of Mpl in platelets and megakaryocytes is independent of thrombopoietin. *Blood* 93:2859–2866.
38. Kubota Y, Osawa M, Jakt LM, Yoshikawa K, Nishikawa S (2009) Necdin restricts proliferation of hematopoietic stem cells during hematopoietic regeneration. *Blood* 114:4383–4392.
39. Umemoto T, et al. (2005) p57Kip2 is expressed in quiescent mouse bone marrow side population cells. *Biochem Biophys Res Commun* 337:14–21.
40. Du Q, Zhang Y, Tian XX, Li Y, Fang WG (2009) MAGE-D1 inhibits proliferation, migration and invasion of human breast cancer cells. *Oncol Rep* 22:659–665.
41. Chambers SM, et al. (2007) Aging hematopoietic stem cells decline in function and exhibit epigenetic dysregulation. *PLoS Biol* 5:e201.
42. Metcalf D, et al. (2008) Two distinct types of murine blast colony-forming cells are multipotential hematopoietic precursors. *Proc Natl Acad Sci USA* 105:18501–18506.
43. Lin SM, Du P, Huber W, Kibbe WA (2008) Model-based variance-stabilizing transformation for Illumina microarray data. *Nucleic Acids Res* 36:e11.
44. Smyth GK (2004) Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* 3: Article3.
45. Holm S (1979) A simple sequentially rejective multiple test procedure. *Scand J Stat* 6: 65–70.