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An ENU-induced Recessive Mutation in *Mpl* Leads to Thrombocytopenia with Overdominance

E. Ricky Chan¹, Heather Lavender¹, Geqiang Li², Peter Haviernik², Kevin D. Bunting^{2,3}, and Mark D. Adams^{1,3,*}

¹ Department of Genetics, Case Western Reserve University, Cleveland, OH, USA

² Department of Medicine, Division of Hematology-Oncology, Case Western Reserve University, Cleveland, OH, USA

³ Case Comprehensive Cancer Center, Case Western Reserve University, Cleveland, OH, USA

Abstract

Objective—The aim of this study was to identify and characterize the causative mutation in the thrombocytopenic mouse strain HLB219 that was generated at the Jackson Laboratory as part of a large scale ENU-mutagenesis screen.

Methods—The HLB219 mutation was identified by interval mapping of F2 mice generated from intercross breeding of HLB219 to both BALB/cByJ (BALB) and 129/SvImJ (129/Sv). *Mpl* was identified as a candidate gene and sequenced. The mutation was characterized *in vivo* in mouse hematopoietic stem/progenitor cell assays and in cell culture by expression in Ba/F3 cells.

Results—A novel mutation in the thrombopoietin (TPO) receptor *Mpl* in HLB219 mice caused a Cys→Arg substitution at codon 40 in the extracellular region of the receptor. Mice homozygous for the *Mpl*^{hlb219} mutation had an 80% decrease in the number of platelets in comparison to the wild type C57BL/6J strain, low numbers of bone marrow megakaryocytes, high TPO levels, and decreased competitive repopulating ability, consistent with a loss-of-function mutation in the receptor. Mice heterozygous for *Mpl*^{hlb219} however, showed an overdominance effect with a significant increase in platelet number. Functional analysis *in vitro* demonstrated that Ba/F3 cells expressing the mutant MPL^{hlb219} protein failed to activate ERK and STAT5, but proliferated in the absence of TPO and required constitutive phosphorylation of AKT for cytokine-independent growth.

Conclusion—Thrombocytopenia in HLB219 mice is caused by a recessive mutation in *Mpl* that abrogates MAPK-ERK and JAK-STAT signaling.

Keywords

Receptors; Thrombopoietin; Thrombopoiesis; Models; Genetic

*Corresponding Author Mark D. Adams, Ph.D., Department of Genetics, BRB-624, Case Western Reserve University, 10900 Euclid Ave, Cleveland, OH 44106-4955, 216-368-2791, 216-368-3432 FAX, mda13@case.edu.

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INTRODUCTION

A steady state level of circulating platelets must be maintained in order to prevent thrombosis or uncontrolled bleeding as a result of a platelet excess or deficiency, respectively. The cytokine thrombopoietin (TPO) and its receptor MPL have been identified as the two major regulators of platelet production and have been shown to be required for the differentiation and proliferation of megakaryocytes both in humans and in mouse models [1,2]. In humans, the autosomal recessive disease congenital amegakaryocytic thrombocytopenia (CAMT) is caused by mutations in *MPL* [3]. CAMT patients have thrombocytopenia as well as elevated TPO levels in the blood due to the inability of MPL to bind and internalize its ligand [3–6]. Mice deficient in *Mpl* show a similar phenotype [1,7,8]. The *Mpl* knockout mouse has been instrumental in demonstrating the importance of this receptor not only in megakaryopoiesis but in many aspects of hematopoiesis [9]. MPL is a member of the hematopoietic growth factor receptor superfamily, characterized by conserved cysteine residues and a WSXWS motif [10]. TPO binding has been shown to trigger signaling by MPL through three main pathways including JAK-STAT, MAPK-ERK, and PI3K-AKT-BAD [11–13]. However, the information about the cell-type specificity and mechanism of activation for these downstream signals is still incomplete.

The thrombocytopenic mouse strain HLB219 was obtained as part of a large ENU mutagenesis screen at the Heart, Lung, Blood and Sleep Disorder Center at The Jackson Laboratory (JAX-HLBS) [14]. ENU (N-ethyl-N-nitrosourea) induces point mutations at a rate of approximately 1 base pair change per million bases and has proven to be a powerful tool in generating novel monogenetic models for genetic studies of complex diseases [15–17]. The screen was conducted on the C57BL/6J (B6) background and a three-generation breeding scheme enabled the identification of recessive mutations. Progeny of mutagenized mice were assessed for a variety of cardiovascular, pulmonary, and hematologic phenotypes. Characterization of HLB219 mice revealed a platelet level approximately 20% of that seen in the parental B6 strain and an autosomal recessive inheritance pattern.

We have identified the molecular defect in the HLB219 strain as a mutation in *Mpl* that disrupts megakaryopoiesis. Heterozygous HLB219 mice have a significant overdominance effect in which platelet levels are elevated relative to wild type mice, a phenomenon that has not been observed in other *Mpl* mutants. Expression of the mutant protein results in growth-factor independent activation of survival pathways in cell culture assays and long-term proliferation competence, suggesting that some features of MPL signaling are maintained in the mutant protein.

There is still much debate over how the processes of megakaryopoiesis and platelet production are controlled and managed [18]. The characterization of the HLB219 strain adds to the expanding knowledge of the mechanism of action of the MPL protein, and provides a resource for future studies of the role of this key protein in hematopoiesis.

MATERIALS AND METHODS

Mapping

The HLB219 mouse strain was obtained from JAX-HLBS [14]. The phenotype and heritability were confirmed. Blood was drawn retro-orbitally from 8–10 week old animals and a complete blood count (CBC) with differentials was obtained by the Marshfield Laboratory. A single HLB219 male was mated to both BALB/cByJ (BALB) and 129/SvImJ (129/Sv) female mice to generate F1s which were then intercrossed to generate F2 mice. 103 (HLB219x129/Sv)F2 and 163 (HLB219xBALB)F2 mice were phenotyped. The map location reported by JAX-

HLBS was confirmed by genotyping 86 (HLB219xBALB)F2 mice using microsatellite markers D4Mit348, D4Mit9, and D4Mit308.

Platelet lifespan assay

Blood was drawn retro-orbitally from 8–10 week old mice. Red blood cells were removed using RBC lysis solution. Platelets were collected by centrifugation. Cells were blocked in PBS with 5% normal mouse serum. After blocking, cells were washed with PBS and resuspended in PBS with 2% fetal bovine serum and stained with CD41 antibody conjugated to phycoerythrin. Cells were washed in PBS and resuspended in Retic Count (thiazole-orange dye) solution (Becton Dickinson) [19]. Cells were then analyzed using a BD LSR1 flow cytometer.

Sequencing

Primers were designed using the Primer3 program [20] for the 12 exons of *Mpl* with flanking intronic sequence. Exons were amplified by PCR, sequenced and run on an Applied Biosystems 3730xl DNA Analyzer. Trace files were analyzed using Vector NTI software. Sequences were compared to the published B6 reference sequence [21].

Circulating TPO concentration

Blood was drawn retro-orbitally from eight mice each of the following strains: HLB219, B6, BALB, and (HLB219xBALB)F1. The blood was allowed to clot at room temperature before centrifugation. Serum was extracted and tested for circulating TPO levels using a QuantikineM Murine TPO ELISA kit (R&D Systems).

Assay for colony forming units of hematopoietic progenitors

Bone marrow cells were plated in methylcellulose media (MethoCult M3334; Stem Cell Technologies, Vancouver, BC, Canada) supplemented with 50 ng/ml rmSCF, 20 ng/ml rmIL-3 and 50 ng/ml rhIL-6. Duplicate cultures from three mice were plated for each genotype (HLB219, (HLB219xB6)F1, and B6). After 7 days the colonies containing at least 30 cells were scored under the inverted microscope. Detection of colony-forming units (CFU)-Mk was done by plating bone marrow cells in collagen based media (MegaCult-C; Stem Cell Technologies, Vancouver, BC, Canada) supplemented with 50 ng/ml rhTpo, 10 ng/ml rmIL-3, 20 ng/ml rhIL-6, and 50 ng/ml rmIL-11. Four aliquots were plated in slide chambers. Duplicate cultures of bone marrow cells from three mice for each genotype (HLB219, (HLB219xB6)F1, and B6) were incubated at 37 °C. After 7 days the slides were dehydrated and fixed in acetone. Slides were stained with acetylthiocholineiodide and counterstained with Harris' hematoxylin solution. Colonies containing at least three brown cells were scored under the inverted microscope.

Competitive repopulation assay

1:1 cellular equivalents of bone marrow from HLB219 or B6, which express CD45.2 on the surface of differentiated hematopoietic cells, and a congenic control strain B6.SJL-Ptprca^a Pep3^b/BoyJ (CD45.1) [22] were injected into lethally irradiated CD45.1 mice. Two months after transplantation, fluorescently-labeled antibodies, specific to the CD45 isotype of either the test strain or CD45.1 strain (Ly5.1 and Ly5.2 respectively), were used to determine the proportion of repopulated cells derived from the test and control strain by flow cytometry. Five recipient animals were tested for each strain. HLB219 bone marrow was also transplanted into irradiated mice to examine the ability to transfer the bone marrow defect.

Construction of *Mpl* or *Mpl^{hlb219}* vectors

Mpl cDNA was kindly provided by Tony Blau (University of Washington). The HLB219 mutation was generated by site-directed mutagenesis of the wild type *Mpl* cDNA using GeneTailor (Invitrogen). Forward primer 5'-tgggcacagagcccctgaaccgcttctcca-3' and reverse primer 5'-gttcaggggctctgtgcccaggccagcaa-3' were used for the mutagenesis reaction. Mutant plasmids were transformed into DH5α cells. Six colonies were picked for expansion and confirmed by sequencing of the cDNA. The GFP coding sequence was excised from MGIN (a vector with a *neo*-selection cassette and GFP expressed under the control of the MSCV promoter) and replaced by a ~1.9 kb EcoRI-XhoI fragment of mutated or wild type *Mpl* cDNA. The MGIN vector was kindly provided by Linzhao Cheng (Johns Hopkins University).

Stable transformation of Ba/F3 cells with *Mpl*, *Mpl^{hlb219}*, or MGIN

Ba/F3 cells, a progenitor B-cell line [23], were resuspended in RPMI 1640 media (Invitrogen). Plasmid with the *Mpl*, *Mpl^{hlb219}*, or GFP inserts was added to the media at a concentration of 10 µg/5x10⁶ cells. The cells were incubated at 4 °C. The cells were pulsed on a GenePulser Xcell electroporation system at 300 V and 960 µF for 70 milliseconds in a 4 mm cuvette. Cells were allowed to grow at 37 °C in growth media for 2 days (RPMI 1640, 10% FBS, 10% IL-3 conditioned media, 1% PSA). Cells were spun down and selected on growth media supplemented with 800 µg/ml G418 (CalBiochem). Cells were maintained on selective media (RPMI 1640, 10% FBS, 10% IL-3 conditioned media, 1% PSA, 400 µg/ml G418). Clonal selection was done by serial dilution to ~1 cell per 100 µl in 96 well culture plates. 5 Ba/F3 clonal lines of each construct (*Mpl*, *Mpl^{hlb219}*, or the MGIN control vector) were chosen for expansion.

Proliferation assay

Approximately 300,000 cells from Ba/F3 lines expressing *Mpl*, *Mpl^{hlb219}*, MGIN, or no vector, were placed in 3 ml cultures in RPMI 1640 media supplemented with 10% FBS, 1% PSA and subjected to the following conditions: no cytokines, 10% IL-3 conditioned media, 0.01 ng/ml, 0.1 ng/ml, 1 ng/ml, or 10 ng/ml TPO (PeproTech) in a 6-well culture plate. 100 µl samples of each condition were quantified for proliferation in response to TPO at 24 hour intervals, over the course of 5 days, using a colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide) assay (Chemicon). Extended proliferation in the absence of growth cytokines was also examined by trypan blue staining and a hemacytometer over the course of 60 days. Three replicates of each cell line were analyzed.

Downstream signaling pathways

Ba/F3 cells expressing *Mpl*, *Mpl^{hlb219}*, or MGIN were washed free of serum and growth factors. Cells were then placed on starvation media (RPMI 1640) with no growth cytokines for 10 hours. After starvation, cells were resuspended in RPMI 1640 and stimulated with 10 ng/ml TPO for 0, 15, 30, and 60 minutes. Cells were then lysed and protein lysates were separated by electrophoresis using an SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane. Membranes were blocked and incubated overnight with antibodies recognizing pERK, ERK, p-AKT, AKT, p-STAT5, STAT5, and MPL (Upstate Antibodies). Detection was performed using an ECL kit. Growth in response to rapamycin was examined over a course of 5 days [24,25]. Cells expressing either *Mpl* or *Mpl^{hlb219}* were placed in media (RPMI 1640) supplemented with 30 ng/ml TPO, IL-3 conditioned media, or no cytokine. Cells were then treated with 1 ng/ml of rapamycin. Each cell line and condition was assayed in duplicate. Cell density was determined by trypan blue staining and a hemacytometer.

RESULTS

Phenotypic Characterization of the HLB219 Strain

Complete blood counts (CBCs) confirmed HLB219 as thrombocytopenic with a platelet level reduced by approximately 80% in comparison to B6 (Table 1). Although there were no obvious signs of excessive bleeding, there was a significant increase in tail vein bleeding time (data not shown). In addition to the decrease in circulating platelets, HLB219 mice also have significantly fewer red blood cells and lower hemoglobin and hemocrit levels indicative of mild anemia. Mutant animals tend to have lower white blood cells counts than wild type (Table 1). Other than platelet counts, which are elevated relative to B6, heterozygous mice have a blood cell profile that more closely matches mutant animals than wild type, including a significant reduction in red blood cells and hemoglobin. This suggests that expression of the mutant receptor may have an effect at multiple stages of hematopoiesis. Microscopic examination of blood smears stained with Wright-Giemsa showed normal morphology of platelets and no signs of platelet aggregation (Fig. 1A, B). Platelets extracted from HLB219 and (HLB219xB6)F1 peripheral blood showed no difference in percentage of reticulation after staining with thiazole orange compared to B6 platelets, indicating that the platelets have comparable turnover rate (data not shown). Necropsy of two of three mice showed minor splenomegaly.

Identifying the mutation

The JAX-HLBS had mapped the HLB219 mutation to a 13 centimorgan region on chromosome 4 between D4Mit153 and D4Mit52 (<http://pga.jax.org/reports/30941.html>). Three microsatellite markers were used to confirm this location using progeny of an F2 intercross between an HLB219 mouse and BALB/cByJ and 129/SvImJ (Fig. 2A). F2 mice showed a slight reduction from the expected Mendelian pattern of inheritance for the platelet phenotype with 56/266 mice exhibiting platelet levels $<200 \times 10^3$ cells/ μ l, but the difference was not significant. Association of the low platelet phenotype with the three markers on 86 (HLB219xBALB)F2 mice showed the strongest linkage between the phenotype and the distal marker D4Mit308. This result suggested that the mutation region should be widened to include into consideration the *Mpl* gene.

DNA sequencing of exons from HLB219 genomic DNA revealed a single point mutation in the *Mpl* gene at codon 40 (Fig. 2B). The mutation is a T→C transition yielding an amino acid change from a conserved cysteine to an arginine. The mutation was named *Mpl*^{hlb219} to reflect the mutant mouse strain name. Confirmatory sequencing was also done on the (HLB219xBALB)F2 and (HLB219x129S1/SvImJ)F2 mice. Low platelet counts were perfectly correlated with mice homozygous for the mutation. Interestingly, mice heterozygous for the mutation showed an overdominance effect with a significantly higher platelet count compared to B6 mice (Fig. 3). The overdominance effect was even more striking in coisogenic heterozygotes: HLB219xB6 F1 mice have a 35% higher platelet count than wild type B6 mice ($p < 0.001$; Table 1), confirming that the effect is due to the mutation and not genetic modifiers in the outcross strains.

Supporting evidence for a mutation in *Mpl*

Previous studies suggest that loss-of-function mutations in *Mpl* result in an increase in TPO levels due to the inability of the receptor expressed on platelets to bind and internalize TPO for degradation [26]. Circulating TPO levels in HLB219 were approximately ten times higher than in B6 (Table 1C). Heterozygous animals had normal TPO levels.

A colony forming unit (CFU) assay was used to test the ability of bone marrow cells from HLB219 to differentiate into the various myeloid lineages. Studies of *Mpl* knockout mice have

demonstrated a reduction not only in megakaryocyte colony-forming cells CFU-Mks, but also progenitor cells of other hematopoietic lineages [27]. HLB219 bone marrow cells gave rise to significantly smaller CFU-GEMM, CFU-GM, CFU-G, and CFU-Mk populations compared to bone marrow from B6 mice (Fig. 4A). Consistent with the CFU-Mk assay, femur sections stained with H&E revealed a decreased number of megakaryocytes in the bone marrow (Fig. 1C, D). In contrast, there was a significant increase in the progenitor CFU-GM, CFU-M, and CFU-Mk populations in heterozygous mice compared with B6 (Fig. 4A). Despite the elevated CFU-Mk counts, there was no significant difference in megakaryocyte counts in bone marrow sections between B6 and (HLB219xB6)F1 heterozygous mice (data not shown).

As an *in vivo* test of HLB219 hematopoietic stem cell function, a competitive repopulation assay was performed. Equivalent numbers of bone marrow cells from HLB219 and an isogenic B6-derivative expressing CD45.1 were mixed and transplanted to a lethally-irradiated CD45.1 mouse [22]. As a control, B6 bone marrow cells were also mixed with CD45.1-derived cells and transplanted. After two months, blood was collected and the proportion of cells originating from each donor population was determined by flow cytometry using fluorescently labeled antibodies specific for distinct CD45.1 and CD45.2 isoforms. Hematopoietic stem cells from HLB219 bone marrow were unable to contribute effectively to the repopulation of blood cell lineages, suggesting that HLB219 progenitor cells have a general hematopoietic defect (Fig. 4B). Non-competitive transplantation of HLB219 bone marrow into an irradiated CD45.1 host showed an overall decrease in progenitor frequency of all lineages in subsequent CFU assays (data not shown).

***Mpl*^{hlb219} supports TPO-independent proliferation in Ba/F3 cells**

To demonstrate causality of the *Mpl*^{hlb219} mutation, we assessed the ability of the mutant receptor to promote TPO-dependent proliferation of Ba/F3 cells [28]. Ba/F3 is an immortalized B-cell line that has been used extensively in the characterization of *Mpl* mutants [29]. The mutation was introduced into the wild type *Mpl* cDNA and inserted into the MGIN retroviral vector backbone and stably transduced into Ba/F3 cells. Cultures were obtained following selection in G418. The MGIN vector expressing GFP was used as a control. Ba/F3 cells expressing *Mpl*^{hlb219}, *Mpl*⁺, or the control MGIN vector were examined for MPL expression by Western blot analysis using an antibody that is specific for an epitope in the intracellular region of MPL. Cell lysates from the Ba/F3 clones expressing the wild type receptor showed two bands correlating with the glycosylated and unglycosylated MPL protein, whereas the lysates from the three Ba/F3 clones expressing the mutant receptor had a strong band for the unglycosylated protein only, suggesting that the mutant protein may not be correctly localized within the cells (Fig. 5).

Each Ba/F3 clone was tested for TPO-dependent proliferation using the MTT assay (Fig. 6). Ba/F3 clones transfected with the control vector showed no growth in response to TPO and served as a negative control. Ba/F3 cells expressing *Mpl*⁺ showed TPO-dependent growth with increased proliferation in response to escalating concentrations of TPO with an effective concentration ≥ 0.1 ng/ml. Ba/F3 cells expressing the mutant receptor were able to proliferate in the absence of IL-3 conditioned media, albeit at a reduced rate, and this proliferation was independent of TPO concentration. Furthermore, Ba/F3 cells expressing the mutant receptor were able to proliferate indefinitely in the absence of any cytokine. The cells were maintained in culture and have continued to grow with a doubling time of approximately twelve hours for over two months.

***Mpl*^{hlb219} downstream signaling**

Mpl has been shown to activate three major signaling cascades in response to TPO stimulation, including the SHC-RAS-RAF-ERK, JAK-STAT, and PI3K-AKT-BAD pathways [11–13].

The ability of wild type and mutant MPL proteins to cause phosphorylation of key intermediates in each pathway was examined in Ba/F3 cells. Western blots of select representatives from these pathways showed that expression of *Mpl*^{hlb219} resulted in a pattern of phosphorylation that is distinct from cells expressing the wild type receptor (Fig 5). Unlike wild type *Mpl*, there was no phosphorylation of STAT5 or ERK observed in cells expressing *Mpl*^{hlb219}. Since activation of the JAK/STAT pathway is essential for megakaryopoiesis [30], the inability of the mutant protein to activate this pathway is consistent with low megakaryocyte and platelet counts in HLB219 mice. In contrast, *Mpl*^{hlb219} expression resulted in a constitutive phosphorylation of AKT, a serine-threonine kinase involved in BCL2 survival pathways [31].

Rapamycin, an inhibitor of the AKT effector protein mTOR [24,25], was used to test whether the constitutively active AKT signaling plays a role in the factor-independent growth of Ba/F3 cells expressing *Mpl*^{hlb219}. As has been shown previously [32], proliferation of Ba/F3 cells was not dependent on the AKT pathway, as demonstrated by their ability to grow in IL-3 conditioned media with rapamycin regardless of the MPL receptor expressed (Table 2). However, Ba/F3 cells expressing *Mpl*^{hlb219} grown in the absence of cytokines showed a significant reduction in growth ($p < 0.05$, Student's two-tailed t-test) in the presence of rapamycin (Table 2). This suggests that the factor-independent growth of these cells is AKT dependent.

DISCUSSION

A novel mutation in the *Mpl* gene has been identified in HLB219 mice. In addition to thrombocytopenia, HLB219 mice show similarities to previously described *Mpl* knockout mice including elevated TPO levels, reduced numbers of megakaryocytes and hematopoietic progenitors, and defects in hematopoietic progenitor cells [1,7,9]. These HLB219 phenotypes underscore the established role that the MPL receptor plays in general hematopoietic development.

The HLB219 mutation results in substitution of a conserved cysteine to arginine at codon 40 of the MPL protein (Figure 2B). The C40R mutation is in the extracellular domain of the protein and affects one of eight cysteine residues that are thought to form disulfide bonds as part of a highly conserved structural motif for ligand binding in a large superfamily of hematopoietic cytokine and growth-factor receptors [33]. Our original hypothesis based on the phenotype of the homozygous mice, was that the C40R mutation would disrupt TPO binding and that Ba/F3 cells expressing the *MPL*^{hlb219} protein would be unable to proliferate in the presence (or absence) of TPO. *Mpl*^{hlb219}-expressing Ba/F3 cells did in fact grow at a slower rate than Ba/F3 cells expressing the wild type receptor in the presence of TPO. However, the growth rate was unaffected by the presence or absence of TPO or IL-3. This factor-independent proliferation demonstrates the disruption of the receptor-ligand interaction, due either to an inability of the mutant receptor to bind TPO or to be localized to the cell surface or both. The presence of a band on the Western blot that is consistent with non-glycosylated MPL suggests that the mutant protein is improperly processed. The loss of MAPK-ERK and JAK-STAT signaling, which are required for thrombopoiesis, is consistent with the thrombocytopenia observed in HLB219 homozygous mice [11, 12].

Studies on the relationship between the structure of the receptor and the ability to promote signaling through downstream pathways are complicated by the limited cell lines available as experimental systems. For example, deletion of ten amino acids of the intracellular domain of MPL, adjacent to the transmembrane region has been shown to eliminate JAK-STAT signaling without affecting proliferation in B-cell-derived Ba/F3 and myeloid-derived 32D cells, but in the leukemia cell line UT-7, the same mutation was defective in signaling through the MAPK

and PI3K pathways and was unable to promote differentiation into megakaryocytes [34]. We show that although *Mpl*^{hlb219} can promote factor-independent growth of Ba/F3 cells, this activity is insufficient to promote thrombopoiesis in homozygous HLB219 mice. These immortalized cell lines therefore may not fully reflect the function of MPL in hematopoietic progenitors.

The elevated platelet levels in heterozygous mice may be the result of a low level of TPO-independent activation of AKT in addition to the normal MPL signaling of the wild type receptor. AKT activation might be expected to result in a reduction in platelet apoptosis [25, 35]. However, platelets from HLB219xB6 F1 and HLB219 mice showed no difference in turnover rate as measured by thiazole orange incorporation compared to platelets from B6 mice (data not shown). Progenitor frequencies representing several hematopoietic lineages are skewed in heterozygous mice relative to B6 in the CFU assay. Elevated CFU-Mk are consistent with elevated platelet counts in heterozygous mice. However, the relationship between increased CFU-GM and CFU-M in the heterozygous mice and blood cell counts is less clear, since the CBC profile of heterozygous mice more closely resembles HLB219 than B6, with lower mature cell counts in all lineages. Mice that are heterozygous (or homozygous) for a knock-out mutation of *Mpl* have similar RBC and WBC profiles to wild type mice [7], suggesting that loss of one copy of the *Mpl* gene is insufficient to cause the differences observed between B6 and HLB219 heterozygotes and homozygotes. Further studies will be necessary to determine the role of the mutant receptor and AKT signaling throughout hematopoiesis.

Most mutations in the human *MPL* gene are associated with a loss-of-function phenotype [36,37] with no indication of overdominance, although mild thrombocytosis in heterozygotes could be under-reported. However, a few MPL mutations in the extracellular, transmembrane and intracellular regions have been identified that lead to the constitutive activation of the receptor in cell culture experiments [38–42]. A polymorphism in humans designated MPL Baltimore results in a substitution at amino acid 39 (K39N) and is associated with thrombocytosis [43]. This polymorphism is restricted to African Americans with a heterozygote frequency of 7%. *In vitro* studies of the MPL Baltimore mutation showed TPO-independent proliferation in 32D cells, despite incomplete processing and reduced protein expression [43]. This amino acid change is adjacent to the C40R mutation in HLB219. Interestingly, N39 is the wild type allele in mice, precluding study of the MPL Baltimore mutation in the context of the mouse protein. A point mutation in the intracellular region of MPL (W508S) showed factor independent growth of Ba/F3 cells and constitutive activation of the RAS-MAPK, JAK-STAT, and PI3K-AKT-BAD pathways [42]. In comparison, Ba/F3 cells expressing the HLB219 mutant receptor had only constitutive AKT activation and a loss of JAK-STAT and MAPK-ERK signaling.

TPO and its receptor have been well studied, however, there are still many questions regarding interactions of MPL with a diverse set of signaling pathways and their role in promoting megakaryocyte differentiation and proliferation and the development and function of mature platelets. The use of *in vivo* models such as HLB219 will continue to be an important adjunct to *in vitro* studies of receptor function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. HLB219 mice have low levels of platelets and megakaryocytes

Blood smears from B6 (A) and HLB219 (B) were stained with Wright-Giemsa stain and images captured at 100x magnification. The white arrow shows an example of a platelet. HLB219 blood smears had markedly reduced numbers of platelets. Bone marrow sections from B6 (C) and HLB219 (D) were stained with H&E and images captured with a 20X objective. A representative megakaryocyte is indicated by the black arrow.

Figure 2. Identification of the HLB219 mutation in *Mpl*
(A) The mapped region reported by the JAX-HLBS was confirmed and extended using three microsatellite markers; D4Mit348, D4Mit9, and D4Mit308 on 86 (HLB219xBALB)F2 mice.
(B) A mutation in the *Mpl* gene results in a cysteine to arginine substitution at codon 40. This is a highly conserved cysteine in the extracellular domain of the receptor.

Figure 3. Phenotypic and genotypic correlation of the F2 mouse population

Sequencing of the (HLB219xBalb)F2 (O) and (HLB219x129Sv)F2 (X) mice revealed an overdominance effect in which mice heterozygous for the mutation had a significantly higher platelet average ($p \leq 0.001$) compared to $Mpl^{+/+}$ mice using a Student's two-tailed t-test, considering both strains together or (HLB219xBalb)F2 and (HLB219x129Sv)F2 strains individually. Mice homozygous for the mutation have the lowest platelet counts.

Figure 4. HLB219 progenitor cells have defects in committed and multipotent progenitor cells (A) The ability of bone marrow cells from HLB219, (HLB219xB6)F1 and B6 to form colonies in methylcellulose media in response to specific cytokines was tested using a CFU assay. Averages and standard deviations of 3 mice of each strain are shown. Asterisk indicates $p \leq 0.05$ using a Student's two-tailed t-test. (B) Repopulation of hematopoietic lineages in lethally irradiated mice was tested by mixing bone marrow cells from HLB219 (CD45.2) and isogenic CD45.1 mice (see Methods). After two months, the relative contribution of each strain to the repopulation of nucleated hematopoietic lineages was measured by flow cytometry using strain specific CD45 isoform antibodies. Each bar represents the average and standard deviation of percentages from five animals.

Figure 5. *Mpl^{h1b219}* constitutively activates the AKT pathway

Ba/F3 cells were starved of cytokines for 6 hours before stimulation with 20 ng/ml of TPO. Cell lysates were probed with a MPL antibody and antibodies for total (T) and phosphorylated (p) ERK, AKT, and STAT5. Cells expressing *Mpl^{h1b219}* show constitutive phosphorylation of AKT and loss of ERK and STAT5 activation.

Figure 6. Ba/F3 cell expressing *Mpl*^{h1b219} proliferate factor-independently

The growth of stably transformed clones expressing wild type *Mpl*, *Mpl*^{h1b219} or the MGIN vector were tested for proliferation ability using an MTT assay in the presence of increasing concentrations of TPO. Three clones were tested for each condition and the average and standard deviation are plotted.

Table 1

Hematologic profile and TPO levels of HLB219 mice.

(A) Blood profiles					
Blood Type	HLB 219	B6	(HLB219xB6)F1	BALB	129/Sv
Platelets (x10 ³ /ul)	108.4 ± 61.2 *	724.8 ± 211.0	980.6 ± 79.8 *	856.0 ± 134.5	523.1 ± 137.9
RBC (x10 ⁶ /ul)	9.8 ± 1.1 *	10.4 ± 0.7	8.7 ± 0.2 *	10.5 ± 0.3	10.7 ± 0.6
Hemoglobin (g/dL)	14.9 ± 1.5 *	16.1 ± 1.0	14.5 ± 0.3 *	16.3 ± 0.4	16.4 ± 0.4
Hematocrit (%)	45.0 ± 4.2	48.1 ± 3.4	44.6 ± 1.6	47.1 ± 2.4	47.8 ± 2.2
WBC (x10 ³ /ul)	9.2 ± 3.6	10.0 ± 3.3	8.7 ± 0.9	9.6 ± 2.7	8 ± 1.2
Lymphocytes (x10 ³ /ul)	7.9 ± 3.1	8.4 ± 2.8	7.6 ± 0.7	8 ± 2.3	6.7 ± 1.1
Neutrophils (x10 ³ /ul)	0.9 ± 0.8 *	1.2 ± 0.7	0.8 ± 0.2	1.3 ± 0.5	1 ± 0.3
Eosinophils (x10 ³ /ul)	0.1 ± 0.1 *	0.2 ± 0.2	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1
Monocytes (x10 ³ /ul)	0.2 ± 0.2	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
N	107	28	10	8	7

(B) Serum thrombopoietin levels.	
Strain	TPO (pg/ml)
HLB219	1588 ± 290 *
(HLB219xBALB) F1	184 ± 50
BL6	139 ± 65
BALB	250 ± 151

* significantly different (p<0.01) from B6 using a Student's two-tailed t-test

Table 2

Response of Ba/F3 cells to rapamycin

	Cytokine	Rapamycin	Day 1 (cells/ μ l)	Day 3 [†] (cells/ μ l)	Day 5 [†] (cells/ μ l)
<i>Mpl^{flb219}</i>	No Cytokine	-	50	139 $\pm$ 24	239 $\pm$ 39
		+	50	28 $\pm$ 8	6 $\pm$ 8
	IL-3	-	50	328 $\pm$ 118	1839 $\pm$ 55
		+	50	311 $\pm$ 47	1289 $\pm$ 534
	TPO	-	50	111 $\pm$ 47	200 $\pm$ 47
		+	50	22 $\pm$ 0	6 $\pm$ 8
<i>Mpl⁺</i>	No Cytokine	-	50	6 $\pm$ 8	0 $\pm$ 0
		+	50	6 $\pm$ 8	0 $\pm$ 0
	IL-3	-	50	333 $\pm$ 16	2211 $\pm$ 409
		+	50	256 $\pm$ 0	1911 $\pm$ 47
	TPO	-	50	122 $\pm$ 16	872 $\pm$ 86
		+	50	72 $\pm$ 39	106 $\pm$ 39

[†] values are mean $\pm$ standard deviation from three independent cultures.