Published in final edited form as:

Leukemia. 2011 April ; 25(4): 655–662. doi:10.1038/leu.2010.301.

The PlagL2 transcription factor activates Mpl transcription and signaling in hematopoietic progenitor and leukemia cells

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Abstract

Cytokine signaling pathways are frequent targets of oncogenic mutations in acute myeloid leukemia, promoting proliferation and survival. We have previously shown that the transcription factor PLAGL2 promotes proliferation and cooperates with the leukemia fusion protein Cbfβ-SMMHC in acute myeloid leukemia development. Here we show that PLAGL2 upregulates expression of the thrombopoietin receptor Mpl, using 2 consensus sites in its proximal promoter. We also show that Mpl overexpression efficiently cooperates with Cbfβ-SMMHC in development of leukemia in mice. Finally, we demonstrate that PlagL2-expressing leukemic cells show hyper-activation of Jak2 and downstream STAT5, Akt and Erk1/2 pathways in response to Tpo ligand. These results show that PlagL2 expression activates expression of Mpl in hematopoietic progenitors, and that upregulation of wild type Mpl provides an oncogenic signal in cooperation with CBFβ-SMMHC in mice.

Keywords

acute myeloid leukemia; PlagL2; MPL; Cbfb-MYH11; gene regulation; transcription factors

Introduction

Acute myeloid leukemia (AML) results from the accumulation of genetic alterations in hematopoietic progenitors that alter survival and differentiation programs. The study of these alterations is key for understanding the mechanism of leukemia development. The chromosome 16 inversion inv(16)(p13;q22), called inv16, is a frequent mutation found in AML cases, which creates the leukemia fusion gene *CBFB-MYH11* (1). The Cbfβ-SMMHC protein encoded by the fusion gene creates preleukemic myeloid progenitors unable to

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The authors declare no conflict of interest in relation to the work described in this manuscript.

differentiate, which transform into full-blown leukemia in synergy with mutations that promote proliferation and survival (2, 3).

A fraction of inv16-AML samples also present activating mutations in genes encoding components of the receptor tyrosine kinase signaling pathways, including KIT and FLT3, and the their downstream GTPases NRAS and KRAS (4-7) (8). These mutations produce ligand-independent constitutively active signaling that enhances the expansion and survival of leukemic blasts. We have previously shown that the zinc finger PLAG transcription factors, Plag1 and PlagL2, have similar capacity to induce proliferation of hematopoietic progenitors and cooperate with Cbfβ-SMMHC in acute myeloid leukemia development (9, 10). The PLAG factors bind to the GRGGC$(N)_{6-8}$RGGK consensus site in regulatory regions of target genes to activate transcription, such as the promoter 3 of the insulin-like growth factor 2 gene (11). The PLAG oncogenic activity has also been reported in chronic lymphocytic leukemia, breast cancer, and salivary gland tumors (12-14). However, little is known on how PLAG induces transformation in hematopoietic progenitors and leukemia blasts.

In this study, we use gene expression profile analysis of hematopoietic progenitors and leukemic cells expressing PLAGL2 to identify genes that are consistently deregulated by PLAGL2. We identify the thrombopoietin receptor Mpl as a downstream target, using gene profile analysis, and validated its expression levels using quantitative RT-PCR and flow cytometry. Furthermore, we identify two PLAG binding sites conserved in mammals in Mpl proximal promoter using luciferase reporter and electrophoretic mobility shift assays. We determined that Mpl is a key downstream mediator of PLAGL2 leukemogenesis as overexpression of wild type Mpl efficiently cooperates with CBFβ-SMMHC in leukemia development using transplantation assays. The leukemic cells expressing PLAGL2 exhibit sensitivity to TPO ligand as evidenced by increased phosphorylation of Jak2, Erk1/2, Akt, and Stat5. Together, these results demonstrate that PLAGL2 regulates Mpl expression, and that upregulation of wild type Mpl cooperates with CBFB-MYH11 in leukemia development in mice.

Materials and Methods

Microarray analyses

Sample preparation—For infected BM-HP cells, 129SvEv *Cbfb*$^{+/56M}$*;Mx1Cre* mice were treated with 150 mg/kg 5-FU, and BM cells were harvested 5 days later. Cells were subjected to red-blood-cell lysis solution (Purgene, Gentra Systems, Minneapolis, MN), and spin-infected with 2 rounds of retrovirus (MIG or MIG-PLAGL2) as previously described (10). The GFP-positive cells were sorted by FACS and total RNA was immediately isolated (samples wt-MIG1, wt-MIG2, wt-P1, and wt-P2). The mouse AML cells were generated using *Cbfb-MYH11* conditional knock-in mice as previously described (3). AML cells were isolated from spleen of 3 independent leukemic mice expressing MIG-PLAGL2 (samples AML-P1, AML-P2, and AML-P3) and from 3 independent leukemic mice with low *Plagl2* levels (AML-x1, AML-x2, AML-x3). In all cases, RNA was extracted with Trizol (Invitrogen, Carlbad CA) and cleaned with RNA-Easy columns (Qiagen, Valencia CA). Complementary-DNA was synthesized using the Superscript II (Invitrogen, Carlbad CA),

and Biotin-labeled cRNA was subsequently synthesized using RNA-transcript labeling kit (Affymetrix, Santa Clara, CA). Ten μg of labeled cRNA was fragmented and hybridized to Affymetrix Mouse Genome 430 2.0 Chip Arrays (Affymetrix, Santa Clara, CA).

Data Analysis—To identify transcripts that were differentially expressed in MIG-PLAGL2, AML-PLAGL2 and AML samples, we used the GeneSpring (version 7.3; http://www.silicongenetics.com) and GENECLUSTER (version 2.0; http://www-genome.wi.mit.edu) software packages. The expression values of each probe set in each of the MIG-PLAGL2, AML-PLAGL2 and AML samples were normalized to that of the MIG control number one or number two samples. For a given transcript to be considered as differentially expressed in MIG-PLAGL2, AML-PLAGL2 and AML samples, it had to meet the following three criteria. First, the signal values in these samples and the MIG control sample had to exhibit a relative difference of at least a factor of 2 and an absolute difference of 100 units. Second, changes of comparable magnitude had to be reproduced in 2 out 2 independent experiments for the MIG-PLAG2 sample or 3 out 3 independent experiments for the AML-PLAGL2 and AML samples when compared to MIG controls. Third, when statistical group comparisons (Welch's approximate t test) were applied to the raw signal values of the MIG control samples and the MIG-PLAGL2, AML-PLAGL2 or AML samples, it yielded a significance value of $p \leq 0.05$. Transcripts differentially expressed in MIG-PLAGL2, AML-PLAGL2 or AML samples were subjected to hierarchical clustering analysis using the GeneSpring (version 7.3) software package. For this analysis, the signal value of each transcript in each of the samples was normalized to the corresponding value of the MIG control number one sample. The resulting ratios were subjected to two-dimensional clustering by using the average linkage algorithm and the standard correlation coefficient as a distance metric.

Reverse transcriptase and quantitative RT-PCR analyses

RNA from mouse BM and AML cells was extracted with Trizol (Invitrogen, Carlsbad CA). First-stand cDNA was generated by using 2 μg RNA, 1 U Superscript-II reverse transcriptase (Invitrogen, Carlsbad, CA), and 0.5 μM oligo-dT primer in a 20-μL reaction. SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) was used for qPCR according to the manufacturer's instructions. *Mpl* primers were *Mplx1* and *Mplx2* and β-actin primers were *ActbF1* and *ActbR1* (supplementary Table 1). QPCR was performed in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Samples were normalized to β-actin transcript levels, and relative values were determined using standard curve method.

Retroviral production

The *MIG-Mpl* construct was generously provided by Harvey Lodish (Massachusetts Institutes of Technologies, Cambridge MA). The *MIG-MplY* mutant construct was generated by PCR using primers *pMSCV1* and *MplY112rev*, cut with EcoR1 and Xho1, and cloned into MIG. Phoenix packaging cells (Gary Nolan, Stanford University, Stanford CA) were co-transfected with 2 μg retroviral constructs, 2 μg Psi-Eco packaging plasmid with Effectene (Qiagen, Valencia CA) according to manufacturer's protocol. Retrovirus supernatants were taken at 48, 56, and 72 hrs and titered in by GFP-FACS analysis.

Bone marrow transduction and transplantation (tBMT)

Endogenous *Cbfb-MYH11* expression was induced in *Cbfb*$^{56M/+}$*Mx1Cre* conditional knock-in mice (3) using the Mx1Cre transgenic system (15). Briefly, mice were injected with 3 doses every other day of polyinosinic-polycytidylic acid (pIpC; Sigma, St Louis, MO) at 3- to 5-weeks of age, treated with 150 mg/kg 5-fluorouracil (5FU), and BM cells were harvested 5 days later. Efficiency of Cre-mediated *Cbfb-MYH11* induction was consistently above 90% (3). The BM progenitor cells were spin-infected twice with retrovirus supernatant, and 5×10^5 to 1×10^6 BM cells transplanted by intravenous injection (iv.) into 4- to 6-week old sublethally irradiated (650 rads) 129SvEv wild-type recipient mice. Mice were under daily observation for early signs of leukemia. These signs included limited motility, pale paws, and dehydration. At first signs of illness, peripheral blood was analyzed for the presence of immature cells. FACS analysis of peripheral blood was performed by using antibodies to cell-surface markers Gr-1, CD11b, B220, CD3, Ter119, and c-kit (BD Biosciences, San Diego CA). For flow cytometry analysis of Mpl receptor expression, a polyclonal rabbit anti-Mpl extracellular domain antibody (provided by Harvey Lodish, Whitehead Institute for Biomedical Research, Cambridge MA) and a PE-anti rabbit secondary antibody were used. For secondary transplantations, leukemic cells were harvested from the BM or spleen of affected mice in RPMI 1640 and 20% FBS (both from (Invitrogen, Carlsbad, CA), and 1×10^6 single-cell suspension aliquots were transplanted iv. into sub-lethally irradiated 4- to 6-week-old 129SvEv recipients.

Immunoblot analyses

Cryopreserved AML cells were thawed and immediately serum starved in RPMI media with 1% BSA for 60 min at 37°C (Invitrogen, Carlsbad CA). Cells were then incubated with RPMI media with 0.1% BSA and 0, 5, 25, or 50 ng/ml Tpo (Peprotech, Rockyhill NJ) for 10 min. Cells were washed with PBS and resuspended in RIPA buffer with protease-inhibitor-cocktail III (Calbiochem, Darmstadt Germany). Antibodies included anti-Jak2 (cat#sc-294; Santa Cruz Biotechnology Inc., Santa Cruz, CA), and anti-phospho-Jak2-Tyr221 (cat#3774), anti-phospho-Stat5-Tyr694 (cat#9351), anti-Stat5 (cat#9310), anti-phospho-Akt-Ser273 (cat#4058), anti-Akt (cat#9272), anti-phospho-Erk1/2 -Thr202/Tyr204 (cat#9101), and anti-Erk1,2 (cat#9102; all from Cell Signaling Technology, Danvers MA).

DNA Sequence Analysis

The search for PLAG consensus site GRGGC(6-8)RGGK was analyzed using the UCSC Genome Browser on Mouse July 2007 (NCBI37/mm9) Assembly (http://genome.ucsc.edu). Promoter regions were defined as 1000 base pairs upstream and 200 base pairs downstream of transcription start site, with this site as position 1000 (Supplementary Table 1). Evolutionary conservation of PLAG sites among 5 mammal species (human, orangutan, mouse, rat, dog and horse) was estimated. The sequence conservation analysis at the Mpl proximal promoter was performed using ClustalW-Alignment Analysis from MacVector9.5.2 (MacVector Inc.).

Luciferase assays

A 300 bp fragment of the *Mpl* proximal promoter was amplified with primers *MPLFLPromkpnF* and *MPLFLpromBglR* using PFU (Stratagene, La Jolla CA), and the amplicon was digested with KpnI and BglII and cloned into pGL3-Basic (Promega, Madison WI). Mutants were constructed by overlapping PCR with primers *Mpls1F* and *Mpls1R* for "site-1", *Mplps2F* and *Mplps2R* for "site-2", and *Mplps3F* and *Mplps3R* for "site-3" (Supplementary Table 1). The mutant primers were used with primers *MPLFLpromkpnF* and *MPLFLpromBglR* to amplify overlapping fragments that were annealed, digested with KpnI and BglII and cloned into the PGL3-basic vector. Constructs were transfected into NIH3T3 cells along with MhCD4 or MhCD4-PLAGL2 and PRL-TK with Effectene (Quiagen Valencia CA). Cells were analyzed for luciferase levels with the Dual-Luciferase Reporter Assay System (Promega, Madison WI).

Electrophoretic mobility shift assay

The electrophoretic mobility shift assay was performed as previously described (16). Briefly, 3T3 cells transfected with MIG-PlagL2 were used for isolation of nuclear proteins and added to protein-DNA binding reactions with double stranded DNA oligos representing *PlagL2* binding sites in the *Mpl* promoter. The sequence for DNA oligos *P1ss* and *P1as*, *P2ss* and *P2as*, *P3ss* and *P3as*, *mutP1ss* and *mutP1as*, *mutP2ss* and *mutP2as*, and *mutP3ss* and *mutP3as* are shown in Supplementary Table 1.

The *P1ss*, *P2ss* and *P3ss* oligos were radioactively labeled with ^{32}P using OptiKinase (USB, Cleveland OH), and the excess ^{32}P-γATP was removed by purifying the labeled oligos using G-25 Mini Quick Spin Oligo columns (Roche, Basel, Switzerland). The oligos were annealed with equal amounts of antisense oligos (*P1as*, *P2as*, *P3as* respectively) to obtain a double stranded labeled oligo with a *PlagL2* binding site. 100 pg of the oligo were used for each binding reaction. Cold competitors and mutant competitors were obtained the same way, but without labeling the oligos. When using cold competitors (wild type or mutant), a 1:200 molar ratio of labeled and cold oligo was used. Constant DNA amount was maintained by adding scrambled oligos *Sss* and *Sas*. The protein-DNA binding reactions were loaded onto a 4% polyacrylamide gel, ran until unlabeled ^{32}P-γATP went out into buffer, dried and exposed for 24 hrs.

Cytology Analysis

Cells from peripheral blood smears were stained with Wright-Giemsa. Pictures were taken using a Zeiss Axioskop 40 microscope using 100x lenses and a Zeiss AxioCam MRc camera and software.

Results

Mpl is upregulated by the transcription factor PLAGL2 in hematopoietic progenitors and AML blasts

We have previously shown that the transcription factor PLAGL2 induces AML in synergy with Cbfβ-SMMHC in mice (10). In order to identify PLAGL2 target genes that participate in leukemia development, we defined a group of genes consistently deregulated by PLAGL2

by assessing those genes consistently deregulated in bone marrow (BM) hematopoietic progenitors (HP) and AML blasts expressing PLAGL2. The HPs from *Cbfb*$^{+/MYH11}$ conditional knock in mice expressing *Cbfb-MYH11* were transduced with a pMSCV-IRES-GFP (MIG) or pMSCV-PLAGL2-IRES-GFP (PL2-MIG) retrovirus (Supplementary Figure 1a). The RNA from GFP sorted cells was used in a gene profile analysis using 2 independent samples per group, and represent the "early deregulated gene-set". In addition, the expression profiles of 6 mouse AML samples expressing *Cbfb-MYH11*, 3 expressing (P +1AML to-P+3AML) and 3 not expressing PLAGL2 (P-1AML to-P-3AML), were analyzed (experimental design in Supplementary Figure 1b). Two-dimensional hierarchical clustering of the 10 samples was performed using GeneSpring® software (Supplementary Figure 1c). Thirty-three probes identified 23 genes consistently upregulated over 2-fold in *PLAGL2* expressing samples (Figure 1A). These included upregulation of transcripts for the thrombopoietin receptor Mpl, the G-protein couple receptors Gpr56, *Prokr1* and Gpr124, the endothelial protein-C receptor, the vitamin-D receptor, endoglin, and collagenase genes *Col18a1* and *Col4a2*. In addition, the transcripts for the extracellular matrix proteins tenascin-XB and Spondin-2, the ubiquitin ligase Cullin-7, the actin-associated *Cyb5r3*, and Janus kinase Jak3 were consistently increased by PLAGL2. Consistent with the reported transcription activation role of PLAGL2, this analysis did not identify down-regulated genes (17, 18). The presence of PLAG consensus GRGGC(8-6)RGGK sites (11) was searched in a 1,200 base pairs window (1000 base pairs upstream and 200 base pairs downstream of the gene transcription start), with the start site as position 1,000 (Supplementary Table 2). The conservation of these sites in 5 mammal species was analyzed using the UCSC Genome Sequence. From the 23 identified genes, 3 genes (*Mpl*, *Ccdc8* and *Eng*) showed high conservation in all sites, while 4 genes (Hif3a and 3 genes with unknown function) have at least 1 conserved site.

Considering that PLAGL2 induces proliferation and replating capacity of hematopoietic progenitors (10), and the high conservation of the three PLAG sites in its promoter region, we focused our study on the cytokine thrombopoietin receptor *Mpl*. Quantitative RT-PCR (qRT-PCR) analysis revealed that the level of *Mpl* transcript was increased over 100-fold 48 hours after transduction (Figure 1B, lanes 1 and 2). A similar increase (30- to 500-fold) was observed in AML cells expressing PLAGL2 when compared to AML cells not expressing PLAGL2 (Figure 1B, lanes 3 to 10). In addition, the levels of Mpl receptor in membrane were markedly increased in AML cells expressing PLAGL2 using flow cytometry (Figure 1C).

PLAGL2 induces expression of the *Mpl* proximal promoter

The Mpl gene consists of 12 exons highly conserved in mammals and its expression is regulated from a proximal promoter spanning approximately 200 bp upstream of the translation start site (Figure 2A, top). This region is highly conserved in the mammalian genomes (30 way multiz-alignment and conservation; USCS-Mouse genome Browser; Figure 2A, middle). The *Mpl* proximal promoter (−189 to +3 from translation start site) is highly conserved in mammals, including human, monkey, mouse, dog, horse, and cow genomes (ClustalW sequence alignment; Figure 2A, bottom). This region has 3 PLAG consensus binding-sites. To test whether PLAGL2 could directly activate *Mpl* expression,

we tested PLAGL2 responsiveness of a luciferase reporter carrying the *Mpl* proximal promoter in NIH3T3 cells. PLAGL2 activated the reporter over 30-fold relative to control (Figure 2B, lanes 1 and 2). The introduction of previously described (11) point mutations in PLAG binding sites 1 (m1) and 2 (m2) resulted in a 2- and 5-fold reduction in luciferase activity respectively (Figure 2B, columns 3-6), indicating that these sites are critical for PLAGL2 induced *Mpl* transcription. Conversely, mutation of the third PLAG site (m3) did not change significantly the PLAGL2 responsiveness, suggesting that this site may not participate in the *Mpl* promoter activity (Figure 2B, columns 7 and 8). Importantly, mutation of all three PLAG binding sites totally obliterated PLAGL2 responsiveness of the reporter, confirming that binding of PLAGL2 to the promoter is necessary for transcriptional activation (Figure 2B, columns 9 and 10). To validate PLAGL2 binding to these sites, we performed electrophoretic mobility shift assays using oligonucleotides covering each of the three Mpl binding sites (o), and a combination of labeled oligonucleotides with cold competitor (c), or mutated competitor (m) oligonucleotides (Figure 2C). These assays show that P1 and P2 sites bind to PLAGL2 with specificity, but P3 site binding is poorly competed with cold competitor. These results suggest that PLAGL2 can directly activate the *Mpl* proximal promoter using the P1 and P2 binding sites.

Overexpression of Mpl induces AML in cooperation with CBF fusion genes

Since PLAGL2 induces expression of Mpl in HPs and AML cells, we asked whether Mpl expression is a key event triggered by PLAGL2 in leukemia development. We tested whether co-expression of *Cbfb-MYH11* and *Mpl* is sufficient for AML development in a BM transplantation assay (Figure 3A-B, and “Materials and Methods”). All of the mice transplanted with BM cells expressing *Cbfb-MYH11/MIG-Mpl* (n=14) readily developed leukemia between 4 and 10 weeks post transplantation (Figure 3C, blue line). Control groups remained healthy over 5 months, including *Cbfb-MYH11/MIG* (n=10) or *MIG-Mpl* (n=8) transplanted mice (Figure 3C, black line). Secondary transplantation of AML cells into sublethally irradiated recipients succumbed with leukemia with a median latency of 4 weeks (Figure 3C, green line; n=11) confirming the leukemic nature of the disease. The pathology of disease in *Cbfb-MYH11/Mpl* AML mimics that of *Cbfb-MYH11/MIG-PLAGL2* leukemia previously described (9, 10, 19). Briefly, we found a significant increase of GFP(+), Kit(+) immature cells in peripheral blood (Figure 3D), confirming the presence of the MIG-Mpl retrovirus, and were lineage negative (Lin: CD3, Gr-1, B220, Ter119, and Mac-1). These cells were blast-like cells with dark and pigmented cytoplasm, and monocytic-like cells with lobulated nucleus (Figure 3E, red arrow and asterisks respectively). Moribund mice also displayed infiltration of leukemic cells into the spleen, causing a significant enlargement (Figure 3F). The MPL mutation tyrosine to phenylalanine at amino acid 112 (Y112F) in the carboxyl-terminus largely reduces downstream signaling through the PI3k/Akt, Ras/Erk and Stat3/5 pathways, and is critical for proliferation of hematopoietic cell lines and for transformation of FRE-rat fibroblasts (20-22). To test if downstream MPL signaling was critical for leukemogenesis, we performed BM transplantation assays with BM expressing *Cbfb-MYH11* and transduced with *MIG-Mpl*^{Y112F} retrovirus. Recipient mice transplanted with BM cells expressing *Cbfb-MYH11* and *MIG-Mpl*^{Y112F} mice developed AML with decreased penetrance (3/8) and latency

(range 12 and 16 weeks; Figure 3C, red line). These data suggest that activation of signaling networks by upregulation of Mpl receptor is critical in leukemia development.

CBFb-MYH11-AML cells expressing PlagL2 are hypersensitive to Tpo ligand

The TPO/MPL signaling pathway regulates proliferation and survival of hematopoietic progenitor cells. In contrast to other members of the cytokine-receptor superfamily, MPL lacks intrinsic tyrosine kinase activity. Ligand stimulation of MPL results in the phosphorylation of JAK2 kinase, a required step for phosphorylation of cytoplasmic residues of MPL, which then triggers activation of AKT/mTOR, STAT3/5 and RAS/ERK cascades (23). We analyzed the phosphorylation status of Jak2 in *CBFb-MYH11*-AML cells expressing PLAGL2 (PL2+) or not (PL2−) cultured in presence of Tpo after serum starvation. We found increased phospho-Jak2 levels in presence of Tpo only in samples expressing PLAGL2 (Figure 4A). In addition, the phosphorylation of downstream signals Akt1, Stat5, and Erk1/2 was increased accordingly in response to Tpo in these AML samples (Figure 4B). Of note, phopspho-Akt levels were significantly increased upon Top induction (Figure 4C). These results demonstrate that *Cbfb-MYH11*-AML cells expressing PLAGL2 have an activated Mpl signaling pathway in response to Tpo ligand.

Discussion

The leukemias with core-binding factor inv(16)(p13;q22) frequently present oncogenic mutations in receptor tyrosine-kinase pathway proteins KIT, FLT3 and RAS, which provide proliferation and survival capacity to hematopoietic blasts (8). We have previously shown that the related transcription factors PLAG1 and PLAGL2 cooperate with CBFβ-SMMHC in leukemia development (10). This study shows that the transcription factor PLAGL2 activates Mpl receptor, inducing activation of the Erk, Stat, Akt pathways.

The members of the PLAG zinc finger transcription factors PLAG1 and PLAGL2 have been shown to activate growth factor associated genes, including IGFII and CLF1, in epithelial cells (24). We found that the PLAG factors promote proliferation of hematopoietic progenitors and participate in AML development (9, 10). Here we designed a gene expression profiling approach to identify genes consistently deregulated by PLAGL2 in hematopoietic progenitors as an “early” readout of PLAG function, and in leukemic cells expressing PLAGL2 as a “leukemic” readout. In this analysis, 23 genes were upregulated over 2 fold while no repressed genes were found, highlighting the transcription activation role of PLAG proteins. The sustained upregulation of Mpl levels in membrane by PLAGL2 strongly suggests that Mpl is a major player in PLAG mediated oncogenicity. Consistent with these findings, mutations in the *MPL* and *TPO* genes that promote translation and protein stability have been found in the inherited myeloproliferative syndrome familial essential thrombocythemia (25, 26). In addition, somatic activating mutations in *MPL* are present in a fraction of patients with myelofibrosis with myeloid metaplasia and essential thrombocythemia (27, 28). Future studies should evaluate the levels of PLAGL2 and MPL in myeloproliferative disorders that lack oncogenic mutations in *MPL*.

Mpl expression is regulated by the ETS, RUNX and GATA factors in megakaryocytes (29-31). The Tpo/Mpl pathway is a major regulator of hematopoietic stem cells (32-34).

However, the mechanism of regulation of Mpl expression in HSCs is not well understood. We identified two PLAG consensus sites within a 180 base pair evolutionary conserved region upstream of the *Mpl* translation initiation site. These sites can be bound by PLAGL2 which upregulates transcription, strongly implicating PlagL2 as regulator of *Mpl* transcription. Importantly, RUNX and ETS binding sites are also located within the Mpl proximal promoter, suggesting that Mpl expression could be regulated by the combinatorial role of multiple transcription factors in megakaryocytes, stem cells and leukemic cells. Future studies should unravel the specific PLAG, RUNX and ETS factors that interact to regulate Mpl expression in different cell types. In addition to the oncogenic role of PLAG1 and PLAGL2, PLAGL1 (the third member of the PLAG family) has been implicated as a tumor suppressor in multiple types of cancer, including breast and ovarian cancer and neck squamous cell carcinomas (reviewed in (35)), and has recently been found repressed in diffuse large B-cell lymphoma (36). Since PLAGL1 binds to the first GC-block, it is also possible that MPL expression in hematopoietic progenitors and leukemia may be affected by PLAGL1-loss of expression. Coexpression analysis of available gene expression datasets of human AML (www.oncomine.org) suggest no correlation between PlagL2 and Mpl transcript levels. However, further study of the three PLAG proteins and other factors as modulators of Mpl signaling in human AML is warranted.

The present study demonstrates that PLAGL2 activates expression of Mpl, using two PLAG-consensus binding sites in its proximal promoter, and activates its downstream signaling in hematopoietic progenitors and leukemic cells. This activation is at least one of PLAGL2 induced oncogenic signals that promotes leukemia development in cooperation with CBFβ-SMMHC in mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We wish to thank Julie Zhu and Stephen Baker for the statistical analyses of the expression data. This work was supported by National Institutes of Health Grant CA09683 to L.H.C, and Core resources were supported by the Diabetes Endocrinology Research Center Grant DK32520. L.H.C is recipient of a Scholar Award from the Leukemia & Lymphoma Society.

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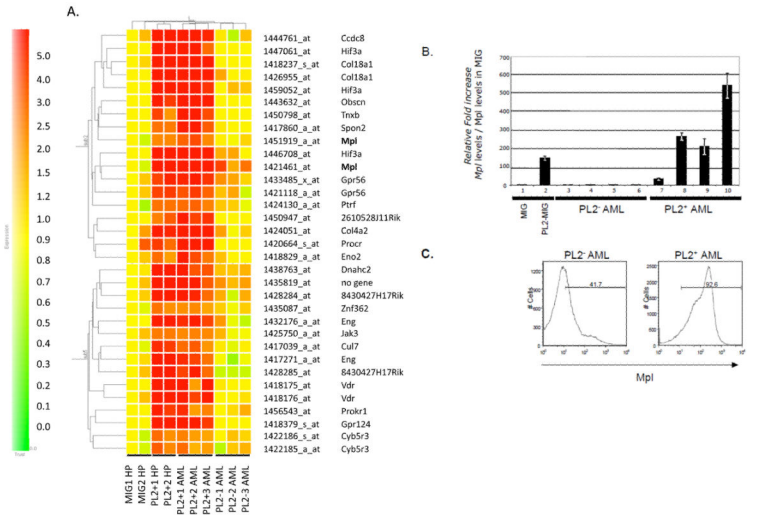

Figure 1. Identification of PLAGL2 target genes in leukemia development using microarray analysis

(A) Cluster of genes specifically up-regulated in PLAGL2+ cells from two-dimensional hierarchical clustering analysis. Hematopoietic progenitor samples infected with MIG (MIG1-HP and MIG2-HP), HPs infected with PLAGL2-MIG (PL2+1HP and PL2+2MIG), AML samples expressing PLAGL2 (PL2+1, PL2+2, and PL2+ AMLs), and AML samples not expressing PLAGL2 (PL2−1, PL2−2, PL2−3 AMLs). All samples express *Cbfb-MYH11*. (B) Relative *Mpl* transcript levels of *Cbfb-MYH11*/hematopoietic progenitor (HP) transduced with MIG (lane 1) or MIG-PLAGL2 (lane 2) retrovirus, and of PLAGL2-negative (PL2−; lanes 3-6) and PLAGL2-positive (PL2+; lanes 7-10) *Cbfb-MYH11* AML samples using quantitative RT-PCR analysis. (C) Expression of Mpl receptor in AML cells with non-detectable PLAGL2 expression (left panel) or expressing PLAGL2 (right panel) using FACS analysis. Percentage of cells expressing Mpl is shown relative to negative control.

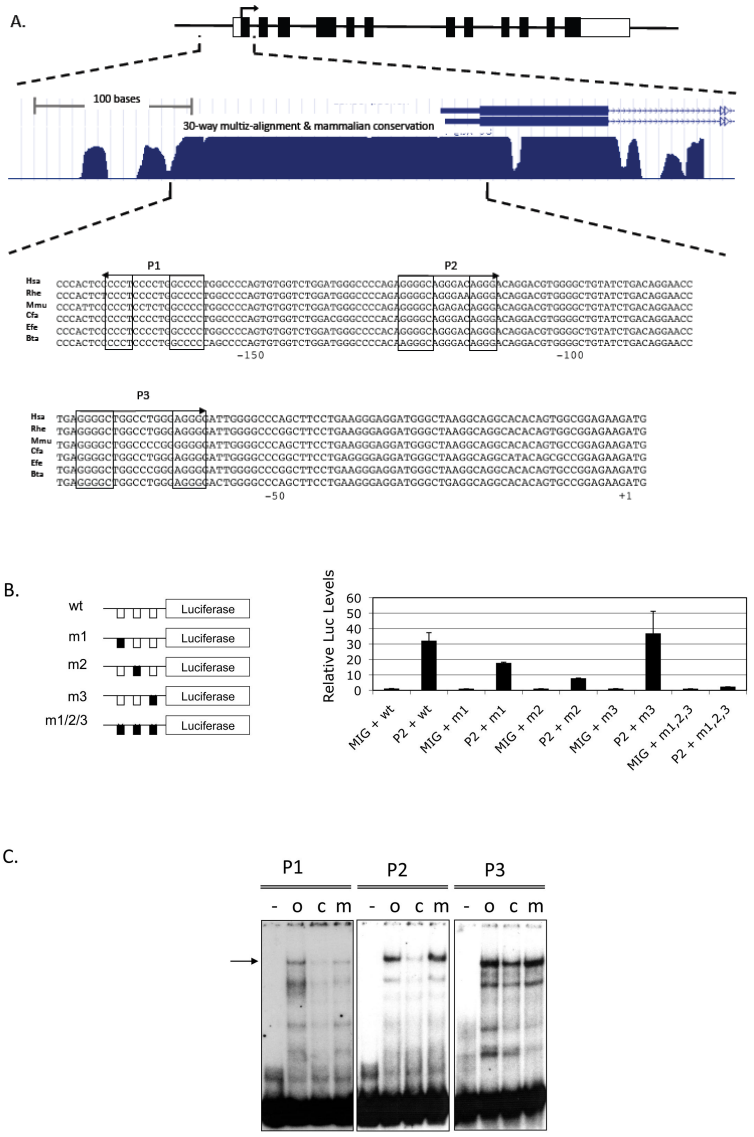

Figure 2. Mpl expression is upregulated by PLAGL2 in hematopoietic cells

(A) Top: depiction of the Mpl gene structure, including untranslated exon sequences (blank boxes), coding regions (black boxes), translation start (arrow). Middle: zoom-in of the proximal promoter, exon 1 (black box) and section of intron 1 (arrowed line), and 30-way multiz-alignment & mammalian conservation analysis of the mouse genome segment chr4:118,129,933-118,130,333; using the UCSC Genome Browser on Mouse July 2007 (NCBI37/mm9) Assembly. Bottom: ClustalW sequence alignment of the *Mpl* proximal promoter (−189 to +3 from translation start site) including human (Hsa), monkeys (Rhe), mouse (Mmu), dog (Cfa), horse (Efe), and cow (Bta). The PLAGL2 consensus binding sites GRGGC(6-8)RGGK (P1 and P2) and a third site (P3) with an $(N)_9$ linker are shown in boxes. Arrows indicate orientation of binding site. (B) Analysis of PLAGL2 activation of the *Mpl* proximal promoter using luciferase reporters in NIH3T3 cells. The luciferase reporters with 300 bp of the *Mpl* proximal promoter (wt) and respective mutants ablating one or more sites (m1, m2, m3, m1, 2, 3) are illustrated on the left panel. Fold activation

relative to wt reporter and no PLAGL2 (MIG + wt) of each construct with (PL2) or without (MIG) PLAGL2 is shown on right panel. (C) Electrophoretic mobility shift assay of PLAGL2 consensus sites P1, P2, and P3 in the *Mpl* proximal promoter, using labeled wild type oligo (o), unlabeled oligo (c), or labeled oligo with point mutation in the core box of the Consensus PLAGL2 site (m). Arrow indicates PLAGL2:DNA binding.

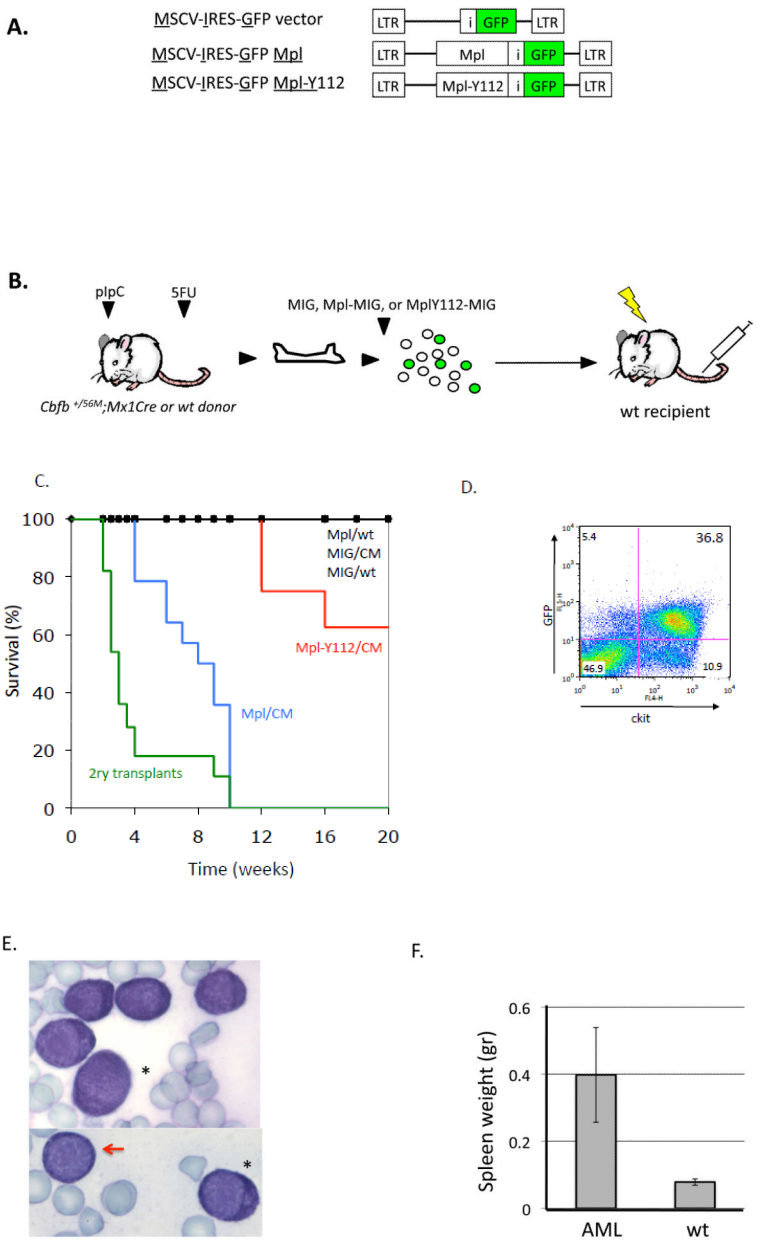

Figure 3. Mpl expression cooperates with Cbfβ-SMMHC in leukemia development

Schematic representation of viral vectors (A) and experimental strategy (B) used in BM transplantation assay. Hematopoietic progenitors from wild type and *Cbfb*$^{+/56M}$*;Mx1Cre* mice (expressing *CBFb-MYH11*) were infected with MIG, MIG-Mpl, or MIG-Mpl-Y112F retrovirus and transplanted into sub-lethally irradiated recipient mice. (C) Kaplan Meier survival curves for mice transplanted with wild type (wt) or *Cbfb*$^{+/56M}$*;Mx1Cre* (CM) cells infected with MIG-Mpl (Mpl), MIG-MplY112, or MIG retroviruses. (D) Representative FACS analysis of peripheral blood white blood cells from a leukemic mouse expressing Cbfβ-SMMHC and MIG-Mpl (GFP+cKit+ cells). (E) Representative cell morphology of 2 AML samples from peripheral blood of CM/Mpl leukemic mice, depicting balstlike (red

arrow) and monocyticlike (asterisks), with magnification 100X. (F) Spleen weight of leukemic (n=11) and control (n=6) mice.

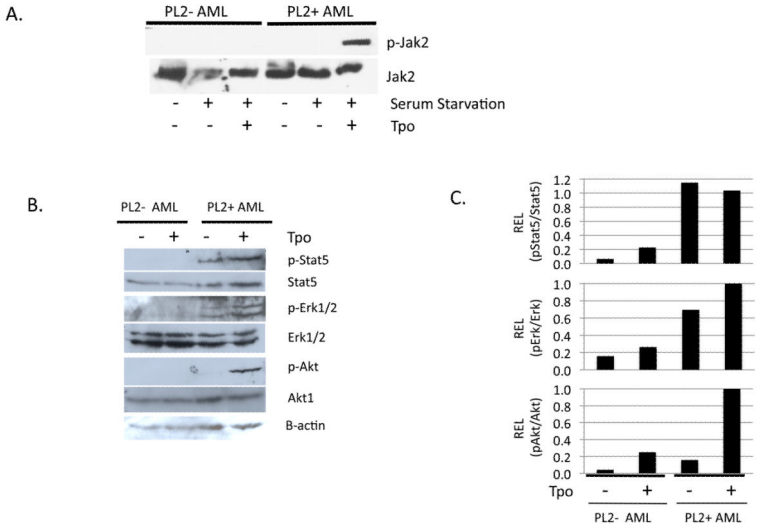

Figure 4. AML cells expressing *Cbfb-MYH11* and PLAGL2 are hypersensitive to Tpo ligand

(A) Phosphorylation analysis of Mpl target Jak2 protein in *CBFb-MYH11* AML samples expressing PLAGL2 (PL2+ AML) or not (PL2− AML), after serum starvation and incubation with Tpo ligand. (B) Expression of phospho-Stat5, Stat5, phospho-Erk1/2, Erk1/2, phospho-Akt, Akt, and B-actin by immunoblot analysis after serum starvation and incubation with Tpo ligand. (C) Densitometric quantification of phospho-protein levels shown in B.